

The Development of a Non- Invasive Short Synacthen Test



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*'Science appears calm and triumphant when it is
completed;
but science in the process of being done is only
contradiction and torment, hope and disappointment.'*

P. Émile Roux, Bacteriologist

1853-1933

Pasteur Institute, Paris

Authenticity of the work

This thesis is the author's own work. Many people have contributed and assisted from its conception to the preparation of the thesis itself. Dr Trevor Johnson performed the expert pharmacokinetic analysis using specialised software. Martin Loxley, laboratory manager at Royal Hallamshire Hospital, was invaluable in supervising the Synacthen assay validation work. NHS clinical chemistry laboratories processed the serum and salivary cortisol samples. The small projects designed to delineate local and national practice were conceived and supervised by the author but carried out by other medical staff in the Department of Endocrinology at Sheffield Children's Hospital (SCH). The illustrated figures in the introduction and clinical studies chapters were designed by the author but the artistic talents of Steve Jones, research assistant at SCH, and Kelly-Marie Nelson, research healthcare assistant at SCH, were used to produce them.

Charlotte Elder, 30th May 2014

Abstract

Patients receiving long-term corticosteroid treatment may develop adrenal insufficiency (AI) with failure of an adequate cortisol surge during stress, potentially resulting in serious illness and death. There is growing concern about inhaled corticosteroids, used in the treatment of asthma, causing AI.

The Short Synacthen Test (SST) is a popular diagnostic investigation for AI. It requires intravenous cannulation and blood sampling to measure the adequacy of the cortisol response. Meta-analytical level evidence supports a lower dose SST, using 1 microgram (mcg) rather than the standard 250 mcg. The SST is invasive, time-consuming and resource-intensive.

This work aimed to develop a non-invasive alternative to the intravenous 1 mcg SST, with Synacthen given nasally and saliva used to measure cortisol response. The initial doses of 25 and 100 mcg were derived from extrapolated animal data and showed some absorption but were not equivalent to the 1 mcg intravenous dose. To aid nasal absorption an increased dose and the addition of a nasal drug enhancer, chitosan, were investigated. Bioavailability and cortisol response were both heightened but dose escalation improved the absorption of nasal Synacthen more than the addition of chitosan. The 500 mcg and the 100 mcg with chitosan formulations showed a good and comparable cortisol response to

the 1 mcg i.v dose. Nasal administration of the 500 mcg tetracosactide with chitosan formulation resulted in the highest bioavailability.

The use of salivary cortisol and cortisone samples to detect the adrenal response to Synacthen had a close and reliable relationship with serum samples. Salivary cortisone was the more sensitive marker of adrenocortical response at lower values.

Nasally administered 500 mcg tetracosactide (Synacthen) with chitosan and salivary cortisone sampling at 60, 75 and 90 minutes may provide a useful alternative to the low-dose SST and help establish which children with asthma are at risk of AI.

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List of abbreviated terms

11 β -HSD-2 - 11 β -hydroxysteroid dehydrogenase type 2

Ab – antibody

ACTH – adrenocorticotrophic hormone

ACTH(1-24) - Synacthen

ACTH(1-39) – endogenous ACTH

ADR – adverse drug reaction

Ag – antigen

Ag* - radiolabelled antigen

AI – adrenal insufficiency

AIDS – acquired immunodeficiency syndrome

AUC – area under the curve

AVP – arginine vasopressin

BDP – beclomethasone dipropionate

BNFc – British National Formulary for Children

BMI – body mass index (kg/m^2)

BMS – biomedical Scientist

BSA – body surface area (m^2)

BTS – British Thoracic Society

CAH - congenital adrenal hyperplasia

cAMP – cyclic adenosine monophosphate (second messenger)

CAR – cortisol awakening response

CBG – corticosteroid-binding globulin

CCRF – children’s clinical research facility (based at Sheffield Children’s Hospital)

CIAs - chemiluminescent immunometric assays

CL - clearance

C_{max} – maximum concentration of a drug in plasma

CNS – central nervous system

CRH – corticotrophin-releasing hormone

CRH-R1 - corticotropin-releasing hormone receptor-1

CRH-R2 - corticotropin-releasing hormone receptor-2

CTIMP - (clinical trial of investigational medicinal product)

CV – coefficient of variation

DHEA – dihydroepiandrosterone

ELISAs - enzyme-linked immunosorbent assays

EMA – European medicines agency

F – bioavailability

FIAs – fluorescent immunoassays

FP – fluticasone propionate

g – grams

GABA - gamma-aminobutyric acid

GlcN - glucosamine

GlcNAc – N-acetyl glucosamine

GP - general practitioner

GR – glucocorticoid receptor type II

hACTH – human ACTH

HDS - hydroxysteroid dehydrogenase e.g. 11 β -HSD

HMG-CoA reductase – 3-hydroxy-3-methyl-glutaryl-CoA reductase

HPA – hypothalamic-pituitary-adrenal axis

HPLC - high performance liquid chromatography

¹²⁵**I** – radioactive iodine

ICS – inhaled corticosteroids

IL - interleukin

i.m – i.m

IMP - investigational medicinal product

i.n – intranasal

IRMAs – immunoradiometric assays

ITT – insulin tolerance test

IU – international units

i.v – intravenous

KIU/ml – kilo international units per millilitre

LC/MS-MS – liquid chromatography tandem mass-spectrometry

LDL – low-density lipoprotein (cholesterol)

LDSST – low dose (1mcg) Synacthen test

LoD - limit of detection

μ Ci - microcurie

mcg - microgram

MAD - mucosal atomizer device

MCC - mucociliary clearance

MC2R – melanocortin receptor type 2 (ACTH receptor)

mcl – microlitre

mg - milligram

mins - minutes

ml – millilitres

MHRA – Medicines and Healthcare products Regulatory Agency

MR – mineralocorticoid receptor (Glucocorticoid receptor type I)

mRNA – messenger ribonucleic acid

MW – molecular weight

ng – nanogram

ng/l – nanogram per litre

nmol/L – nanomol per litre

OMT - overnight metyrapone test

ONS – Office of National Statistics

PBS - phosphate buffer saline

PBS BSA - phosphate buffer saline and bovine serum albumin

PD - pharmacodynamic (the effect of the drug on the body)

pg/ml – picograms per millilitre

PI – principal investigator (Dr Charlotte Elder)

PIL – participant information leaflet

PK – pharmacokinetic (the effect of the body on the drug)

PKA - protein kinase A

POMC - pro-opiomelanocortin

PSUR - Periodic Safety Update Report

PVN – paraventricular nucleus (located in the hypothalamus)

QCs - quality controls

R+D – research and development

REC – research ethics committee

RHH – Royal Hallamshire Hospital (part of Sheffield Teaching Hospitals NHS Foundation Trust)

RIA – radioimmunoassay

SCH – Sheffield Children’s Hospital (Sheffield Children’s NHS Foundation Trust)

SD – standard deviation

SOP - standard operating procedure

SSST – standard (250mg) short Synacthen test

SST – short Synacthen test

StAR - steroidogenic acute regulatory (transport protein for cholesterol in the mitochondrion)

Synacthen – synthetic analogue of ACTH (1-24 ACTH)

TNS - tetracosactide Nasal Solution

Tmax – time at which drug is at maximal levels in plasma

UHSM - University Hospital of South Manchester

UK NEQAS – United Kingdom National External Quality Assessment Service

V1b – AVP receptor

Vd – volume of distribution

ZO - zona occludens

For more descriptive definitions of the pharmacokinetic parameters in please refer to chapter 4, section 3.5.1.

Chapter 1

Introduction

1 Rationale for the research

The advent of inhaled corticosteroids (ICS) for the treatment of asthma allowed their anti-inflammatory actions to be targeted at the lung in the hope that reduced doses might minimise the problematic systemic side effects of steroids: poor linear growth, reduced bone mineral density and hypothalamic-pituitary-adrenal axis (HPA) suppression. In the UK, national asthma guidelines published within the last two decades (1997), prescribing off-label high doses of ICS, were recommended for difficult to control cases (British Thoracic Society 1997). However during the last decade there has been increased recognition and concern about the possibility of adrenal suppression in children taking high-dose ICS (Todd, Acerini et al. 2002). The Commission on Human Medicines and British Thoracic Society/Scottish Intercollegiate Guidelines Network have issued guidance suggesting children who require prolonged courses of high-dose ICS should be provided with a steroid treatment card and consideration given to checking their adrenal function (MHRA 2006, BTS/SIGN 2008). What remains unclear is what dose of inhaled steroids may potentially cause adrenal suppression, whether age, sex, pubertal status and ethnicity play a significant role and therefore whom we should be screening and with what screening test. There is no national consensus and a questionnaire study of UK tertiary paediatric respiratory centres demonstrated considerable variation in practice (Brodie and McKean 2008).

Asthma is the most common chronic disease of childhood and worldwide. The UK has the highest prevalence of children with asthma (Asthma UK 2013). There are an estimated 1.1 million (1 in 11) children with asthma in the UK (Asthma UK 2013), a large proportion of whom are treated with ICS, therefore the number of children who may require evaluation or investigation is significant. Almost 50% of respondents to a recently published questionnaire survey of British Paediatric Endocrinologists have noted a marked increase in referrals from other clinicians for the investigation of potential adrenal suppression in children with asthma prescribed ICS (Elder, Sachdev et al. 2012).

Traditionally the investigation of choice to assess adrenal insufficiency/suppression in most paediatric units has been the Short Synacthen Test (SST). Synacthen is a synthetic analogue of naturally occurring adrenocorticotrophic hormone (ACTH). Both Synacthen and ACTH act on the adrenal gland to stimulate cortisol production. The test requires intravenous (i.v) cannulation (or intramuscular (i.m) injection) to administer the Synacthen and subsequent blood sampling to quantify the cortisol response. The dose of Synacthen used in the SST is a controversial area as some studies have advocated that a lower dose (e.g. 1 microgram (mcg)) may be more physiological than the licenced 250 mcg.

The SST may be unpleasant for the patient and is labour-intensive, requiring skilled personnel and a day-care admission. There is also a paucity of normative data for the SST in children with a single paediatric study using 250 mcg of

Synacthen, performed over 20 years ago, on relatively small numbers and using now out-dated analytical techniques (Lashansky, Saenger et al. 1991). There is no normal paediatric reference range data for the low-dose 1 mcg SST (LDSST). It remains unclear what the most appropriate cut-offs are for paediatric practice (Mushtaq, Shakur et al. 2008) and the questionnaire survey of British Paediatric Endocrinologists has shown considerable variation in the definition of adrenal insufficiency (Elder, Sachdev et al. 2012).

Cortisol can be readily measured in saliva, providing an alternative to venous blood sampling. If the plasma levels required to maximally stimulate the adrenal gland can be reached by nasal administration of Synacthen, a non-invasive SST would be possible. A non-invasive SST would enable research involving sufficient children of different ages, sex and pubertal status to be recruited, allowing normative data to be established and subsequently investigate which children with asthma are at particular risk of adrenal insufficiency. Ultimately this may allow stratification of patients on ICS into risk groups, with their adrenal monitoring tailored to their risk, negating unnecessary investigation of low-risk patients and allowing close monitoring for high-risk individuals. Additionally if this data were available it would enable national consensus guidelines to be written improving consistency of practice.

This chapter will outline the physiology of the HPA-axis, its component parts and how they interact, adrenal insufficiency and its relationship to corticosteroid use (including ICS), how to test the integrity of the HPA-axis, with particular emphasis

on the SST, nasal drug administration and the measurement of corticosteroids in particular cortisol, in saliva.

2 Hypothalamic-Pituitary-Adrenal (HPA) axis

The HPA-axis is integral both to the maintenance of baseline homeostasis and the body's response to stress. Within the hypothalamus both corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are synthesised and then travel in the hypothalamo-pituitary-portal circulation to act on the anterior pituitary gland and cause release of adrenocorticotrophic hormone (ACTH) which in turn acts on the adrenal cortex to stimulate biosynthesis and release of a number of steroid hormones, including cortisol (*figure 1.1*).

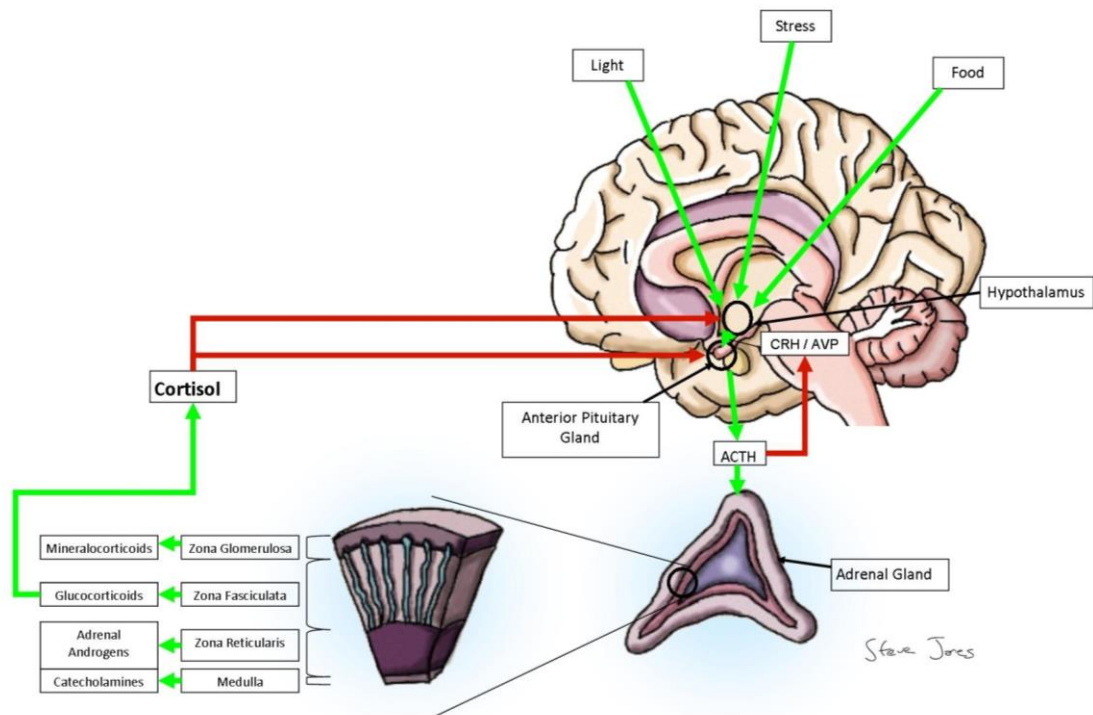


Figure 1.1: Schematic diagram of the Hypothalamic-Pituitary-Adrenal axis. Green arrows depict stimulating inputs and red arrows negative feedback. Kindly illustrated on the author's instruction by Steve Jones (research Assistant, Sheffield Children's Hospital (SCH)).

2.1 Corticotropin-releasing hormone (CRH) & arginine vasopressin (AVP)

Corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are synthesised in the hypothalamus. Corticotropin-releasing hormone is synthesised within the nerve cell bodies of the parvocellular neurosecretory neurons, and AVP in the magnocellular division, of the paraventricular nucleus (PVN). They

travel via axons to the median eminence where they are released into the hypothalamo-pituitary portal system (Majzoub 2006, Miller 2009). Corticotropin-releasing hormone axons terminate in the anterior pituitary and the majority of AVP axons in the posterior pituitary gland (Miller 2009).

The hypothalamus receives input from central (brainstem, amygdala and hippocampus) and peripheral (sympathetic and parasympathetic) sources when triggers, including stress, food and light are encountered. These stimuli cause the synthesis and release of both CRH and AVP (Chrousos, Kino et al. 2009) (*figure 1.1*). A number of neurotransmitters have a role in the regulation of CRH: acetylcholine, noradrenaline, serotonin and histamine, all stimulate release. Gamma-aminobutyric acid (GABA), benzodiazepines and opiates inhibit release (Majzoub 2006, Chrousos, Kino et al. 2009). Corticotropin-releasing hormone biosynthesis is reduced by gene transcription down-regulation when glucocorticoid levels are high (Majzoub 2006).

Corticotropin-releasing hormone is a 41-amino-acid peptide hormone whose mechanism of action, when acting via its type-1 receptor (CRH-R1), is stimulation of adenylate cyclase to increase cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) and thus promote gene transcription of the precursor hormone of adrenocorticotrophic hormone (ACTH). There are two CRH receptors, CRH-R1 and CRH-R2. The R2 receptor is further divided into two subtypes: CRH-R2 α and CRH-R2 β . Both CRH receptors are transmembrane G-protein-coupled cell surface receptors but with different patterns of tissue

expression and effecting different functional roles within the stress system. Corticotropin-releasing hormone-R1 is expressed in the brain, predominantly in the anterior pituitary corticotrophs, similarly CRH-R2 α is found in the brain but with a wider distribution. Corticotropin-releasing hormone-R2 β is expressed in the periphery, predominantly the heart and skeletal muscle. Corticotropin-releasing hormone-R1 mediates the stimulation of the HPA-axis whereas CRH-R2 may have effect on vasodilatation and the regulation of blood pressure (Majzoub 2006).

Arginine vasopressin acts on its receptor, V1b (different from AVP receptors in other locations e.g. kidney), a seven-transmembrane domain G-protein-coupled receptor which stimulates gene expression of the precursor polypeptide, POMC (pro-opiomelanocortin), and therefore ACTH biosynthesis by the activation of protein kinase C and an increase in intracellular calcium (Melmed 2011). Without CRH, AVP has minimal ACTH secretagogue activity, but has a potent synergistic role and both CRH and AVP have a positive effect on the production of the other releasing hormone (Miller 2009).

2.2 Adrenocorticotrophic Hormone (ACTH)

Pituitary ACTH is a 39-amino acid peptide hormone and is synthesised in the corticotroph cells of the anterior pituitary gland. It is one of the cleavage products of the 241-amino acid pro-opiomelanocortin (POMC) (*figure 1.2*). Pro-opiomelanocortin is not found exclusively in the brain but the amounts in the testes, liver, kidney and placenta are not thought to contribute significantly to

circulating ACTH levels (Miller 2009). It acts through its receptor on the adrenal cortex to regulate glucocorticoid and adrenal androgen secretion and participates in the control of aldosterone production.

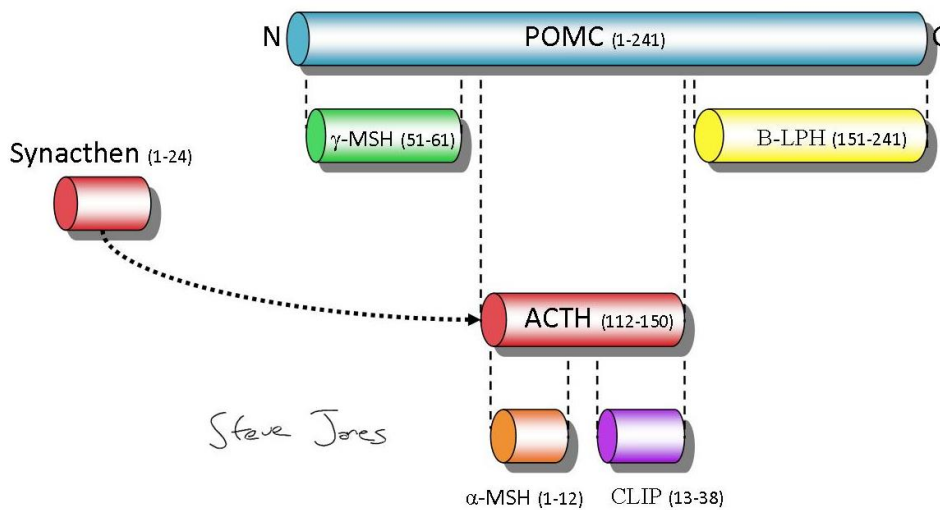


Figure 1.2: Schematic diagram of the main cleavage products of POMC. N and C termini depicted. Synacthen is also shown but is a synthetic product and not derived from POMC. Not all end products are shown. POMC = pro-opiomelanocortin, MSH = melanocyte stimulating hormone, β-LPH = β-lipotrophic hormone, CLIP = corticotropin-like intermediate peptide. Kindly illustrated on the author's instruction by Steve Jones (research Assistant, SCH).

Adrenocorticotrophic hormone is a peptide hormone and is therefore polar, water-soluble and unable to cross lipophilic cell membranes. Unlike steroid hormones e.g. cortisol, which are synthesised from cholesterol and non-polar, and therefore can cross cell membranes to reach intracellular receptors, peptide hormones exert their effects by attaching to cell surface receptors. Adrenocorticotrophic hormone promotes steroidogenesis by binding to the melanocortin-2 receptor (MC2-R), a 7-transmembrane, G-protein-coupled

receptor, which causes an increase in cAMP, affecting LDL (low density lipoprotein) receptor biosynthesis and LDL cholesterol uptake. Low-density lipoprotein cholesterol acts as a substrate for steroid production (*figure 1.3*). Adrenocorticotrophic hormone is also responsible for a small amount of *de novo* steroid synthesis by promoting transcription of HMGCoA reductase, the rate-limiting stage in cholesterol biosynthesis (Miller 2009). The rapidity of the cortisol response to ACTH is in part due to the number of ACTH receptors in the adrenal cortex, (each adrenocortical cell contains approximately 3,500 MC2-receptors), and the role of ACTH (via cAMP) and glucocorticoids in the upregulation of MC2-R gene expression. Melanocortin type 2 receptors are also found in skin and adipocytes, stimulating lipolysis during times of stress (Papadimitriou and Priftis 2009).

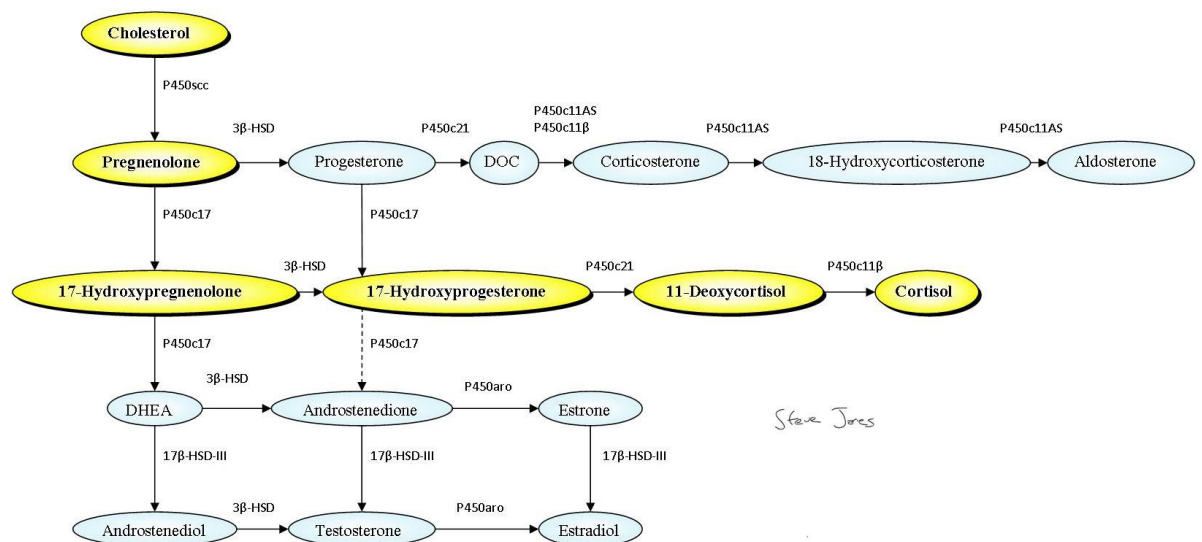


Figure 1.3: Schematic flow diagram of adrenal steroid biosynthetic pathways. DHEA = dihydroepiandrosterone, HSD = hydroxysteroid dehydrogenase, DOC = deoxycorticosterone. Kindly illustrated on the author's instruction by Steve Jones (research Assistant, SCH).

There are only small amounts of cortisol stored in the adrenal gland, thus the actions of ACTH are to promote steroid synthesis by increasing intracellular cholesterol, maintaining levels of free cholesterol and facilitating transport of cholesterol onto the inner membrane of the mitochondria, by the CYP11A1 enzyme, for conversion to steroid hormones (Papadimitriou and Priftis 2009) (*figure 1.4*). These actions all occur within minutes thus soon after an ACTH peak a corresponding surge in cortisol is seen. ACTH has a half-life of approximately twenty minutes (Keenan, Roelfsema et al. 2004). Adrenocorticotrophic hormone has an additional role within the fetal adrenal gland where it stimulates growth by its role in the production of local growth factors including insulin-like growth factor 2 and epidermal growth factor (Miller 2009) and increases the adrenal weight in children and adults by both hyperplasia and hypertrophy (Papadimitriou and Priftis 2009).

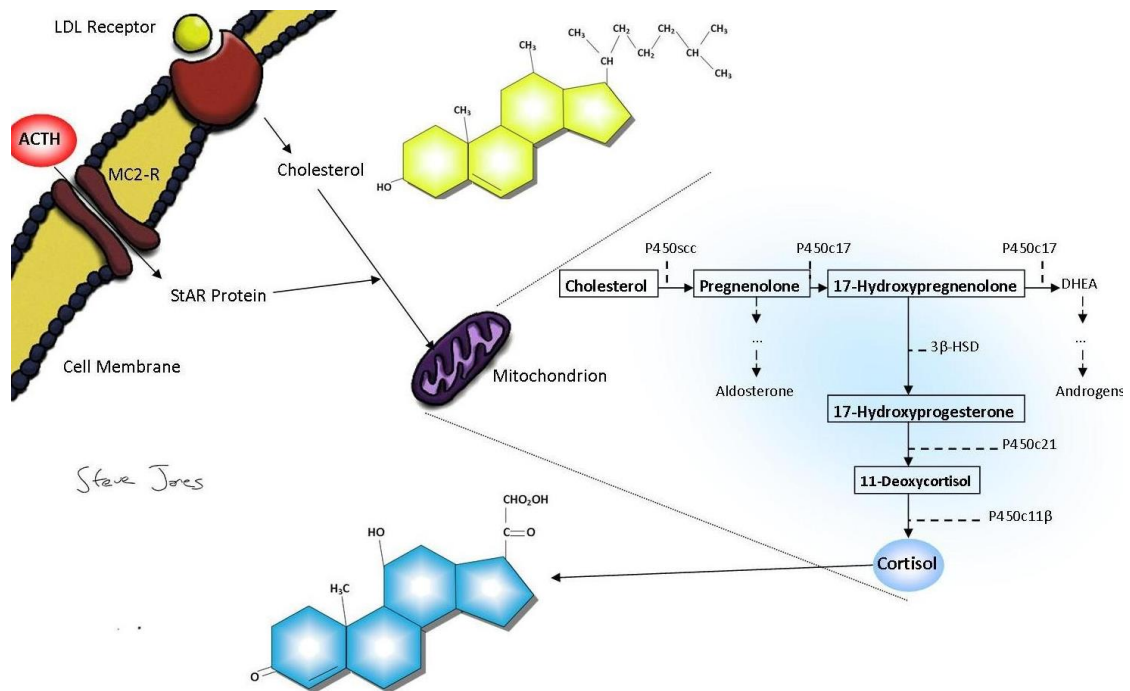


Figure 1.4: Schematic diagram of the intracellular cortisol biosynthesis pathway, with molecular structures depicted for cholesterol and cortisol. LDL = low-density lipoprotein, MC2-R = melanocortin receptor type 2 (ACTH receptor), StAR = steroidogenic acute regulatory protein, DHEA = dihydroepiandrosterone, 3β-HSD = 3β-hydroxysteroid dehydrogenase. Kindly illustrated on the author's instruction by Steve Jones (research Assistant, SCH).

Cholesterol is the precursor of all steroid hormones and in steroidogenic tissues (i.e. those that contain P450_{scc}¹, which catalyses the conversion of cholesterol to pregnenolone), it is stored as an ester, in lipid droplets. Adrenocorticotrophic hormone acts to further increase the amount of free cholesterol within the cell by promoting the activity of cholesterol esterase and inhibiting cholesterol ester synthetase. It is free cholesterol that acts as a substrate for mitochondrial P450_{scc} and its transporter, StAR (steroidogenic acute regulatory) protein, is

¹ P450_{scc} – “scc” denotes the side chain cleavage of cholesterol

synthesised and phosphorylated under the control of ACTH (Miller 2009) (*figure 1.4*).

2.3 Cortisol (Hydrocortisone)

The adrenal cortex forms the outer part of the gland and contains three distinct zones: the outermost zona glomerulosa, responsible for mineralocorticoid production (mainly aldosterone); the zona fasciculata, the main site of glucocorticoid synthesis; and the inner most zona reticularis, which produces androgens (mainly DHEA, (dehydroepiandrosterone)) (*figure 1.1 and 1.4*). The only steroids produced in appreciable quantities by the adrenal cortex are DHEA, cortisol, corticosterone and aldosterone, with cortisol being the dominant glucocorticoid (adult secretion is about 10-20 mg/24 hours) (Miller 2009). All steroid hormones have the same basic structure as they derive from cholesterol (*figure 1.3*). Cortisol biosynthesis and secretion are predominantly under the control of ACTH, however adrenal steroidogenesis is complex with many influencing regulatory factors, including other hormones, growth factors, cytokines (TNF- α (tissue necrosis factor) and leptin) and neurotransmitters (Papadimitriou and Priftis 2009).

Cortisol, like all glucocorticoids, acts through the glucocorticoid receptor (GR), which is expressed by every nucleated cell in the body. Unlike the CRH (CRH-R1) and ACTH (MC2-R) receptors, which are both transmembrane G-protein-coupled receptors exerting their effect via the adenylate cyclase-cAMP-PKA pathway, GRs

are cytosolic receptors, which function as ligand-dependent transcription factors, whose forms are very different depending on the presence or absence of ligand (glucocorticoids). When no ligand is bound the GR is found as a complex of proteins in the cytoplasm however on binding ligand it dissociates, moves into the nucleus, where there is interaction with transcription factors such nuclear factor- κ B (NF- κ B) or direct binding to the glucocorticoid response elements (GRE) of the promoter region of target genes that up- or down-regulates their expression (Chrousos, Kino et al. 2009, Papadimitriou and Priftis 2009).

Glucocorticoids themselves are pleiotropic and central to life-maintaining and -sustaining processes. Their effects are both immunological and metabolic. They are potent immunosuppressors that down-regulate pro-inflammatory proteins and up-regulate the expression of anti-inflammatory proteins. The metabolic effects of cortisol are numerous but all serve to increase available energy sources. Glucocorticoids act to maintain or increase blood glucose by stimulating gluconeogenesis. They enhance expression of enzymes in the gluconeogenic pathway and counteract the effects of insulin, inhibiting glucose uptake in adipose tissue and muscle. Additionally glucocorticoids promote lipolysis and protein breakdown releasing substrates for gluconeogenesis.

The enzyme 11- β -HSD (11- β -hydroxysteroid-dehydrogenase) converts cortisol to a more inactive form, cortisone, and thus determines the level of cortisol activity in different tissues (*vide infra chapter 1, section 2.3.2*).

The majority of cortisol is bound (total cortisol) and has a half-life of approximately 50 minutes. The remaining 5% is free and has a half-life of only 2 minutes (Keenan, Roelfsema et al. 2004). The vast majority of cortisol is metabolised by the liver to more soluble forms before urinary excretion. Only 1% is excreted, unchanged, in the urine (Daidoh, Morita et al. 1995, Miller 2009).

2.3.1 Cortisol Binding Globulin (CBG)

Approximately 95% of cortisol in plasma is bound, the majority (90%) to its specific carrier protein, corticosteroid-binding globulin (CBG) and the remaining 5% to generic binding proteins such as albumin and α_1 -acid glycoprotein (Perogamvros, Keevil et al. 2010). The carriers differ in their binding capacity and affinity for cortisol: CBG has a very high affinity but low binding capacity, albumin has the opposite characteristics and α_1 -acid glycoprotein falls in-between. Traditionally it was believed that only the unbound steroid (free cortisol) is biologically active, this is now thought over-simplistic. Whilst serum free cortisol defines most of the biological activity the bound fraction is not rendered biologically inactive but rather retains some, albeit significantly reduced, potency (Miller 2009). It appears that the effect of binding cortisol allows a reasonably equal distribution throughout tissues and thus reduces the deleterious effects that large peaks and troughs in cortisol might otherwise have (Miller 2009).

Cortisol Binding Globulin is synthesised in the liver and is regulated by oestrogens. Certain states increase CBG levels, most commonly hyper-

oestrogenism e.g. pregnancy and women on the combined oral contraceptive pill, or hyperthyroidism and hereditary CBG excess. The reverse is also the case in hypo-oestrogenic states e.g. hypothyroidism, hypoproteinaemic conditions and familial CBG deficiency. In conditions where CBG levels are high the unbound portion of cortisol is thought to remain at similar levels to a normal CBG state whilst the total serum cortisol levels are increased. This can lead to confusion clinically, where total serum cortisol is routinely measured rather than the unbound fraction (Zollner 2007, Perogamvros, Aarons et al. 2011).

2.3.2 Cortisone

After secretion from the *zona fasciculata* within the adrenal cortex, cortisol is converted to the much less biologically potent cortisone in the peripheral tissues, by the enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD). Two isoforms of 11 β -HSD exist. 11 β -hydroxysteroid dehydrogenase-1 which catalyses both oxidase activity (conversion of cortisol to cortisone) and the reverse reductase activity and is found in glucocorticoid-responsive tissues such as the lung and liver. 11 β -hydroxysteroid dehydrogenase-2 only catalyses oxidation and is expressed in mineralocorticoid-responsive tissues. The role of 11 β -HSD-2 is protective by reducing the potent effects of cortisol in tissues where mineralocorticoid activity needs to predominate, such as the kidney (Miller 2009). Cortisol Binding Globulin has a lower affinity for cortisone compared with cortisol, probably because the protective role of CBG in moderating the

potentially adverse effects of large amounts of circulating free serum cortisol is not required (Wood 2009).

2.4 Negative feedback system

Regulation of the HPA-axis is via a classical endocrine feedback system with cortisol having a suppressive effect on both the hypothalamus and the anterior pituitary and similarly ACTH on CRH and AVP (*figure 1.1*). The negative feedback system serves to protect the body from the harmful catabolic, lipogenic, anti-reproductive and immunosuppressive effects of excessive exposure to glucocorticoids (Chrousos, Kino et al. 2009).

There are two glucocorticoid receptors in the central nervous system: glucocorticoid receptor type I (mineralocorticoid receptor, MR) and glucocorticoid receptor type II (GR). The mineralocorticoid receptor responds to low levels of glucocorticoids and stimulates production whereas GR is activated both when cortisol levels are low and when concentrations are high, suppressing further production and is therefore the sole effector of negative feedback (Chrousos, Kino et al. 2009).

The mechanism of feedback differs depending on whether the stress exposure is acute or chronic. Acute negative feedback disables the release of ACTH and CRH from their respective secretory granules and occurs within minutes. The chronic exposure negative feedback for ACTH and CRH/AVP is mediated by the direct

inhibitory action of glucocorticoids on POMC gene transcription in the anterior pituitary gland and mRNA synthesis and in the hypothalamus respectively (Miller 2009, Papadimitriou and Priftis 2009).

2.5 The secretory patterns of components of the HPA-axis

Each of the main components of the HPA-axis (CRH, AVP, ACTH and cortisol) is secreted in a pulsatile, circadian manner. A circadian rhythm not only repeats itself every 24-hours (diurnal) but is endogenous (sustained even in the absence of the usual external cues (e.g. light, food)) and yet entrainable e.g. adjustment following travel to a different time-zone (Papadimitriou and Priftis 2009). Circadian rhythm is orchestrated from the suprachiasmatic nucleus in the hypothalamus, and causes CRH release (Debono, Ghobadi et al. 2009). This diurnal pattern of secretion is influenced by a number of factors including light, activity, food and stress and the intrinsic rhythmicity of both the adrenal gland and hypothalamus (Miller 2009). Stressors, such as significant illness or major surgery, stimulate an increase in the amplitude of AVP and CRH, their synchronisation and a reduction in the time lag between their release and their effect on ACTH (Chrousos, Kino et al. 2009). The action of CRH on the anterior pituitary gland and ACTH on the adrenal cortex is amplified many thousands of times such that a few pg/ml of ACTH results in serum cortisol release of mcg/dl (Oelkers 1996).

The use of more sensitive assays to measure CRH, ACTH and cortisol has shown the HPA-axis to be active throughout the 24-hour period, with episodic pulses of its components released every 30-120 minutes. Corticotropin-releasing hormone and AVP pulse amplitude is characteristically at its height at 04.00, with ACTH peaking between 04.00 and 06.00 and the resultant cortisol surge seen at 08.00. In both children and adults the frequency and amplitude of the HPA-axis components pulses increase during peak secretion seen in the early morning and similarly reduce during the quiescent period of approximately 5 hours in the night (23.00 to 04.00) (Wallace, Crowne et al. 1991, Miller 2009).

Estimations of the number of peaks in 24 hours vary between 10 and 40 dependent on the sampling interval used and the sensitivity and specificity of the assays employed (Gallagher, Yoshida et al. 1973, Horrocks, Jones et al. 1990, Veldhuis, Iranmanesh et al. 1990). Some episodes of ACTH secretion are not succeeded by cortisol bursts, generally because the ACTH peaks are so closely temporally related as to result in a single, sustained elevation of cortisol secretion (Gallagher, Yoshida et al. 1973). Between half and three quarters of the total cortisol peaks occur between midnight and 09.00, with increasing higher concentrations of ACTH and cortisol per peak and increasing pulse frequency seen to crescendo until maximum levels are reached approximately an hour before awakening (Krieger, Allen et al. 1971) (Keenan, Roelfsema et al. 2004).

It is unclear whether circadian rhythm is different between the sexes. One study reported more pulses (18 versus 10), higher mean peak amplitude, greater mean

level and a larger area under the curve over 24 hours in males but no difference in cortisol leading the authors to surmise that the adrenal cortex may be more sensitive to the effects of ACTH in the female (Horrocks, Jones et al. 1990). Other studies have found circadian rhythm not to be affected by age or sex in adults (Krieger, Allen et al. 1971, Keenan, Roelfsema et al. 2004). In children there are on average 24-34 peaks of both ACTH and cortisol in a 24-hour period and no significant differences with respect to gender or pubertal status (Wallace, Crowne et al. 1991). Circadian rhythm is absent in newborn babies and starts to establish itself during infancy but may not fully mature until three years of age or older (Miller 2009).

2.5.1 Cortisol awakening response (CAR)

It was observed over 40 years ago that cortisol reaches its acrophase² between 30 and 120 minutes after waking (Krieger, Allen et al. 1971). More recently a sharp increase in cortisol, prior to the acrophase and maximal 30 minutes after waking, known as Cortisol Awakening Response (CAR), has been demonstrated (Pruessner, Wolf et al. 1997).

There is a large inter-individual variation in early morning cortisol values, usually sampled between 08.00 and 09.00, which may cause overlap and clinical confusion with samples taken from patients with low cortisol conditions. This may in part be due to the sharp decline in cortisol levels seen following the

² Acrophase – the time at which the peak of a rhythm occurs

acrophase and the variability in individuals' time of waking. The cortisol awakening response is more individually specific as it has been reliably shown to increase cortisol values by 38-75% within the first 30 minutes following waking in both sexes and appears not to be affected by age (Wust, Wolf et al. 2000). The response seems unaffected by the quality or length of sleep or activity following waking (Pruessner, Wolf et al. 1997) but does seem to be altered by stress and time of waking (Fries, Dettenborn et al. 2009, Clow, Hucklebridge et al. 2010). Recently CAR has been shown to be present in toddlers and young children (Baumler, Kirschbaum et al. 2013).

2.6 The Stress System

A significant stressor is one that threatens an individual's life and therefore likelihood of successful reproduction and gene propagation. The response to a stressor is tripartite: behavioural (e.g. fear, increased vigilance, anorexia), autonomic, mediated by the sympathetic nervous system, (e.g. increase in pulse and respiratory rate, bronchodilation, increase cerebral and musculoskeletal blood flow and maintenance of normoglycaemia) and hormonal, mediated through the HPA-axis, which releases the energy reserves necessary to fuel the stress response (Majzoub 2006).

The HPA-axis has a number of different neurological inputs, both central (hypothalamus and brainstem) and peripheral, which enable it, in conjunction with the sympathetic nervous system, to play the central role in controlling the

biological responses to stress. Following an acute stressor these higher centre inputs cause greater synchronicity and amplitude of CRH and AVP and thus increase their release from the PVN in the hypothalamus. In addition secretagogues of CRH, AVP or ACTH such as magnicellular AVP and angiotensin II are released (Chrousos 1995). Furthermore in stressors such as febrile illness, inflammation and cytokines stimulate cortisol production by increasing CRH (IL-1 (interleukin) and IL-6 mediated) and ACTH (IL-2 and tumour necrosis factor mediated) (Chrousos 1995).

In addition to catecholamines, cortisol is the final common pathway of these complex interactions and triggers the “flight, fight and fright” response. This enables rapid release and transport of energy sources to vital organs: heart, brain and muscles and, by an increase in blood pressure, cardiac output and respiratory rate, optimum delivery of oxygen and nutrients is achieved. The end result is a body primed for maximum activity with increased concentration and cognition, faster motor reflexes, a higher pain threshold and less appetite for food and reproduction. The stress system is not quiescent during rest and remains responsive to circadian rhythms and other neurosensory inputs. Cortisol is both responsible for priming these mechanisms and tempering them to prevent harm (Majzoub 2006, Papadimitriou and Priftis 2009).

3 Adrenal insufficiency (AI)

3.1 Definition and causes of AI

Adrenocortical failure may be primary or secondary and can present acutely or chronically. It results in hypocortisolism, which may be non-specifically or overtly symptomatic or sub-clinical. Primary adrenal failure occurs when there is destruction of the adrenal gland itself e.g. by autoimmune antibodies, haemorrhage or tumour or a disturbance in its ability to manufacture steroids e.g. congenital adrenal hyperplasia (CAH). Secondary failure describes a disruption of the hypothalamic or pituitary component of the HPA-axis, and tertiary hypothalamic failure leading to reduction in steroidogenesis. Previously in all parts of the world tuberculosis was the commonest cause of AI and it remains an important cause in developing world, however in the developed world autoimmune adrenalitis (Addison's Disease, first described by Thomas Addison in 1855) accounts for 80-90% of the adult cases (Arlt and Allolio 2003). Many of the causes of AI have their onset in adulthood, making the genetic causes, CAH, and withdrawal from glucocorticoids relatively more common in childhood. Reported experience over 20 years in Montreal, Canada, showed that 72% of the 103 children with primary AI had CAH, 13% autoimmune AI and remaining 15% were either unexplained or had one of the rarer causes such as Triple A, Zellweger syndrome or adrenoleucodystrophy (Perry, Kecha et al. 2005). Some examples of causes of primary and secondary AI are given in *table 1.1*.

Table 1.1: Causes of Adrenal Insufficiency (Oelkers 1996, Arlt and Allolio 2003, Miller 2009).

Primary AI	Secondary AI
Autoimmune adrenalitis	Withdrawal from corticosteroid therapy
Autoimmune polyglandular syndromes	Hypopituitarism
Infectious adrenalitis	Tumours
Tuberculosis	Craniopharyngioma
AIDS	Hypothalamic
Fungal infections	Pituitary
Sepsis	Metastases
Adrenal haemorrhage/necrosis	Post CNS irradiation
Adrenal infiltration	Idiopathic intracranial hypertension
Primary xanthomatosis, sarcoidosis	Severe head injury
metastases	Lymphocytic hypophysitis
Iatrogenic	Pituitary infiltration
bilateral adrenalectomy	TB
drug induced	Sarcoid
Genetic conditions	Pituitary infarction: Sheehan's syndrome
IMAGe	Genetic disorders
Allgrove syndrome,	POMC deficiency
Adrenoleukodystrophy	Septo-optic dysplasia
Congenital adrenal hyperplasia,	
Smith-Lemli-Opitz syndrome	
Congenital lipoid hypoplasia	

3.2 Incidence and prevalence of AI

Incidence and prevalence figures may be difficult to interpret due to the covert and insidious nature of the clinical presentation of AI. Prevalence figures vary but it is thought 150-280 per million are affected by secondary AI and 93-140 per million people by primary AI, with an incidence in white populations of 4.7-6.2 per million (Arlt and Allolio 2003). Although not stated it is likely these figures based on adult estimates. Both primary and secondary AI occur more commonly in women, with primary diagnoses peaking in the fourth decade and secondary in the sixth (Arlt and Allolio 2003). Iatrogenic AI from corticosteroid use is the commonest cause of secondary AI, although due to its transient aetiology its

prevalence may be lower than other causes. Incidence and prevalence figures are lacking in the paediatric population but CAH, as the commonest form of primary AI, has an incidence of 1 in 10,000 to 18,000 (White and Speiser 2000). A British Paediatric Surveillance Unit (BPSU) survey has been recently conducted to ascertain the prevalence of AI in children and its report is awaited.

3.3 Symptoms and presentation of AI

Diagnosing AI is challenging for clinicians due to the numerous and non-specific nature of the symptoms, causing a delay in diagnosis and most patients seeking multiple opinions with half only being diagnosed following an episode of adrenal crisis (Arlt 2008). The symptoms are predominantly due to the glucocorticoid deficiency, but others may be because of mineralocorticoid deficiency (symptoms attributable to electrolyte disturbance) or adrenal androgen deficiency (lack of adrenarche or pubarche in children or loss of pubic and axillary hair in women), or derangement in POMC cleavage products (skin colour changes secondary to excess melanocortin) (Arlt and Allolio 2003).

Chronic symptoms of AI include fatigue, weakness, arthralgia, anorexia, abdominal pain and the patient may feel nauseous or vomit. Adults and older children may experience weight loss with younger children failing to thrive. If the pathogenesis includes mineralocorticoid deficiency (e.g. the genetic mutation in CAH is expressed in the cells of the *zona glomerulosa*) those affected may experience postural hypotension and symptoms associated with hyponatraemia

and hyperkalaemia (Arlt 2008). More insidious signs include pale skin in secondary AI and hyperpigmentation in primary AI due the lack of negative feedback driving an increase in POMC and its cleavage products (*figure 1.2*) which stimulate the melanocortin receptors in the skin (Arlt and Allolio 2003).

Acute adrenal crisis carries an appreciable mortality and may occur when undiagnosed, or undertreated chronic insufficiency is further exacerbated by stress such as trauma, surgery or infection. Adrenal crisis usually presents with the patient hypotensive, in hypovolaemic shock, with severe abdominal pain and vomiting. Children, especially young ones, are more likely to present with hypoglycaemia and consequent seizures or coma (Shulman, Palmert et al. 2007).

3.4 AI secondary to corticosteroid use

In 1952, a decade after the medical landmark discovery of cortisone as a treatment for inflammatory conditions, the first case of adrenal crisis and death in a patient on glucocorticoid therapy undergoing surgery was described (Fraser, Preuss et al. 1952) and thereafter a spate of such cases were published (Lewis, Robinson et al. 1953, Salassa, Bennett et al. 1953) and recommendations as to how these patients should be investigated and managed followed (Bayliss 1958, Sampson, Brooke et al. 1962). It is now widely acknowledged that patients on long-term corticosteroids require increased steroid cover at times of stress, such as major surgery and significant febrile illness, and a gradual tapering of the dose if the medication is to be stopped. Although permanent adrenal failure

secondary to prolonged corticosteroid usage may occur the effects on the HPA-axis can usually be reversed and normal HPA-axis activity restored over many months or possibly years with carefully managed tapering of long-term glucocorticoid therapy (Stewart 2003).

3.4.1 Pathophysiology of AI secondary to corticosteroid usage

Exogenous glucocorticoid use may cause hypothalamic and pituitary suppression only, with normally functioning adrenal glands; complete HPA-axis suppression with adrenal atrophy; or not cause HPA-axis suppression at all. Adrenal suppression secondary to exogenous corticosteroid use causes reduction in CRH and ACTH with eventual atrophy of the adrenal cortex (*figure 1.5*). Whilst exogenous steroids are being administered on a regular basis this atrophy is of no clinical consequence however if there is an abrupt cessation to the exogenous steroid supply or a requirement for a significant increase in glucocorticoids in times of stress, then the adrenal insufficiency may become clinically manifest, as adrenal crisis.

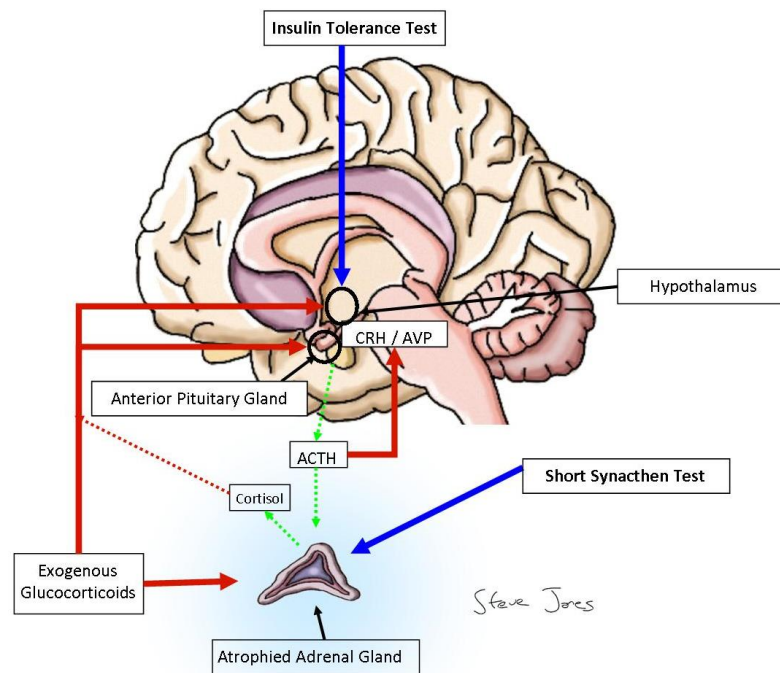


Figure 1.5: Schematic diagram of the HPA-axis with the consequences of exogenous steroids on the adrenal gland and the sites of action of two tests of adrenal function shown (blue arrows). Kindly illustrated on the author's instruction by Steve Jones, research assistant SCH.

It is thought that corticosteroids cause secondary AI by a number of mechanisms. The lack of cortisol production in the adrenal gland leads to adrenal atrophy but also to a reduction in the expression of adrenal ACTH receptors. Adrenocorticotrophic hormone has been shown to up-regulate its own receptor and thus down-regulation is thought to occur in times of ACTH deficiency (Lebrethon, Naville et al. 1994). Pituitary corticotroph cells atrophy when chronically exposed to exogenous glucocorticoids and POMC gene transcription is suppressed, thus ACTH levels reduce and the ability to synthesise ACTH

diminishes. There is also an effect on CRH with reduced synthesis and storage and fewer receptors in the pituitary gland (Miller 2009).

3.4.2 Predicting AI in patients on corticosteroids

The risk of HPA-axis suppression is in part dose-dependent but a number of different factors influence the risk for an individual. Different exogenous steroid preparations alter the risk of AI with systemic corticosteroids and those with the highest potency posing the greatest risk. Of the commonly prescribed glucocorticoids hydrocortisone has the least suppressive effect, prednisolone is moderately suppressive and dexamethasone has the most suppressive effect (*table 1.2*). Other contributors to the overall risk include how many times a day they are taken and evening prescriptions of long acting preparations, as this further suppresses the endogenous early morning ACTH surge (Krasner 1999). Parenteral and enteral preparations have been described as causing AI with routes of delivery as innocuous sounding as dermal, inhaled and intranasal all having been reported. Duration of corticosteroid therapy and cumulative dose do not appear to be reliable predictors of AI as there appears to be considerable individual variation with some individuals developing AI on relatively short courses and small doses others seeming more resistant (Mackenzie and Wales 1991). This may be due to individual protein binding and pharmacokinetics of glucocorticoid clearance (Krasner 1999).

Table 1.2: Corticosteroid equivalent potencies quoted in BNFc 2013 (Paediatric Formulary Committee 2013)

Corticosteroid	Relative anti-inflammatory potency	Equivalent anti-inflammatory dose	Half-life (hours)
Prednisolone	4	5 mg	18-36
Dexamethasone or betamethasone	25-50	750 mcg	36-54
Hydrocortisone	1	20 mg	8-12
Methylprednisolone or triamcinolone	5	4 mg	18-36

A 4-week course of corticosteroids (0.2mg/kg oral dexamethasone) in children with leukaemia caused HPA-axis suppression in all at the end of the course and for 2 months after discontinuation in 3 out of 10 patients (Felner, Thompson et al. 2000). Generally it is believed that in most adult individuals doses of more than 7.5 mg prednisolone equivalent or more, for greater than four weeks is likely to cause AI (Arlt and Allolio 2003).

3.4.3 Inhaled corticosteroids (ICS) and adrenal insufficiency

In the UK asthma is the most common chronic disease of childhood and has a prevalence of 17-23% (NICE 2007). Since their introduction in 1972 inhaled corticosteroids (ICS) have become the mainstay of preventative treatment and 70% of asthmatic children are prescribed them (Office for National Statistics 1998). They are first line treatment in anything but the most mild asthma (BTS/SIGN 2008). ICS have not only improved asthma symptoms with fewer side effects compared with oral preparations, but have also significantly reduced

hospital admissions and deaths (Blais, Ernst et al. 1998, Lanes, Garcia Rodriguez et al. 2002). However, at higher doses, side effects may become apparent, especially in atopic children who may be receiving intranasal steroids for rhinitis, topical steroids for eczema and courses of oral steroids during acute exacerbations of their asthma.

Possible HPA-axis suppression with ICS has concerned doctors for 40 years. Early studies in children showed low doses, beclomethasone (BDP) up to 400 mcg daily or equivalent, to be safe but impairment of normal HPA-axis response to provocation testing at higher doses (Vaz, Senior et al. 1982, Law, Marchant et al. 1986). The current regimen of maintenance with ICS and short courses of oral steroids during acute exacerbations was first recommended in the 1970s following the deaths of three children from adrenal failure who had had oral steroids stopped (Mellis and Phelan 1977). Although there is intra-individual variation in sensitivity to steroids it is the use of high-dose ICS which naturally causes the most concern and guidelines recommend prescribing the lowest effective dose of ICS (BTS/SIGN 2008). A survey of British GPs showed 4.5% of all children are prescribed ICS. Of those on ICS, 10% of 5-11 year olds and 5.6% of the under 5s were prescribed doses above the recommended maximum for age (*table 1.3*) and over double the recommended maximum dose in 4.9% and 3.9% respectively (Thomas, Turner et al. 2006).

Table 1.3: Maximum licensed dose of ICS in children quoted in BNFC 2013 (Paediatric Formulary Committee 2013).

	Maximum dose	Age
Beclomethasone dipropionate/ Budesonide	400 mcg bd	5-12 years
	1 mg bd	12-18 years
Fluticasone propionate	200 mcg bd	5-12 years
	500 mcg bd	12-18 years
Mometasone furoate	400 mcg bd	12-18 years

Cases of AI or crisis on high dose ICS began to be reported in the 1990s but the issue was further compounded by the introduction in 1993 of fluticasone propionate (FP) (Wong and Black 1992, Zwaan, Odink et al. 1992). Fluticasone is an ICS with approximately twice the potency of either budesonide or beclomethasone but with a high therapeutic index (a large difference between the therapeutic and toxic dose of a drug). The high therapeutic index encouraged clinicians to prescribe off-licence doses in difficult to control asthma causing a number of cases of AI and a death, attributable to fluticasone (Todd, Dunlop et al. 1996, Patel, Wales et al. 2001, Drake, Howells et al. 2002, Macdessi, Van Asperen et al. 2003). These cases prompted the authors of one series to conduct a national questionnaire survey of all paediatricians and adult endocrinologists in the UK requesting cases of adrenal crisis in asthmatic patients on ICS. They identified 33 patients, 28 of whom were children, one of whom had died. The majority of those identified were on doses above licence, yet recommended in national guidelines, and 94% were on fluticasone (Todd, Acerini et al. 2002). This prompted several bodies to revise their ICS dosage guidance and recommend management by a specialist, specific written advice to be given to the

patient/family, steroid treatment cards be issued and consideration given to periodic testing of the adrenal gland (MHRA 2006, BTS/SIGN 2008, MHRA/CSM 2008).

Following this heightened awareness there have been numerous attempts to delineate the prevalence of AI in asthmatic children on high dose ICS. This has been beleaguered by a lack of consensus about what test to use and difficulty in knowing what diagnostic threshold to adopt. A summary of some of the studies is shown in *table 1.4*.

Table 1.4: Summary of prevalence studies of adrenal suppression in children with asthma treated with ICS (FP = fluticasone propionate, BDP = beclomethasone dipropionate, LDSST = low-dose SST, SSST = standard SST (250 mcg))

Study	Method	Outcome
Blair <i>et al</i> (Blair, Lancaster et al. 2013).	Prospective study of 246 children on any ICS for >3 months who all underwent a LDSST.	Peak cortisol was <500 nmol/L in 37.5% and <350 nmol/L in 4.5%.
Paton <i>et al</i> (Paton, Jardine et al. 2006)	Children recorded as receiving more than 500 mcg FP daily underwent LDSST. 192 children met the criteria and had LDSST.	2.8% had flat adrenal responses (peak cortisol <500 nmol/L, increment of <200nmol/L and basal am cortisol <200 nmol/L). 39.6% had impaired responses (peak cortisol <500 nmol/L).
Sim <i>et al</i> (Sim, Griffiths et al. 2003)	Cross-sectional prevalence study. Children with asthma prescribed more than 1000 mcg FP daily for more than six months. 50 children met criteria and had SSST performed if morning serum cortisol < 400 nmol/L.	72% had low (<400nmol/l) morning serum cortisol levels. 17% were found to have AI (12% of the initial cohort) on SSST (peak cortisol <550 nmol/L, <2x increase from baseline).

Eid <i>et al</i> (Eid, Morton et al. 2002)	Observational study. 62 children with asthma on FP followed for 2 years. Morning cortisol performed after approximately 8 months.	36% of children had abnormal early morning cortisol (<150 nmol/L), 17% on low dose FP and 43% taking more than 880 mcg daily.
Fitzgerald <i>et al</i> (Fitzgerald, Van Asperen et al. 1998)	Randomised, double-blind cross-over study comparing budesonide (1500 mcg) and fluticasone (750 mcg) daily for 12 weeks. 24 hour urinary free cortisol at 8+12 weeks and LDSST at 12 weeks.	68% had abnormal results (baseline cortisol <200 nmol/L and peak <500 nmol/L) on LDSST, but no difference between the groups.
Ninan <i>et al</i> (Ninan, Reid et al. 1993)	Children prescribed ICS doses of more than 400µg underwent an am cortisol and SSST. 49 children on budesonide. 28 children on BDP. 23 controls (non-asthmatic children under investigation for short stature).	Mean basal (normal > 200 nmol/L) and stimulated cortisols (normal >430 nmol/L) lower in children on ICS compared with controls. 33% budesonide and 25% BDP groups had suboptimal basal cortisol response. 8% and 10% respectively had abnormal results on SSST.

A systematic review and meta-analysis of placebo-controlled randomised dose-response studies of more than 4 weeks duration in adults examined the data in 732 patients and reported AI in 3.9%, with the odds of abnormality increasing by 1.38 for a 500 mcg/day increase in fluticasone (Masoli, Weatherall et al. 2006). No such study exists in children although some have attempted to identify a dose at which children are at significant increased risk and a cohort study by Vaz *et al* demonstrated 100% absolute risk of HPA-axis suppression in children with asthma taking doses of beclomethasone between 250-600 mcg/m²/day for 6-42 months (Vaz, Senior et al. 1982).

A large prospective study of adrenocortical function in children on ICS recently published reported a dose related suppressive effect, which was more evident in males than females. Basal cortisol levels were seen to increase with age (10 nmol/L with each year of life) and peak cortisol, quantified following stimulation with 1 mcg Synacthen, was lower in boys and as age increased (mean decrease of 6 nmol/L per year of life) (Blair, Lancaster et al. 2013), similar to that found in a study following administration of 250 mcg Synacthen (Lashansky, Saenger et al. 1991).

Adrenal insufficiency with ICS other than fluticasone occurs (Ozbek, Turktas et al. 2006, Raux Demay MC 2006) but fluticasone appears to have higher dose-related AI than other ICS. This is not entirely due to its greater potency as most studies compare therapeutically equivalent doses and is therefore likely to be due to pharmacokinetic properties of fluticasone (Lipworth 1999). Additionally whilst AI with ICS is thought to be a dose related class effect there are idiosyncrasies (delivery device, formulation, adherence, aspects of the patients' disease and genetic factors), causing individuals to be affected to different extents making patient specific prediction of systemic side effects very difficult (Anthracopoulos, Priftis et al. 2008). In children some have found impaired cortisol responses to be more pronounced in children on higher doses of ICS (Nickson, Wilson et al. 2008, Blair, Lancaster et al. 2013), others have reported no correlation between daily dose of ICS and results of a low-dose Short Synacthen Test (LDSST) (Broide, Soferman et al. 1995). Adrenal insufficiency occurs with all the ICS routinely used in the treatment of childhood asthma but prevalence rates and severity vary

depending on the drug, dose, adrenal function test, diagnostic cut-offs employed and the study methodology.

4 Tests of adrenocortical function

There are a number of different diagnostic tests of adrenocortical function, each attempting to emulate “natural” stress enabling physicians to assess whether or not an individual is able to withstand a stressful event or whether corticosteroid replacement is required. There is a fine balance between adequate test sensitivity and specificity, with none of the available tests classifying all patients correctly. The lack of a safe, easy and reliable test of adrenocortical function contributes to a number of controversies: who to test, with which test, at what dose, what times to sample and what diagnostic cut offs to use.

4.1 A summary of the different tests

Investigating the HPA-axis usually requires dynamic testing because of the pulsatile nature of CRH, ACTH and cortisol secretion and thus a high false positive rate when using unprovoked tests. Some physicians measure random cortisol but the peaks and troughs of normal circadian rhythm renders low values difficult to interpret. A very low early morning cortisol (<100 nmol/L) or one that is reassuringly high (>500 nmol/L) may be of help but there is diagnostic uncertainty about values in between. An early morning cortisol was performed in

a study of 32 children with symptoms of AI. In order to achieve 100% sensitivity in children with a normal response to both a low-dose (LDSST) and standard dose SST (SSST) a cut-off of 500 nmol/L was required, but at the expense of specificity which was only 33%, indicating that an early morning cortisol is helpful if high but low values need to be further investigated with a dynamic test (Agwu, Spoudeas et al. 1999). More recently the relationship between time of waking and the upsurge in cortisol has been investigated and cortisol awakening response (CAR) has been suggested as a replacement for 8am cortisol. This may be especially pertinent in children who may have very varied and early times of awakening (Baumler, Kirschbaum et al. 2013).

Similarly early morning paired ACTH and cortisol can be difficult to interpret. It is popular in adult practice, where primary adrenal disease is more common, and the combination of high ACTH with low cortisol may be diagnostic and has even been shown to be more sensitive than SST (Blevins, Shankroff et al. 1994). This is less commonly the case in paediatric practice, where secondary causes of AI predominate.

Serum cortisol profiling (measuring cortisol at 15-30 minute intervals) is time and resource expensive and often impractical in children, as are some of the less invasive alternatives which may be difficult to perform in children and therefore unreliable. Although 24-hour urinary free cortisol has been shown to correlate with the results of the LDSST, it may not discriminate normal from abnormal clearly enough in conditions of hypocortisolaemia (Broide, Soferman et al. 1995)

and is dependent on accurate urine collection, thus generally felt to be impractical in children.

The dynamic tests include the CRH test, overnight metyrapone test (OMT), Insulin Tolerance Test (ITT) and the Short Synacthen Test (SST). The CRH test is rarely performed in children, as it produces unpleasant side effects; is thought by some to produce sub-maximal cortisol responses and is not well standardised between centres (Orth, Jackson et al. 1983, Oelkers 1996). However it has been shown to correlate closely with the ITT (Schlaghecke, Kornely et al. 1992) and has a role in investigation of some pathologies. Metyrapone inhibits the conversion of 11-deoxycortisol to cortisol by blocking adrenal 11 β -hydroxylase and normal HPA-axis feedback mechanisms result in a high 11-deoxycortisol the morning after administration. Although thought of as one of the gold standard tests by some metyrapone can precipitate adrenal crisis, is unpleasant to take and can be difficult to source, as can 11-deoxycortisol assays and is therefore rarely used in paediatric practice.

The gold standard for investigation of the HPA response to stress is the ITT, which evaluates the cortisol response to hypoglycaemia, causing central activation and therefore examining the entire axis (*figure 1.2*). It gained credence when it was seen to correlate closely with the adrenal response seen in times of surgical stress (Plumpton and Besser 1969). The standard cut-off is a cortisol peak of 500 nmol/L (Grinspoon and Biller 1994), although some advocate 550 nmol/L (Arlt 2008) as cases of clinical AI have been missed. Although the it is still

performed in adults and a few paediatric centres, most have rejected it as unpleasant and dangerous as it has been responsible for a number of deaths (Shah, Stanhope et al. 1992). No test of adrenal function is perfect and, despite being considered a standard, healthy controls have been known to fail both the OMT and ITT and their adrenal crisis predictive accuracy has not been established (Kazlauskaite, Evans et al. 2008). The average inter-subject variability in peak cortisol for the ITT has been reported as 8-12%, although others have found it to be as much as 42% (Vestergaard, Hoeck et al. 1997, Pfeifer, Kanc et al. 2001).

The standard and most widely used investigation of adrenocortical function in paediatric practice is the SST. This involves i.v (or i.m) administration of the ACTH analogue, Synacthen, and the subsequent measurement of plasma cortisol levels, usually at 0, 30 and 60 minutes. Most physicians look both for an incremental rise from baseline (usually more than 200nmol/l) and a peak cortisol of more than 500nmol/L or 550nmol/L or 580nmol/L, although some advocate higher levels (Grinspoon and Biller 1994, Bangar and Clayton 1998, Abdu, Elhadd et al. 1999, Arlt 2008). In children two mainstream paediatric endocrinology textbooks advocate sampling at 0 and 60 minutes, following 250 mcg i.v Synacthen, and consider a cortisol response of over 830 nmol/l in the pre-pubertal and 690 nmol/l in pubertal children as normal (Miller, Achermann et al. 2008, Miller 2009). More recently, as cortisol assay specificity has improved, there has been a call to reduce the diagnostic cut-off and determine local

reference ranges to avoid excess false positive results (Blair, Lancaster et al. 2013, Chitale, Musonda et al. 2013, Elder, Somerset et al. 2013).

Although, as a foreign protein, Synacthen is potentially anaphylactogenic it rarely causes any adverse reactions (*vide infra, chapter 1, section 4.2.2*) and is therefore widely regarded as a safe and reliable way to assess adrenal function.

In primary AI the adrenal cortex is already maximally stimulated and as such the administration of an exogenous ACTH analogue does not usually provoke a further rise in glucocorticoid production. In secondary AI the adrenal gland is usually atrophic and unable to synthesis and release adequate cortisol (*figure 1.5*). Appropriate application of these tests is important; an SST performed too early for the assessment of acute onset secondary AI, e.g. in the first post-operative week after pituitary damage or resection, will not have allowed sufficient time for diminished endogenous ACTH to have reduced the responsiveness of the *zona fasciculata* (Thaler and Blevins 1998, Nieman 2003). It is thought that 8-12 days of reduced ACTH action on the adrenal gland is enough to detect changes in responsiveness of the cortex (Hjortrup, Kehlet et al. 1983, Kehlet, Lindholm et al. 1984).

4.2 Tetracosactide (Tetracosactrin, Synacthen™ Cosyntropin™)

4.2.1 Chemistry, pharmacology and mechanism of action of Synacthen

Synacthen is a linear 24-amino acid peptide with a free amino group at the N-terminus and a free carboxyl group at the C-terminus. The counter ion³ acetate is bound in ionic form to the basic groups of the peptide molecule. All amino acid residues except the achiral glycine are in the L-configuration.

Chemical name:

L-Seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophanyl-glycyl-L-lysyl-L-prolyl-L-valyl-glycyl-L-lysyl-L-lysyl-L-arginyl-L-arginyl-L-prolyl-L-valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline, acetate salt

Abbreviated chemical name:

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH, acetate salt

The 1-24 amino acid component of endogenous ACTH(1-39) confers the same biological activity on Synacthen but does not contain the remaining immunologically active 15 amino acids of endogenous human ACTH. Its biological activity is associated with the N-terminal 1-18 amino acid sequence and the immunogenic portion of the protein resides in the N25-33 positions, the sequences involved in species differences. Anaphylactic reactions to porcine, bovine, ovine and human ACTH were fairly common until the advent of synthetic ACTH and the removal of the immunogenic portion of the molecule in the early

³ Counter ion is the accompanying ion e.g. acetate in order to allow maintenance of electric neutrality.

1960s. The C-terminal amino acid sequence 19-39 additionally affects the half-life, with native ACTH having the longest, accounting for the different half-lives for the ACTH analogues (Alia, Villabona et al. 2006). Its synthetic manufacture also allowed precise amounts to be administered by weight and studies investigating the required amount for minimal biological activity and maximal secretion of cortisol from the adrenal gland following administration of ACTH.

Two forms of Synacthen are marketed, an i.v form for diagnostic purposes and a depot i.m form for therapeutic purposes and occasional diagnostic use. Following i.v administration Synacthen exhibits triphasic pharmacokinetics. The first phase is rapid elimination from plasma and distribution to the adrenal glands and kidneys (half-life approximately 7 minutes). It then undergoes a process of metabolism into free amino acids by endopeptidases and aminopeptidases (half-life approximately 37 minutes) and is finally excreted in the urine (half life approximately 3 hours). Almost all (95-100%) of the i.v dose is excreted within 24 hours. Synacthen has a volume of distribution of 0.4 L/kg and a log p value⁴ of -7.9 (Alliance Pharmaceuticals 2011).

As would be expected Synacthen's mechanism of action mimics that of ACTH(1-39), binding to its receptor on the plasma membrane of adrenocortical cells and activating adenylate cyclase to increase cAMP levels thus promoting the synthesis of corticosteroids from cholesterol (Alliance Pharmaceuticals 2011).

⁴ Log p value describes the partition coefficient, a measure of lipophilia

4.2.2 Synacthen safety

Synacthen can rarely cause serious allergic reactions including anaphylaxis. Contraindications include allergic conditions (including asthma), psychosis, peptic ulcer, Cushing's syndrome and primary AI. Safety information was sourced from the company with the marketing authorisation for Synacthen in the UK, Alliance Pharmaceuticals Ltd (Wiltshire, UK), and shows serious reactions to be extremely rare.

In the UK from 1965 (release of Synacthen onto the market) to 2008 (43 years) there have been a total of 440 reactions to Synacthen reported to the Committee on Safety of Medicines via the yellow card system: 233 Adverse Drug Reactions (ADRs) and 15 fatalities, 7 from anaphylaxis or anaphylactoid reactions (Gunnberg, Astley et al. 1999, Drug Analysis Print 2008).

The last Periodic Safety Update Report (PSUR), a worldwide perspective on the safety of a medicinal product, for Synacthen covers the 35 years (from Synacthen's launch) to 31st May 2000 during which it was estimated that approximately 25 thousand million ampoules of Synacthen/Synacthen Depot had been distributed worldwide. There were 418 reported cases worldwide of anaphylaxis or anaphylactoid reactions to Synacthen, of which 208 were in asthmatic patients (Novartis 2000).

4.3 Comparison of ITT and SST

Each test of the HPA-axis has those who would advocate its use and those who would not. The ITT has the advantage of mimicking the stress response and therefore with a normal result one can perhaps say with more confidence that a patient will respond appropriately when physiologically stressed. The SST gives information about the incremental rise and peak cortisol achieved when synthetic ACTH acts on the adrenal gland and is as such only a surrogate marker of how an individual may respond to stress.

A number of studies have shown the SST to correlate closely with the ITT (Stewart, Seckl et al. 1988, Abdu, Elhadd et al. 1999), notably in a study of 200 consecutive adult patients with proven or suspected HPA-axis disorders. The 30-minute cortisol level from the SST and peak cortisol in the ITT were significantly correlated ($r=0.83$) (Lindholm and Kehlet 1987). Others have found a close correlation in both healthy subjects and those with established HPA-axis disease, although some discrepancies occur dependent on the serum cortisol cut-offs employed (Stewart, Seckl et al. 1988, Hurel, Thompson et al. 1996). Abdu *et al* compared the standard (250 mcg) SST (SSST), LDSST and ITT in 64 adult patients with suspected or confirmed pituitary disease. They found the cortisol peaks in all tests to be highly correlated ($r=0.83$ to 0.89) and calculated the sensitivities and specificities of the tests with cortisol cut offs of 500 and 600 nmol/L (*vide infra, chapter 1, section 4.4, table 1.5*) (Abdu, Elhadd et al. 1999). There are a number of reported cases of individuals passing one test while failing the other

(Borst, Michenfelder et al. 1982, Cunningham, Moore et al. 1983, Stewart, Seckl et al. 1988, Ambrosi, Barbetta et al. 1998, Abdu, Elhadd et al. 1999).

Surveys of the SST in 1988 and 1994 reported 25% and 69% of adult endocrinologists using it respectively (Stewart, Seckl et al. 1988, Davies and Howlett 1996). It is more popular amongst UK paediatricians, with 100% usage, as it was felt by most, whilst acknowledging its flaws, to be the most appropriate investigation of adrenal function, safer and more pleasant than alternatives (Elder, Sachdev et al. 2012).

4.4 Comparison of the SSST (standard dose SST) and LDSST (low dose SST)

The relationship between the administration of ACTH and maximal cortisol response was first described as a test of adrenal function in 1963, using porcine ACTH (Ney, Nicholson et al. 1963). In 1965 Wood *et al* described the use of ACTH(1-24) in a rapid test of adrenal sufficiency (Wood, Franklan et al. 1965). The standard SST administers 250 mcg, an empirical equivalent to 25 IU of purified porcine ACTH historical utilised (Daidoh, Morita et al. 1995). Although 250 mcg is more than adequate to assess the maximum secretory capacity of the adrenal gland, it may induce ongoing ACTH receptor stimulation and glucocorticoid synthesis, and as such its ability to detect subclinical hypoadrenalism has been questioned (Alia, Villabona et al. 2006).

Despite two of the early studies showing the effectiveness of a low dose of ACTH (Landon, James et al. 1967, Leclercq, Bruno et al. 1972) it was not until the early 1990s that interest shifted to enhancing the potential sensitivity of the SSST by using a lower dose of Synacthen. It had been widely recognised that 250 mcg represented a supraphysiological, pharmacological dose and that lower, more physiological, doses were capable of maximally stimulating the adrenal gland (Graybeal and Fang 1985). Dickstein *et al* were the first to describe a LDSST. They reported that 5 mcg Synacthen was sufficient to achieve maximal adrenal secretion in adults and found no difference in cortisol levels obtained at 30 and 60 minutes for the 250 mcg and 5 mcg tests. The results with 1 mcg were the same at 30 minutes but significantly lower at 60 minutes leading them to conclude that the 1 mcg test offers a more sensitive alternative (Dickstein, Shechner et al. 1991).

Numerous studies have demonstrated in normal healthy adults that the cortisol response at 30 minutes is not significantly different between the SSST and the LDSST, but that the cortisol rises further by 60 minutes in the SSST, where it falls in the LDSST (Dickstein, Shechner et al. 1991, Krishnan, Miller et al. 1993, Daidoh, Morita et al. 1995, Mayenknecht, Diederich et al. 1998, Nye, Grice et al. 1999, Alia, Villabona et al. 2006). Crowley *et al* studied the dose-response relationship between Synacthen and cortisol at different low doses of Synacthen and concluded that 500 ng/1.73m² was sufficient to elicit a rise in cortisol of more than 200 nmol/L (Crowley, Hindmarsh et al. 1991). Further studies have confirmed that 1 mcg of Synacthen is sufficient to produce a maximal, but not

supraphysiological, cortisol response (Daidoh, Morita et al. 1995, Darmon, Dadoun et al. 1999, Nye, Grice et al. 1999) and others have confirmed its reproducibility (Bridges, Hindmarsh et al. 1998, Park, Park et al. 1999, Gupta, Cheetham et al. 2009).

Demonstration of the discriminatory ability of the tests in patients with AI is important when validating their clinical usefulness, as physicians want to avoid false negatives and reduce false positives where possible. Advocates of the LDSST are concerned that the SSST may falsely reassure by over-stimulating partially atrophied adrenal glands, where the reduced serum ACTH levels associated with AI may be sufficient to enable the adrenal cortex to respond to supraphysiological amounts of ACTH, but this does not mimic the physiological stress situation (Streeten 1999). A study of 32 symptomatic children, mostly with hypothalamic-pituitary damage from irradiation, compared their responses to SSST and LDSST. There were eight discrepancies, all “failing” the LDSST but “passing” the SSST, three of whom reported amelioration of their AI symptoms on commencing glucocorticoid replacement (Agwu, Spoudeas et al. 1999). A number of other studies, mainly in adults, have concluded that the LDSST is more sensitive compared with the SSST, detecting subtle degrees of AI missed by the supraphysiological dosing in the SSST (Broide, Soferman et al. 1995, Rasmuson, Olsson et al. 1996, Gonzalez-Gonzalez, De la Garza-Hernandez et al. 1998, Abdu, Elhadd et al. 1999, Agwu, Spoudeas et al. 1999, Tordjman, Jaffe et al. 2000).

The corollary is argued by other authors believing that the increased sensitivity may reflect false positive cases (Oelkers 1998, Stewart and Clark 1999, Mushtaq, Shakur et al. 2008) and some advocate a reduction in cortisol cut off (350 nmol/L) in children undergoing a LDSST on ICS (Blair, Lancaster et al. 2013). A follow up study of 148 patients with low-normal or borderline results helped allay fears that the SSST lacked sensitivity as only two subsequently developed AI (Agha, Tomlinson et al. 2006).

The relative sensitivities and specificities of the two doses have been compared (*table 1.5*). In one study both tests were reported to have excellent sensitivity (100%), but the specificity of the LDSST to be greater (93.3% compared to 90%) (Abdu, Elhadd et al. 1999). In another study the sensitivity of the SSST was seen to improve with higher 30 minute cortisol cut-offs: 18.8% at 500 nmol/L, 75% at 605 nmol/L and 93.7% at 830 nmol/L (Tordjman, Jaffe et al. 2000) (*table 1.5*). A meta-analysis examined 346 subjects undergoing the SSST and 589 undergoing the LDSST. It was reported that a cortisol of less than 440 nmol/L was predictive of AI for both tests and a cortisol value of over 833 nmol/L for the SSST and 600 nmol/L for the LDSST was highly predictive of a normal HPA-axis (Kazlauskaitė, Evans et al. 2008). These data have resulted in some advocating a higher diagnostic cut off for the SSST (Hurel, Thompson et al. 1996, Abdu, Elhadd et al. 1999, Tordjman, Jaffe et al. 2000, Kazlauskaitė, Evans et al. 2008).

Table 1.5: Sensitivities and specificities of the SSST and LDSST, using ITT or OMT as gold standard, reported by Abdu et al. N=42 and Tordjman et al. N= (Abdu, Elhadd et al. 1999; (Tordjman, Jaffe et al. 2000)

	Sensitivity*	Specificity*	Sensitivity**	Specificity**
SSST (cortisol cut off 500 nmol/L)	100%	90%	18.8%	92.2%
SSST (cortisol cut off 600 nmol/L)	100%	77%	75%	78.1%
LDSST (cortisol cut off 500 nmol/L)	100%	93.3%	94.7%	90%
LDSST (cortisol cut off 600 nmol/L)	100%	80%	-	-

*Abdu et al (Abdu, Elhadd et al. 1999) ** Tordjman et al (Tordjman, Jaffe et al. 2000)

Since its introduction there has been much debate as to whether the LDSST represents a viable alternative to both the SSST and ITT. Over the last decade three meta-analyses have been published examining the studies that compare the SSST and LDSST (Dorin, Qualls et al. 2003, Kazlauskaitė, Evans et al. 2008, Magnotti and Shimshi 2008). All three looked at English language studies published over the previous 40 years (*table 1.6*).

Table 1.6: Summary of meta-analyses comparing SST and LDSST (Dorin, Qualls et al. 2003, Kazlauskaite, Evans et al. 2008, Magnotti and Shimshi 2008)

	No of studies included	Exclusion criteria	Inclusion criteria
<i>Dorin et al 2003 (Dorin, Qualls et al. 2003)</i>	29	1. Normal controls	1. Primary/secondary AI 2. ≥ 5 patients 3. ITT/OMT control for secondary AI
<i>Kazlauskaite et al 2008 (Kazlauskaite, Evans et al. 2008)</i>	13	1. Normal controls 2. Critically ill patients	1. Primary/secondary AI 2. ≥ 10 patients 3. ITT/OMT for comparison
<i>Magnotti et al 2008 (Magnotti and Shimshi 2008)</i>	7	None	1. Primary/secondary AI

The controversy about which test is best is partly ameliorated by the conclusions of these meta-analyses. All three agree that there is no superiority of the SSST over the LDSST, and two reported the LDSST to be a better test (Kazlauskaite, Evans et al. 2008, Magnotti and Shimshi 2008). Dorin *et al* found no significant differences in the operating characteristic of the two tests but concluded that, due to low sensitivity of the SSTs, for secondary AI a test with hypothalamic stimulation (ITT or OMT) may be superior (Dorin, Qualls et al. 2003). There continue to be several strong proponents of central dynamic tests in adulthood (ITT and OMT) and of the SSST over the LDSST but it appears the evidence is supportive of a widespread adoption of the LDSST.

4.4.1 ACTH and cortisol dose response

There are a number of different ways to examine the most appropriate dose of Synacthen for assessment of the physiological responsiveness of the adrenal gland to stress. One is to compare the plasma ACTH level produced when stressed (hypoglycaemia during the ITT) with that produced in the SSST and LDSST. The few studies that have measured the plasma ACTH achieved with different doses of Synacthen and ITT found the ACTH level following high doses of Synacthen (1 mcg/kg, 250 mcg) to be significantly higher than with the hypoglycaemic insult of the ITT. The Synacthen dose that most closely mimicked the ITT cortisol response was between 1 and 5 mcg (Graybeal and Fang 1985, Darmon, Dadoun et al. 1999, Nye, Grice et al. 1999). The ACTH level required to near-maximally stimulate the adrenal gland has been quantified as between 60-80 pg/ml, slightly higher than a typical morning ACTH level (Oelkers, Boelke et al. 1988, Darmon, Dadoun et al. 1999). This is considerably lower than plasma levels achieved following administration of 250 mcg Synacthen, which generates levels of between 5800-60,000 pg/ml. In the same studies the levels achieved following 1 mcg Synacthen administration varied between 13-1900 pg/ml and are closer to those doses required to maximally stimulate the gland (Mayenknecht, Diederich et al. 1998, Darmon, Dadoun et al. 1999). The variability seen between studies is likely to be in part due to the employment of different assays, not primarily designed for the detection of Synacthen.

In the only pharmacokinetic study to date examining the relationship between Synacthen and cortisol Alia *et al* reported the responses to both SSST and LDSST

in ten normal subjects (five men). Plasma Synacthen levels were higher in SSST (maximum plasma concentration 1144 nmol/L SSST compared with 960 nmol/L LDSST) but took longer to be achieved (time to maximum plasma concentration 75 versus 52.5 minutes) and elimination of Synacthen was significantly slower in the LDSST. They confirmed identical rates of cortisol production between the two tests (Alia, Villabona et al. 2006).

4.4.2 Timing of cortisol sampling

Most studies comparing the SSST and LDSST have demonstrated a similar cortisol response at 30 minutes but divergence thereafter, with increasing cortisol values in the SSST and diminishing serum levels in the LDSST (Dickstein, Shechner et al. 1991, Krishnan, Miller et al. 1993, Daidoh, Morita et al. 1995, Mayenknecht, Diederich et al. 1998, Nye, Grice et al. 1999, Alia, Villabona et al. 2006, Elder, Somerset et al. 2013). Although there is variability, the recommended sampling times are 0, 30 and 60 minutes for the SSST (Arlt and Allolio 2003). Sampling times for the LDSST tend to be more frequent, including earlier samples, and more variable (Elder, Sachdev et al. 2012). Most studies have identified the cortisol peak between 15 and 30 minutes (Crowley, Hindmarsh et al. 1991, Daidoh, Morita et al. 1995, Nye, Grice et al. 1999). Park *et al* examined the peak cortisol attained following 1 mcg Synacthen in 60 subjects (a mixture of healthy volunteers (N=8, 40 tests) and adrenal patients (N=5, 20 tests)). The peak was seen at 20 minutes in 65% and 23% at 30 minutes. They reported 100% sensitivity and specificity with sampling at 20 and 30 minutes (Park, Park et al.

1999). In other studies the peak cortisol following a LDSST would have been missed using just these times and testing at 0, 15, 25 and 35 minutes has been suggested (Paton, Jardine et al. 2006, Blair, Lancaster et al. 2013).

4.5 Age and sex variability of SST

When interpreting the results of children's SSTs the question of whether age, sex or pubertal status have a role in the diagnostic cut offs employed needs to be considered. Lashansky *et al* examined the responses of children to the SSST by sex, age and puberty groupings (<1 year 8 girls (F), 13 boys (M), 1-5 years 8F 14M, 6-12 years 7F 8M, Tanner stage 2-3 11F 10M, Tanner stage 4-5 8F 13M). They found no differences by age group in basal cortisol levels or between males and females but peak cortisol fell with increasing age in boys and similarly in pre-pubertal girls. They reported greater differences in some of the other products of ACTH stimulation (DHEA, androstenedione, 17-hydroxy progesterone and 17-hydroxy progestogen) and state that age and sex specific normal ranges are required, which may be used to aid diagnosis of genetic causes of primary adrenal insufficiency (Lashansky, Saenger et al. 1991). Currently there is no similar work for the LDSST.

4.6 Current limitations of SST

Although the SST is widely used, especially in paediatric practice, it is not without its limitations. All current versions of the SST require cannulation, i.v or i.m

Synacthen and multiple blood samples. It is unpleasant for the child and labour-intensive, requiring skilled personnel and a brief admission. There is a lack of consensus amongst adult physicians and paediatricians about cortisol timings, acceptable cut-offs for cortisol response and the most suitable dose of Synacthen. There is also marked variability in making up the LDSST and a paucity of normative data in children (Barth, Seth et al. 1995, Elder, Sachdev et al. 2012, Blair, Lancaster et al. 2013).

As for any endocrine investigation assay variability must be considered when interpreting results. A study of the SST using four different cortisol immunoassays reported significant differences in the absolute values of the 5th and 95th percentiles between the four methods (Clark, Neylon et al. 1998). In another study three different assay methods were used and significant differences were observed (Nye, Grice et al. 1999). Other authors have followed suit and all call for assay specific normal ranges to be determined for both sexes (Wood 1998, Stewart and Clark 1999, Chitale, Musonda et al. 2013, Elder, Somerset et al. 2013).

5 Nasal drugs

5.1 Non-invasive alternatives to i.v administration

Intravenous administration allows consistent pharmacokinetics to be achieved and avoids barriers to absorption such as first pass metabolism, but is not without its complications. Cannulation may be painful and a difficult practical procedure, especially in children, requiring repeated attempts. It is therefore resource-intensive as usually trained professionals need to undertake the procedure in a hospital setting. Other adverse effects may occur such as displacement of the cannula causing drugs meant for i.v use being accidentally administered in the subcutaneous tissues causing pain, swelling and with certain drugs tissue damage and long term scarring. All these issues are particularly pertinent in children, where even oral drug delivery may not be straightforward.

Attempts to minimise the distress to a child when they are unwell or need to undergo a procedure are undertaken both for the short term good and because painful experiences have been shown to heighten the perception of pain during future encounters (Taddio, Katz et al. 1997). It is to this end that for many years alternative routes of administration, for many different types of drugs, have been trialled. Routes such as i.m and subcutaneous injection may be as painful as i.v and rectal administration is undesirable in older children and adolescents. Many drugs cannot be given orally due to poor absorption, slow onset of action, degradation by the acid environment of the stomach, first pass metabolism or patient factors e.g. vomiting. Alternative transmucosal routes such as buccal,

sublingual, inhalational and intranasal bypass the enteral system and can be effective providing the taste is palatable and pharmacokinetics appropriate. A study comparing intranasal, sublingual and rectal administration of midazolam found all to be acceptable in the 47 children tested, although sublingual was the most effective and well tolerated (Geldner, Hubmann et al. 1997).

5.2 The nasal route of administration

The intranasal route of drug delivery has many advantages. It requires minimal training to administer drugs in a rapid and tolerable way. There is generally good absorption due to the richly vascular nasal mucosa and thereby high bioavailability, with avoidance of first pass metabolism, and a rapid onset of action. Anatomical proximity to the central nervous system makes it ideal for drugs with central sites of action.

The side effects of intranasal drugs are few and are generally attributable to the drug itself rather than the method of delivery. The commonest adverse effects in children are nasal irritation and crying although these are generally short-lived (McGlone, Ranasinghe et al. 1998, Ljungman, Kreuger et al. 2000, Davis and Illum 2003).

5.2.1 Volume restriction due to nasal anatomy

The nasal mucosa (squamous epithelium transitioning to columnar, pseudostratified, ciliated respiratory epithelium more proximally) is highly

vascularised. It covers a small area (the main chamber is about 5 to 8 cm long in an adult) yet its surface area is considerably increased, to 150-200 cm², by the turbinates (inferior, middle and superior). This means nasal drugs must be administered in small volumes, high concentrations and ideally to both nares in an atomised spray to maximise absorption (Goldman 2006). Volumes of 0.2-0.3 ml per nostril are most suitable and volumes upwards of 1 ml will saturate the nasal mucosa and much of the drug will be lost by dripping back down the nasal cavity or down the back of the pharynx (Wolfe and Braude 2010).

5.2.2 Mechanism of absorption

Ideal characteristics of a nasally administered drug include low molecular weight, lipophilic rather than polar molecules and drugs acting on the central nervous system (Davis and Illum 2003). Drugs with a smaller molecular size have a greater likelihood of effective absorption from the nasal mucosa e.g. morphine (molecular weight (MW) 285 g/mol). Drugs with molecular weights of less than 1000 g/mol do not require the addition of an adjuvant for effective absorption (McMartin, Hutchinson et al. 1987). Some of the most widely used classes of nasal drugs are opiates and benzodiazepines as they have many of the ideal properties and achieve high cerebrospinal fluid levels for their central action. They are effectively administered intranasally for rapid onset of action in cases of seizure control, analgesia and anxiolysis (Wolfe and Braude 2010).

Peptide hormones, like Synacthen (MW 2934 g/mol), generally have low bioavailability by the nasal route, however there is a precedent to administering them. Nasal desmopressin (MW 1069 g/mol), has only 10% the effect of i.v at the same dose, nasal calcitonin (MW 3455 g/mol), insulin (MW 5808 g/mol) and gonadotrophin releasing hormone analogues a 1-3% bioavailability (Davis and Illum 2003). Larger peptide molecules are only absorbed in small amounts due to the endocytotic transport required (Jadhav, Gambhire et al. 2007). These factors and rapid removal via the mucociliary clearance system may be overcome by the addition of drug enhancers capable of modifying the transmembrane transport of drugs (Davis and Illum 2003).

The poor membrane permeability seen with large polar peptides usually precludes them from using transcellular transport mechanisms, exploiting simple concentration gradients, or transfer by specific receptors or vesicular transport. There are two transport mechanisms used by larger peptides: paracellular transport, whereby the molecules pass through the tight junctions between cells and endocytotic transport by which the cell engulfs the molecule using vesicle carriers (Jadhav, Gambhire et al. 2007). Although the most sizable barrier to nasal drug delivery is absorption, mucociliary clearance and enzymatic degradation can additionally affect optimal drug plasma levels.

5.2.3 Mucociliary clearance (MCC)

Within the nasal mucosa reside seromucosal glands comprising of mucus cells, which secrete a thick sticky mucus, and serous cells, which secrete a more watery fluid. There are thought to be 100,000 seromucous glands in the adult human nose, producing 1.5 to 2 litres of mucous daily (Ugwoke, Agu et al. 2005). One of the protective functions of the nasal mucus is mucociliary clearance (MCC), the process of mucus entrapment of potentially noxious particles followed by removal from the nasal cavity to the gastrointestinal tract by ciliary action. Average mucociliary transit time in a healthy adult is approximately 15 minutes, not leaving sufficient time for some drugs to be absorbed (Turker, Onur et al. 2004). Nasal formulations that prolong MCC are more likely to give adequate time for paracellular transport, which is passive and therefore slow.

5.3 Nasal Synacthen

Synacthen administered orally is inactivated in the gastrointestinal tract by proteolytic enzymes and therefore it is given either i.v or i.m. A number of historical studies have examined the potential of nasally administered ACTH analogues as a replacement for depot i.m ACTH, which was studied as a regular therapy in a variety of inflammatory conditions, as an alternative to corticosteroid treatment.

5.3.1 History of intranasal ACTH

It was first demonstrated that ACTH could be usefully absorbed through the nasal mucosa in 1952 (Smith, Dickson et al. 1952). McKendry, Schwarz and Hall performed a control study in nine study volunteers two years later using a purified ACTH(1-39) intranasal formulation “rhinacton”, which was observed to cause a rise in the eosinophil count and urinary 17-ketosteroid (surrogate markers used in the absence of a cortisol assay). There were no significant side effects reported. They went on to describe 17 clinical cases in which Rhinacton had been used and all but 3 patients demonstrated improvement. Of particular interest was the only paediatric case, a 3-year old boy with nephrotic syndrome who had previously responded well to i.m ACTH. He received 40 IU (international units) of intranasal ACTH twice daily for 8 days and once again remitted (McKendry, Schwarz et al. 1954). Although of historical interest, these studies are hard to interpret due to their lack of statistical analysis and use of surrogate markers as endpoints.

By the 1960s significantly more was known about the ACTH molecule. Its amino acid sequence had been elucidated and its biological activity was known to reside in its N-terminus. Work on intranasal ACTH from the late 1960s onwards focused on investigating the properties of modified forms with a view to enhancing its clinical applicability. A number of groups have examined the superior plasma levels and duration of action of analogues such as ACTH(1-18) and ACTH(1-17) over ACTH(1-24), desirable properties in a therapeutic setting but not for the purpose of a one off diagnostic test (Felber, Aubert et al. 1969, Keenan,

Thompson et al. 1971, Baumann, Walser et al. 1976, Adelman, Graef et al. 1984). The short duration of the corticotrophic activity of ACTH(1-24) has limited its therapeutic use and may explain the paucity of research.

The first record of intranasal Synacthen (ACTH(1-24)) was in 1969 when two doses (0.25mg and 1.5mg) were insufflated nasally in six healthy volunteers and fluorogenic corticosteroids measured for five hours afterwards. Both doses resulted in a significant adrenal response at 60 minutes but the effect was maintained for longer in the higher dose. They concluded that this had both diagnostic and therapeutic potential, but no further investigation appears to have taken place for several decades (Keenan and Chamberlain 1969).

The late 1970s saw renewed interest in intranasal administration as this route was already being used for a number of other drugs. Jeffcoate *et al*, during an extensive study, principally comparing ACTH(1-18) with ACTH(1-24), gave 11 healthy volunteers either 1 mg or 5 mg of intranasal ACTH(1-24). There was no significant difference between the two doses, although the individual variation in corticosteroid response was reduced with the higher dose. A peak concentration of ACTH was obtained at 30 minutes and returned to baseline at approximately 2 hours. The corresponding corticosteroid response peaked at 2 hours and was almost back at baseline at 8 hours. Levels of ACTH(1-24) were lower and less well sustained compared with ACTH(1-18) despite this the rise in corticosteroids was almost equivalent. The plasma levels of ACTH were shown to be lower when compared with both the i.v and subcutaneous routes. Of note, three subjects

had undetectable levels of ACTH(1-18) yet still mounted a comparable plasma steroid response with those with higher plasma titres. (Jeffcoate, Phenekos et al. 1977). This may have been attributable to the ACTH assay employed or indicate that relatively small doses of ACTH may be required to generate a suitable adrenal response.

The different pharmacological properties of the shorter ACTH analogues and ACTH(1-24) are thought to be due to proteolytic enzyme activity within the circulation rapidly destroying the steroidogenic potency of ACTH(1-24), but not the shorter analogues. This was demonstrated *in vivo* by a clear dissociation between the bioactive and immunoreactive levels of ACTH(1-24) (Jeffcoate, Phenekos et al. 1977).

In this millennium a Japanese group looked more closely at the effect of intranasal ACTH(1-24) on adrenocortical hormones (Hiroi, Ichijo et al. 2002) and catecholamine secretion (Hiroi, Ichijo et al. 2004) and went on to successfully treat an adult patient with isolated ACTH deficiency (Hiroi, Ichijo et al. 2004). Using 250 mcg ACTH(1-24) both i.v and intranasally (i.n), with saline as a control, they demonstrated that cortisol, aldosterone, DHEA, adrenaline and noradrenaline all rose significantly but DHEA-S (DHEA-sulphate) and dopamine did not. The levels of cortisol and aldosterone were lower following i.n administration than with i.v. As seen in the Jeffcoate *et al* study the cortisol rise peaked at 30 minutes and returned to baseline by 2 hours (Hiroi, Ichijo et al. 2002, Yoshida-Hiroi, Tsuchida et al. 2005).

A pertinent animal study examined nasal absorption in rats and reported that bioavailability was low compared with i.m administration (4.4% compared with 24%) and time to reach peak concentrations (T_{max}) was 10-15 minutes using the i.n route compared with 4 minutes i.m. Two different enhancers were trialled and both significantly changed the pharmacodynamics. Sodium glycocholate decreased the T_{max} and absorption was increased threefold, but this effect was relatively short lived. Bacitracin increased the bioavailability of i.n to i.m levels (Wuthrich, Martenet et al. 1994).

These studies looked at the use of intranasal ACTH from the perspective of discovering an analogue with the pharmacological properties to allow its long-term use in chronic conditions. These are not the same properties required for the SST, where only a single dose is needed, and there are no studies to date looking at the use of nasal Synacthen either in the childhood population or as a one-off diagnostic test. Previous research, though limited, has shown nasal Synacthen to be well tolerated and there have been no significant safety concerns reported.

5.4 Nasal drug enhancers

Nasal absorption enhancers may alter the physiochemical properties of a drug e.g. alter the drug solubility, or exert their effects on the nasal mucosal membrane (Turker, Onur et al. 2004). The addition of formulation additives to

enhance drug absorption offers the opportunity for improved bioavailability without changing the physiochemical properties of the drug and is therefore more commonly employed (Davis and Illum 2003).

5.4.1 Ideal properties of a nasal drug enhancer

A nasal drug enhancer should ideally improve bioavailability but without side effects or change to the drug mechanism of action. It should therefore be pharmacologically inert and non-allergenic, tasteless, odourless and have a transient and reversible effect. It should transport the drug molecule from the apical to basolateral surface of the cell without harming or disrupting the membrane, should improve the mean residence time of the drug (increase the time the drug is in contact with the mucosal surface for maximum absorption) and preferably not be absorbed (although if it is it should be easily and safely metabolised and excreted). The mechanism of action of the promoter should be understood and preferably be enhancing an existing process. Few absorption promoters fulfil all these attributes, although chitosan (*vide infra section 5.4.3*) appears to satisfy them with the additional advantage of being readily available and inexpensive (Davis and Illum 2003).

5.4.2 Types of nasal drug enhancer

There are a number of different nasal drug enhancers that have been investigated in animal models and some in humans. The main classes of which are briefly outlined below (Davis and Illum 2003, Turker, Onur et al. 2004).

- *Microspheres* – e.g. starch or dextran
 - form a gel-like layer when water is absorbed increasing the drug residence time in the nasal mucosa.
- *Liposomes*
 - Phospholipid vesicles composed of lipid bilayer and thus act as drug carriers across the nasal mucosa.
- *Gels* e.g. chitin or chitosan
 - act to slow MCC thereby increasing drug residence time in the nasal mucosa and may facilitate paracellular transport.
- *Cyclodextrins*
 - appear to both affect paracellular transport and have a solubilising effect on some proteins e.g. insulin.
- *Fusidic acid derivatives* e.g. tauro-24, 25-dihydrofusidate (STDHF)
 - act as enzyme inhibitors to slow degradation and increase time for absorption.
- *Phospholipids* - e.g. phosphatidylcholines
 - reside in cell membranes and therefore facilitate cell entry of polar drug molecules.
- *Bile salts and surfactants* – e.g. sodium glycocholate
 - disrupt paracellular and transcellular pathways but in doing so are irritant and can cause mucosal damage.

5.4.3 Chitosan

The term chitosan describes a series of chitosan polymers with differing molecular weights and degrees of deacetylation. Chitosan is a polysaccharide comprising copolymers of glucosamine and N-acetyl-glucosamine and is found in fungi but is more commonly derived by partial deacetylation of chitin from crustacea (i.e. crab and shrimp shells as a by-product of seafood processing) (Davis and Illum 2003).

Commercially, chitosan is made by alkaline *N*-deacetylation of chitin derived from crustacea, due to their abundance. Chitin is a linear polysaccharide consisting of β -(1 \rightarrow 4)-linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-D-glucopyranose (GlcN), chitosan consists nearly entirely of GlcNAc. The heterogeneous deacetylation process, combined with removal of insoluble compound, results in a chitosan product that possesses a random distribution of GlcNAc and GlcN units along the polymer chain. Chitosan is unusual in that it is positively charged at neutral and acidic pH. It is a cationic biopolymer and forms salts with inorganic and organic acids. The dissolution of the soluble polysaccharide results in a positive charge allowing it to demonstrate its bioadhesive, film-forming and gelation properties. Due to its high molecular weight chitosan is not systemically absorbed to any significant degree, greatly minimising its risk of systemic toxicity (Personal communication, Peter Watts, Archimedes Development Ltd). As a group of compounds chitosan may exhibit a range of molecular weights and different salt forms: glutamate (the most common), chloride and lactate. Chitosan displays considerable versatility such

that by altering the degree of deacetylation and the pH or ionic strength of the formulation the solubility and pKa (acid dissociation constant) can be changed (Davis and Illum 2003). The functional properties of chitosan are primarily determined by the degree of deacetylation and molecular weight.

These properties render it of interest for several applications. Chitosan is extensively used in industry as a clarification agent in beverages (fruit juice and beer), a fungicide for the protection of crops and coating apples, a food constituent (especially in Japan), and a nutritional supplement and in the cosmetic, dental and ophthalmic industries. Chitosan is used as a dietary supplement in preparations for weight loss, obesity and hypercholesterolaemia (Illum, Farraj et al. 1994). Biomedically, chitosan is used in bandages and wound dressings and is being explored for applications in tissue engineering (Muzzarelli 2009). Pharmaceutical applications of chitosan which are under investigation include an excipient in tablets, controlled release formulations and gels, an agent for gene delivery, an enhancer of transmucosal drug absorption, and a vaccine adjuvant for nasal vaccine delivery (Saranya, Moorthi et al. 2011, Jabbal-Gill, Watts et al. 2012).

5.4.3.1 Mechanisms of action

Chitosan differs from other enhancers by not exerting an active change on the nasal mucosal membrane, not being absorbed and not being a surfactant-derived material. The nasal mucosa does not absorb hydrophilic compounds,

which generally cannot diffuse across the lipid bilayer, that are larger than the intercellular tight junctions (zona occludens, ZO). It is thought that polar compounds pass through when chitosan facilitates the bioadhesion to and transient (30 to 45 minutes) opening of the membrane's tight junctions (Illum 1998, Dodane, Khan et al. 1999). A model cell membrane has allowed investigation of this process and it appears the chitosan's positive charge enables it to alter the cytoskeleton protein, F-actin, from a filamentous to a globular structure and decrease the ZO-1 proteins, both vital to the opening mechanism of the tight junctions. This alteration is believed to effect ion transport activating intracellular secondary messenger systems and triggering a cascade of events culminating in increased tight junction permeability (Dodane, Khan et al. 1999, Davis and Illum 2003). Additionally there appears from human *in vitro* studies that chitosan also slows mucociliary clearance by three to six fold, using its mucoadhesive properties (Soane, Frier et al. 1999).

5.4.3.2 Chitosan glutamate (PROTASAN™ UP G 213)

Chitosan glutamate is chitosan which has been converted into the glutamate salt form by dissolving chitosan in glutamic acid. Glutamic acid is present at a stoichiometric⁵ amount to the number of GlcN units. Chitosan glutamate has been demonstrated in numerous pre-clinical and clinical studies (phase I-II) to enhance the intranasal delivery characteristics of a wide range of drug compounds and as an excipient in intranasal vaccine formulations. The

⁵ Stoichiometric – quantitative relationship between products and reaction in a chemical substance

PROTASAN grades of chitosan manufactured by FMC BioPolymer (Philadelphia, PA, USA) are characterized by the molecular weight (also measured as viscosity) and the ratio of glucosamine to *N*-acetyl glucosamine (degree of deacetylation). A chitosan glutamate product having a high viscosity and a medium degree of deacetylation would be described by the product name PROTASAN UP G 213 (Personal communication, Peter Watts, Archimedes Development Ltd).

Chitosan glutamate (PROTASAN™ UP G 213) was not, at the time of writing, licensed for use in any pharmaceutical products in Europe. However, it had been extensively evaluated in pre-clinical studies and in human clinical trials in Europe and USA. Intranasal chitosan glutamate-containing products evaluated in humans have included influenza vaccine (Read, Naylor et al. 2005), norovirus vaccine (Atmar, Bernstein et al. 2011) and morphine (Christensen, Cohen et al. 2008).

6 Salivary steroids

The delivery of nasal Synacthen for diagnostic purposes is novel, as is the addition of a nasal drug enhancer, however the second part of a non-invasive SST, the measurement of salivary cortisol, is better established. Salivary cortisol has been shown to have excellent correlation with serum levels (Vining, McGinley et al. 1983) and only the biologically active part of the molecule is measured, thus reducing some variability. Salivary sampling is painless, therefore

less stressful, and relatively inexpensive, as it does not require a hospital setting (Wood 2009). Steroids are not appreciably metabolised in saliva, unlike compounds excreted by the kidney, causing some to advocate salivary steroid hormone analysis over that of urine determination (Groschl 2008). Much of the literature pertaining to salivary cortisol comes from the psychological sciences but increasingly it is being recognised as having a role in clinical endocrinology, particularly in the diagnosis of Cushing's syndrome where it is now recommended as a first-line diagnostic test (Nieman, Biller et al. 2008). Although it is yet to assume widespread adoption in the assessment of hypercortisolism, in part due to the laboratory set up required (Wood 2009), it is in the assessment of hypocortisolism where a paucity of research exists and its clinical application is, as yet, untested.

6.1 Transfer, metabolism and detection of hormones in saliva

6.1.1 Transfer of steroids into saliva

The parotid, sublingual and submaxillary glands all produce saliva, although the character differs between the glands due to the relative abundance of serous or mucinous acinar cells. The parotid glands produce a watery saliva; sublingual a mucousy secretion and submaxillary a combination of the two. Sodium is actively pumped into the acinar end of the glands with water following by osmosis through the tight junctions of the acinar cells. Sodium is then pumped back into the plasma, as saliva travels down the duct, resulting in a hypotonic solution but

a variable sodium concentration as the ductal pumps are flow rate-dependent (Wood 2009).

The salivary acinar cell membrane is a lipid layer and lipophilic molecules such as unconjugated steroids diffuse into the saliva much more readily than hydrophilic molecules like peptides (*figure 1.6*). The majority of steroids are protein bound in serum, with a relatively small, biologically active, free fraction. It is only the free, unconjugated steroids that enter the saliva by diffusion. The alternative passage into the saliva for small, non-protein bound, polar steroids is ultrafiltration, whereby molecules with a weight of less than 1900 g/mol are able to pass with water through the acinar tight junctions (Wood 2009). This is the only entry route for conjugated steroids e.g. DHEA and they are found in very small fractions of their unbound serum concentrations (1%), which falls further at high salivary flow rates. Unconjugated steroids, like cortisol, are present in saliva at equivalent levels to their free fraction in serum (10%) and are unaffected by salivary flow rates (Vining, McGinley et al. 1983).

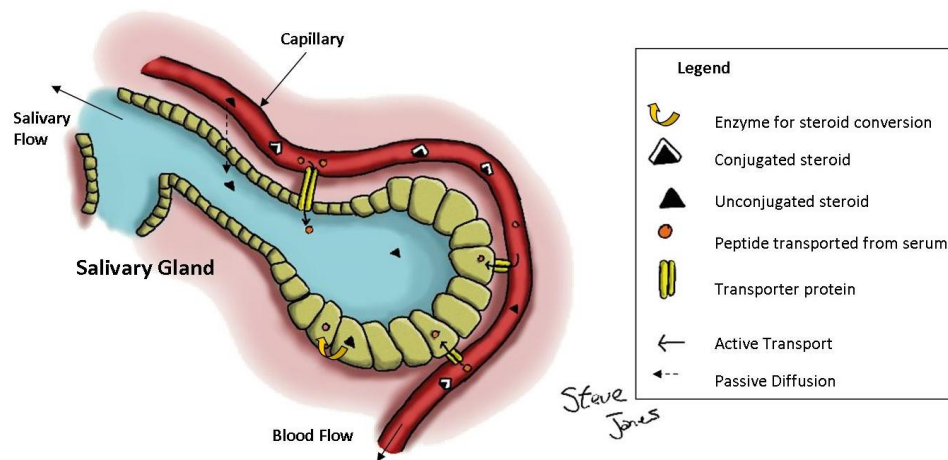


Figure 1.6: Schematic diagram of salivary gland showing transport of molecules into and out of saliva. Kindly drawn on the instructions of author by Steve Jones, research assistant, SCH.

6.1.2 Metabolism of steroids in saliva

The correlation between serum free steroids and salivary values is generally close, however salivary gland metabolism may account for some of the variance. Progesterone, oestrogen, testosterone and androstenedione have all been shown to be metabolised to a greater or lesser extent in saliva but cortisol and its relationship with cortisone is the best-known example. The enzyme which converts cortisol to cortisone, 11β -hydroxysteroid dehydrogenase type 2, (11β -HSD-2), is present in salivary glands and is responsible for the large difference in

the proportion of salivary cortisol: cortisone compared with that in serum (salivary 1:4, serum 8:1 respectively) (Wood 2009).

6.1.3 Analytical methods for quantification of salivary steroids

Immunological methods for determination of salivary hormones have the advantages of requiring small sample sizes, being easy to use and sensitive, however their specificity has prompted an increasing popularity of chromatographic methods; high pressure liquid chromatography (HPLC) or liquid chromatography with tandem mass spectrometric (LC-MS/MS) detection (Baid, Sinaii et al. 2007). Immunoassays, despite their flaws, are still widely employed, most commonly enzyme immunoassays, luminescence and fluorescence assays. Clinical chemists warn of the problems if appropriate extraction and prepurification are not carried out in the preparation phase, techniques which enhance the reliability of the chromatographic techniques (Turpeinen and Hamalainen 2013). Liquid chromatography with tandem mass spectrometry overcomes many of the problems of immunoassay and allows the analysis of a complete profile of salivary steroids in one sample (Groschl 2008). At the time of writing there is no established External Quality Assurance Scheme for salivary steroids but it is hoped that one will be introduced as their popularity and clinical applications grow (Inder, Dimeski et al. 2012).

6.1.4 Saliva collection methods and storage.

Oral saliva is a mixture of secretions from the three main pairs of salivary glands, the minor glands, desquamated epithelial cells, food debris, microorganisms and possible blood from minor oral abrasions. Invasive collection methods exist: suction and cannulation of the ducts, but most look to saliva to access information about blood levels of molecules by a non-invasive method. Published methods include spitting or drooling (active or passive) into a specialised tube, chewing absorbent cotton or polyester and placing cotton wads, pads or eyespears (similar to a specialised cotton wool bud but with absorbent filter paper tip) between the lower buccal membrane and cheek or under the tongue until saturated. The methods involving cotton wool rolls/wads require salivary extraction by centrifugation and concerns have previously been raised regarding the spuriously high levels of salivary cortisol measured when using the cotton or polyester swabs (Shirtcliff, Granger et al. 2001, Kidd, Midgley et al. 2009). Blood, not visible to the naked eye, can be present for 30-minutes after tooth brushing and therefore it is recommended that saliva samples should not be collected within 30 minutes of eating, drinking, chewing flavoured gum, brushing teeth or using dental floss (Wood 2009).

Different patient groups present different challenges when trying to obtain saliva; from the social unacceptability of spitting for the elderly (Nguyen and Wong 2006), to the small size of the mouth in premature infants (Ng, Drury et al. 2013) and the non-compliance of toddlers. A study using marshmallows demonstrated improved procedural adherence in young children, without

cortisol assay interference (Clements, Parker et al. 2007). Detection methods requiring small volumes of saliva can help overcome the problem of obtaining adequate volumes for analysis. Lingual citric acid can increase the amount of saliva produced five to ten fold but has been shown to interfere with immunoassays (Gallagher, Leitch et al. 2006).

Patient preference, ease of collection, volumes of saliva collected and effect on biochemical analysis have all been studied and findings have been varied, on balance favouring absorbent cotton methods (Strazdins, Meyerkort et al. 2005, Groschl, Kohler et al. 2008). Work carried out in the Department of Endocrinology at SCH found the drool technique to be preferred to the chewing of absorbent cotton by both study participants and researchers due to the larger volumes of saliva collected and the closer correlation with serum cortisol (Nickson, Wilson et al. 2008).

Salivary steroids are generally stable and withstand time at room temperature and frozen storage (Clements and Parker 1998). Bacterial growth can cause deterioration in sample quality if stored at room temperature for a week without prior centrifugation (Groschl, Wagner et al. 2001). In the study carried out in the Department of Endocrinology at SCH samples were centrifuged and frozen (N=10) or centrifuged and refrigerated (N=10) or returned to the lab by 2nd class post then centrifuged and frozen (N=10). After one week samples were thawed and analysed. No significant effect on cortisol concentration was seen with any storage method (Nickson, Wilson et al. 2008). Recommended storage for salivary

samples is a month at 4°C and three months at -20 °C, although longer times have been shown to be tolerated for centrifuged samples. Freezing is necessary for long term storage (Wood 2009).

6.2 Salivary cortisol

Lipophilic, unconjugated steroids, such as free, non-protein bound cortisol, pass relatively easily from capillaries to the acinar cells of the salivary glands along a concentration gradient by a method of passive diffusion (*figure 1.6*). Salivary cortisol therefore reflects the unbound, free and biologically active fraction of total serum cortisol, however, due to metabolism within the glands, salivary cortisol concentrations are about half of the corresponding serum free cortisol value (Wood 2009). Passive diffusion results in a stable relationship between serum and salivary cortisol, uninfluenced by salivary flow rate (Wood 2009).

Measuring serum free cortisol requires numerous steps, complex laboratory techniques and considerable time, making it impractical (Perogamvros, Keevil et al. 2010). The disadvantages of measuring serum total cortisol include: reflecting less than 5% of the biological activity of cortisol; changes in levels and binding affinity of CBG in different situations altering the free and biologically active fractions but not the total, and an underestimation of the free cortisol seen in stress when CBG becomes saturated (Perogamvros, Keevil et al. 2010). Measuring salivary cortisol avoids these biochemical pitfalls and as a non-invasive, pain-free alternative would enable diagnosis and monitoring to occur in

the community setting. Additionally it has been shown that, due to the passive diffusion, the salivary concentration of cortisol will reflect that of the serum within minutes making it an ideal method to measure adrenal reserve and responsiveness (Vining, McGinley et al. 1983).

6.2.1 Relationship of serum and salivary cortisol

Cortisol is the best studied of the salivary steroids and appears to have most clinical applicability due to its reliable relationship with serum total and free cortisol levels. Published correlations have demonstrated r values of between 0.85 and 0.97 (Riad-fahmy, Read et al. 1982, Vining, McGinley et al. 1983, Luthold, Marcondes et al. 1985, Gozansky, Lynn et al. 2005, Gallagher, Leitch et al. 2006, Perogamvros, Owen et al. 2010). It is important to note however that correlations are not a measure of relationship and that as these studies are measuring related compounds they would be expected to demonstrate a high degree of correlation. The relationship between total serum cortisol and salivary cortisol has been described as biphasic, with a slow initial phase until Cortisol Binding Globulin (CBG) binding capacity is exceeded (approximately 500 to 600 nmol/L) followed by more rapid rises in salivary cortisol with increasing total serum cortisol concentrations (Vining, McGinley et al. 1983). Others have described this relationship as exponential (Gozansky, Lynn et al. 2005; Perogamvros, Owen et al. 2010). In conditions where CBG levels are high, e.g. pregnant women or those on the oral contraceptive pill, there is a prolonged first phase until CBG saturation (Vining, McGinley et al. 1983). This relationship has

been tested temporally and shown that salivary and serum cortisol correlate closely throughout the circadian rhythm (Dorn, Lucke et al. 2007). When free serum cortisol is plotted against salivary cortisol a linear relationship is observed, with strong a correlation coefficient from 0.89-0.97 (Vining, McGinley et al. 1983); Gozansky, Lynn et al. 2005).

Estimates of the percentage of total serum cortisol represented by salivary cortisol vary and need to be individually determined for each assay (Inder, Dimeski et al. 2012). Perogamvros and colleagues quote 1.66% using LC/MS-MS, which is in keeping with previous work carried out at SCH on an immunoassay (1.65%) (Nickson, Wilson et al. 2008, Perogamvros, Owen et al. 2010).

6.2.2 Salivary Cortisone

Salivary cortisone: cortisol ratios are between 16 and 64 times greater than serum total cortisone: cortisol ratios (Wood 2009). This is the result of two processes: 11β -HSD-2 within the salivary glands enables the one way oxidative conversion of cortisol to cortisone (to protect the glands from the glucocorticoid actions of cortisol) and CBG has a lower affinity for cortisone (the association constant is 10 fold higher for cortisol) and as such more free cortisone is available to diffuse into the saliva (Wood 2009). Salivary cortisone levels are considerably higher than salivary cortisol and it may therefore be the preferable biomarker (Perogamvros, Keevil et al. 2010, Blair, Lancaster et al. 2013). Salivary cortisone has been shown to closely mimic the levels of free serum cortisol and

be independent of CBG levels (Perogamvros, Keevil et al. 2010), however it is not a metric used routinely as yet in clinical practice but is an area of research interest (Raff and Findling 2010).

6.3 Salivary cortisol in the detection of adrenal insufficiency

A single morning cortisol sample, whether serum or salivary, has been found to exhibit poor sensitivity and specificity for the reliable detection of AI (sensitivity 33% for both and specificity 34% for serum, 20% for salivary) (Restituto, Galofre et al. 2008), however others, albeit with smaller numbers (48 versus 189) found it to have 100% sensitivity and 97% specificity (Patel, Shaw et al. 2004).

Those who have studied the use of salivary cortisol as the biochemical endpoint in dynamic tests of adrenocortical function have advocated it as an appropriate and useful alternative to serum cortisol, with many preferring it for its dissociation with CBG. It has been investigated in a number of different diagnostic cohorts, normal volunteers, adults and children and by using the ITT, CRH and Synacthen at 250, 25 and 1 mcg and been found to correlate well with serum cortisol (Laudat, Cerdas et al. 1988, Contreras, Arregger et al. 2004, Gozansky, Lynn et al. 2005, Marcus-Perlman, Tordjman et al. 2006, Arafah, Nishiyama et al. 2007, Cetinkaya, Ozon et al. 2007) Perogamvros, Owen et al. 2010).

6.3.1 The use of salivary cortisol in the assessment of children on ICS

Basal cortisol as a marker for adrenal suppression in children on ICS has been investigated and reported to be significantly lower than controls. The reduction is dose related, however there is considerable overlap with controls and thus is not felt to discriminate adequately enough to be employed as a diagnostic test (Nickson, Wilson et al. 2008, Bakkeheim, Mowinckel et al. 2010). A recent large study reported the results of three consecutive early morning salivary cortisol and cortisone measurements in 269 children on five different types of ICS. They found good negative predictive values for both salivary cortisol (98.8%) and cortisone (99.2%) but poor positive predictive values (9.5% and 30.1% respectively) (Blair, Lancaster et al. 2013).

The utilisation of salivary cortisol in dynamic tests of adrenocortical function in children with asthma on beclomethasone dipropionate compared to controls was described 30 years ago but has not been repeated since. Early morning salivary cortisol was higher in children with asthma, which authors attributed to the stress response of a lowered peak expiratory flow rate. The cortisol response to 250 mcg Synacthen showed a similar pattern in serum and saliva and were not significantly different to controls (Williams, Read et al. 1984).

7 Summary

Asthma is common and there are increasing requests to investigate the adrenal reserve of children on ICS because of the risk of AI. Most of the cases of AI in children prescribed high-dose ICS are sub-clinical, arguably better detected by the LDSST. There are unresolved questions as to the contributory role, if any, of dose, age, sex and pubertal status on AI in children on high-dose ICS. Currently the LDSST is the most commonly used diagnostic test amongst paediatric endocrinologists in UK, yet there is a lack of consensus as to who should be investigated, with which test, at what dose, when to sample and which diagnostic criteria to employ. Research in this area is limited by the invasive nature of the current tests. Synacthen is safely absorbed by the nasal mucosa and cortisol can be readily detected in salivary samples. Salivary cortisol has a predictable relationship with serum cortisol and salivary cortisone. It reflects the free portion of serum cortisol, is stable at room temperature and is unaffected by CBG levels and salivary flow rate. This research project aims to validate a non-invasive LDSST test with nasally administered Synacthen and salivary cortisol/cortisone for future use both as a diagnostic test in a number of clinical settings and for utility as a research tool.

Chapter 2:

Assessment of local and national practice

Whilst reviewing the literature during the set up phase of the first clinical study undertaken for this thesis (NeSST Study, *chapter 4*) it became evident that practice varies between centres and recommendations are often not adhered to, yet little data exists as to what is being done. It was with these deficiencies in mind that a number of small projects were devised to ascertain the following:

- current usage of the low and standard dose SSTs in specialist paediatric endocrine practice in the UK;
- local adherence to recommendations of the assessment of adrenocortical function in children on high-dose ICS;
- local rates of abnormal SSTs in children on high-dose ICS
- local GPs' and paediatricians' knowledge and prescription practises of high-dose ICS in children.

These projects were conceived and supervised by the author, but conducted in the main by medical colleagues within the department of endocrinology at Sheffield Children's Hospital.

1 British Society of Paediatric Endocrinology and Diabetes (BSPED) survey

There are a number of areas of debate and variation in practice in the use and interpretation of the SST to diagnose AI in children. A questionnaire survey of paediatric endocrinologists practicing in UK and Eire was conducted to evaluate its current usage and variability in practice. With an increase in the use of the LDSST, yet only the 250mcg/ml commercial preparation available, respondents

were additionally asked how they made up the low-dose Synacthen. The results of this survey were published in 2012 (Elder, Sachdev et al. 2012).

1.1 Methods

A seven-question survey (*appendix 1*) was emailed to all British Society of Paediatric Endocrinology and Diabetes (BSPED) members (257) in 2009 with a response requested from a representative of each centre (92 UK and Eire centres). This was followed up, one month later, by an emailed request to members of departments who had not yet responded.

The questions sought information on whether the centre currently used the SST and if so what dose was favoured; how the low-dose, if used, was made up; the timings of cortisol sampling and criterion for a normal response. Additionally there was a question pertaining to the centre's experience of request rates for SSTs in light of the concern over AI in children prescribed HDICS and whether they had experience of detecting AI in such children.

1.2 Results

Questionnaire replies were received from 39 of the 92 centres with practicing paediatric endocrinologists in the UK and Eire (42% response rate). Responses were fairly evenly split between secondary and tertiary care centres: 46% (18/39) from District General Hospitals and 54% (21/39) from tertiary centres. All

responding centres employed a form of the SST (100%): 82% (32/39) used a LDSST, 87% (34/39) the SSST, 69% (27/39) used both and 18% (7/39) and 13% (5/39) used exclusively the SSST or the LDSST respectively. The LDSST was the preferred choice when monitoring patients with asthma on high-dose ICS. The majority of LDSST tests were performed using a 1 mcg dose of Synacthen, 44% (14/32), and of the remaining 56% (18/32) seven different doses based on age, weight and body surface area (BSA) were used (*figure 2.1*).

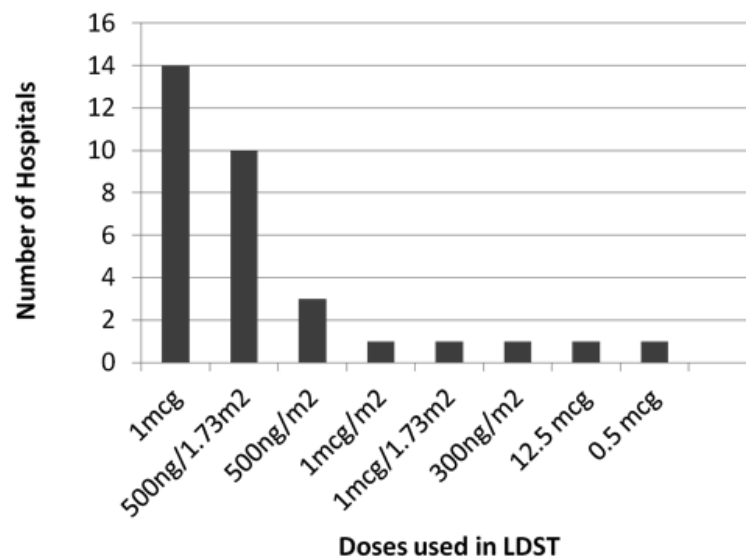


Figure 2.1: Variation in dose employed in the LDSST by different British and Irish paediatric endocrinology units (reproduced with permission).

There was variation in the timings of cortisol sampling. Although all units sampled at 0 and 30 minutes and 69% (22/32) of LDSST and 82% (27/33) of SSST at 60 minutes, there were a number of additional samples taken both in the

LDSST and SSST (*figure 2.2*). The number of samples taken was greater for the LDSST compared with the SSST (mean number of samples per test 4.4 and 3.8 respectively).

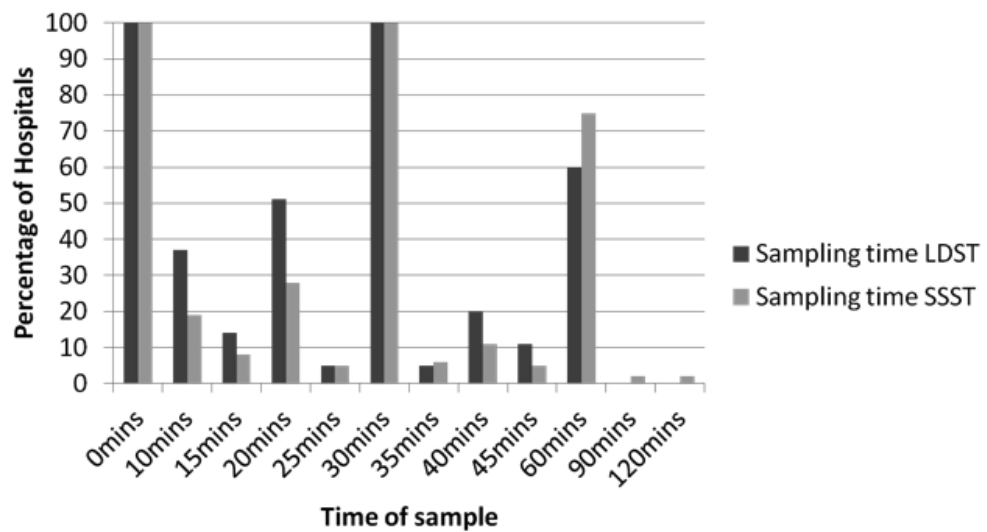


Figure 2.2: Sampling frequency and timing of British and Irish paediatric endocrinology units employing the LDSST (reproduced with permission).

The diagnostic cut-offs used by different centres for a normal SST also varied (*figure 2.3*). Peak cortisol was used on its own as the diagnostic criterion in 36% (14/39), the combination of peak cortisol and rise from baseline was used in 38% (15/39) and 18% (7/39) used peak or rise from baseline but did not require both for a normal result. No centre used rise from baseline as the sole criterion. The remaining 8% (3/39) of centres employed either different criteria depending on

time of day the test was performed, age related cut-offs or used the baseline cortisol value as a component in their decision making.

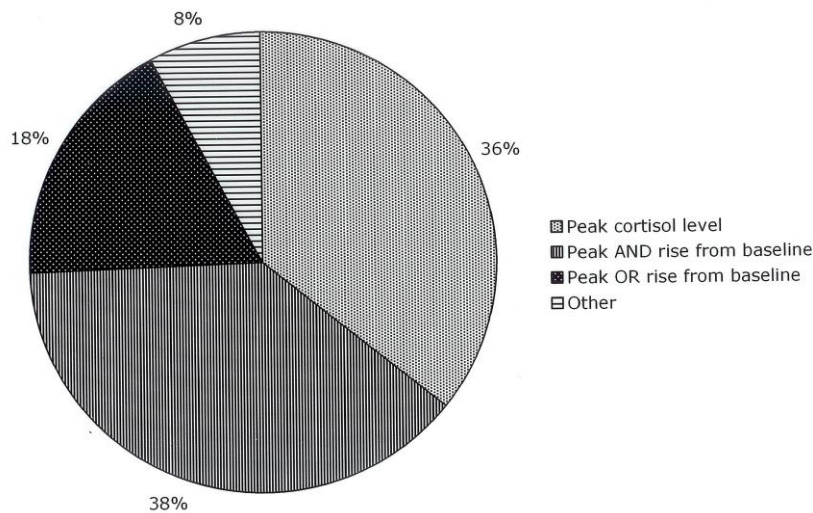


Figure 2.3: Diagnostic criteria used by British and Irish paediatric endocrinology units for a normal the LDSST (reproduced with permission).

Regarding the value required for a normal peak cortisol, 54% (21/39) of responding centres looked for a peak of 500 nmol/l and 44% (17/39) used 550 nmol/l, with one centre employing different criteria based on age. Rise from baseline was defined as more than 200 nmol/l in 67% (26/39), 8% (3/39) used a doubling of the baseline value and one centre used a combination of the two. Of the remaining nine centres, eight did not provide the information and one used an incremental rise of 150 nmol/l.

Marked variability was observed between units in how they reported making up their LDSST, with 14 different methods provided by 23/32 (72%) centres. The most popular method, used by five centres, involved mixing 0.1 ml Synacthen (25

mcg) with 50 ml of 0.9% saline resulting in a concentration of 500 ng/ml, of which 1 ml was given. Single dilution methods, of which eight were described, were used by 74% (17/23) of responding centres. Double or triple dilutions accounted for six of the methods and were used by 26% (6/23) of centres. The diluent volume varied from 10 mls to 1 litre. The volume of Synacthen used varied from 0.1 ml to the whole 1 ml vial of 250 mcg/ml; 0.5 ml or less was used in five of the different methods described.

The final question examined the respondents' knowledge of the current recommendations with regards to monitoring children with asthma on high-dose ICS, whether there had been an appreciable increase in the number of SST requested or performed and a chance to comment on their experience of detecting AI in these children. Only one respondent was unaware of the current recommendations and 44% (17/39) of centres reported an increase in requests for SSTs since their publication. Adrenal insufficiency had been detected in children on high-dose ICS by 67% (26/39) of responding centres and two children's hospitals reported detection rates of between 40% and 50%.

1.3 Discussion

Whilst acknowledging that a response rate of 42% resulted in a survey of fewer than half of UK and Eire centres offering paediatric endocrinology services it was believed to be representative and a good response rate for a questionnaire survey. This survey demonstrated considerable variability in almost all aspects of

the SST: the dose, the timings of cortisol sampling, the diagnostic criterion and cut-offs for AI and the way in which the LDSST was made up. All responding centres employed a form of the SST, however, despite meta-analysis level evidence, 18% did not use a LDSST at all. The variability perhaps in part reflects the paucity of normative data in children and has been noted previously in a UK-wide survey of clinical biochemistry laboratories. That survey reported a lack of consensus with regard to method of Synacthen administration (78% of respondents administering Synacthen i.m), timing of cortisol sampling and acceptable cut-offs for cortisol response (Barth, Seth et al. 1995). Additionally none of the 191 centres adhered to published guidance for interpretation of cortisol responses to Synacthen (Moore, Aitken et al. 1985). In this BSPED members survey the reference source of each centres' cortisol sampling times and diagnostic cut-offs was unknown but no centre adhered to the suggested test in two of the main paediatric endocrinology textbooks: namely 0 and 60 minute sample, following 250 mcg i.v Synacthen, and a cortisol response of over 830 nmol/l in the pre-pubertal and 690 nmol/l in pubertal children for a normal result (Miller, Achermann et al. 2008, Miller 2009). A 30-minute sample is mentioned but the chapters state that best-available evidence is for 60 minute sampling only. The only mention of the LDSST is in relation to the assessment of adrenal recovery from suppression secondary to corticosteroid usage but with no specification of what constitutes a normal response (Miller, Achermann et al. 2008, Miller 2009). Some of the variation noted in this survey may have been due to local protocols written collaboratively with laboratory colleagues,

although the diagnostic cut-offs were the aspect of the test with some of the least variance between centres.

The 14 different methods for making up the LDSST may be causing a marked variation in doses of Synacthen given. Synacthen is an inherently unstable drug, which rapidly degrades in natural light and when not refrigerated. Previous published work has shown Synacthen to bind to plastic, causing significant losses proportionate to the length of the plastic device used for administration (Murphy, Livesey et al. 1998, Wade, Baid et al. 2010). Inaccuracies increase with serial dilutions and the use of larger volume diluents. Diluting Synacthen using ward rather than accurate laboratory equipment and in uncontrolled conditions, with inter-individual differences in mixing strategies, is unlikely to create the desired equilibrium and thus cause variation in the doses being administered. The resultant effect may be of a greater range of cortisol responses and potential false negative results.

2 Short Synacthen Tests performed in children with asthma on high-dose ICS

This project audited how closely Sheffield Children's Hospital (SCH) complied with the recommended guidelines for LDSST in children taking high-dose ICS, including the issuing of steroid treatment cards (MHRA 2006, BTS/SIGN 2008, MHRA/CSM 2008). In addition the local incidence of AI in children on HDICS was

sought and compared to published figures. This work was presented as a poster at the annual BSPED meeting in 2009, held in Reading, UK.

2.1 Methods

A retrospective review over two time periods: pre-recommendation introduction (January 2004 to December 2005) and post- recommendation introduction (January 2007 to December 2008) was carried out of children prescribed high-dose ICS. Eighty-five cases were identified, from which 38 cases were prescribed high-dose ICS before the recommendations were published and 47 cases after. The criteria of a peak cortisol of >500 nmol/l or an incremental rise of >200 nmol/l were selected for a normal LDSST.

2.2 Results

There were 14 requests for LDSST in children on HDICS between 2007 and 2008 compared to 1 between 2004 and 2005, an increase from 2.6% to 30% of patients on HDICS. Using the criteria stated 5/14 (35%) of LDSST were abnormal, 80% of whom were prescribed higher than the recommended dose of ICS. All children receiving high-dose ICS with AI were receiving corticosteroid replacement but there was no documentation of steroid treatment card provision or written advice to carers in the patient notes.

2.3 Discussion

The published recommendations have led to an increase in the monitoring of children with asthma on high-dose ICS. The incidence of AI in the local population of children on high-dose ICS was similar to that reported elsewhere (Ninan, Reid et al. 1993, Eid, Morton et al. 2002, Paton, Jardine et al. 2006, Blair, Lancaster et al. 2013). It was unclear whether children and families were not receiving adequate advice and steroid treatment cards or whether documentation was poor.

3 High-dose ICS and AI at Sheffield Children's Hospital

A project looking to establish practice within the respiratory department at SCH with respect to the issuing of steroid treatment cards to children prescribed doses of ICS higher than those recommended in the British National Formulary for Children (BNFc) 2009 was undertaken (Paediatric Formulary Committee 2009). Additionally knowledge of the link between high-dose ICS and AI amongst medical staff was investigated. This work was presented as a poster at the annual European Society of Pediatric Endocrinology meeting in 2010, held in Prague, Czech Republic.

3.1 Method

A retrospective audit was carried out between April and June 2009 in which cases of children prescribed higher than recommended doses of ICS were identified from the respiratory clinic database. Reviewing the patient notes and telephoning families ascertained information pertaining to steroid treatment cards and advice. In addition knowledge amongst paediatric and Emergency Department medical staff within the Trust was assessed by a 3-question, anonymous questionnaire survey ascertaining seniority, awareness of guidance and what dose of different ICS constitutes “high-dose”.

3.2 Results

Within the database 19 patients were identified, 15 of whom were contactable by telephone. The majority (11/19) were children less than 12 years of age, prescribed >400 mcg fluticasone propionate daily. Steroid treatment cards had been issued to 40% of patients and 47% of families felt they had been made aware of the risks of high-dose ICS. A LDSST had been performed in 16%, all of which were normal (n=3).

Of the 65 questionnaires distributed 19 were returned, a 29% response rate. The majority, 13/19, were completed by junior doctors (Foundation year 1 (FY1), GP trainees and Senior House Officers (ST1-ST3)). Registrars and consultants accounted for only 6/19 responses. The seniority of the doctor affected awareness of the published recommendations, with 8% of junior and 67% of

middle grade doctors reporting they had heard of them. There was also difficulty identifying what constitutes high dose of ICS, especially amongst the most junior medical staff (*table 2.1*).

Table 2.1: ICS corrected identified as “high-dose” by medical staff stratified by seniority.

Age group	% correct responses given by junior doctors (FY1-ST3). N=13	% correct responses given by middle grade and senior doctors (>ST3). N=6
0-4 year olds	74%	93%
5-11 year olds	60%	78%
12-16 year olds	68%	71%

3.3 Discussion

The results were discussed with respiratory colleagues who were concerned about reducing adherence in an already possibly poorly adherent population by “steroidophobia” and had therefore been reluctant to issue steroid treatment cards other than to children on very high doses. Additionally there was a reluctance to perform SSTs in this group because of a belief that most children, despite very high doses of ICS, do not have AI and that the SSTs lack the necessary sensitivity and specificity to predict clinically relevant AI, a view shared by others (Blair, Lancaster et al. 2013).

Some confusion and lack of knowledge about what constitutes “high dose” may have been due to inconsistencies with the published recommendations. The

BNFc used terms for “standard” and “high” doses in their prescribing guidelines but the Medicines and Healthcare products Regulatory Agency (MHRA) recommendations quote maximum licenced doses, which are different to those given in the BNFc (BTS/SIGN 2008, MHRA/CSM 2008, Paediatric Formulary Committee 2009). The British Thoracic Society (BTS) states that suppression may occur at doses >400 mcg BDP/day or equivalent but does not age stratify (BTS/SIGN 2008, MHRA/CSM 2008, Paediatric Formulary Committee 2009).

4 Assessment of GP asthma services and prescription of high-dose ICS

Previously it had been reported that the prescription of high-dose and off-licence ICS was occurring in primary care (Thomas, Turner et al. 2006), and was not in keeping with published recommendations (BTS/SIGN 2008, MHRA/CSM 2008). Local GPs were therefore surveyed to quantify the number of children with asthma in their practice, their awareness of the risk of AI in children on high-dose ICS and their criteria for referral to specialist secondary/tertiary services.

4.1 Method

An 11-question questionnaire (*appendix 2*) was sent to 350 Sheffield GPs requesting information on their awareness of AI in children prescribed high-dose ICS, the doses prescribed and monitoring of children on high-dose ICS, whether

their practice had a designated asthma lead and their referral policy to secondary care. A 5-question questionnaire was sent to 92 GP practice managers for information on total number of children coded as having asthma and those prescribed high-dose ICS.

4.2 Results

There was a 15% response rate from both GPs and practice managers. In the 14 responding practices that supplied information there were 1461 children with asthma, 92.5% of who were over 5 years of age. When asked about prescribing practice 51% prescribed high-dose budesonide, 42% high-dose fluticasone and 8% high-dose beclomethasone. The referral of children with asthma to secondary care was generally prompted by persistent poor control, polypharmacy or multiple admissions and was generally (74%) to a respiratory paediatrician. In contrast to the junior hospital doctors, 96% of GPs responded that they were aware of the association between high-dose ICS and AI.

4.3 Discussion

GPs appear to have good knowledge of the link AI and high-dose ICS and refer appropriately. However there was a low response rate to the questionnaire and it is unknown whether the respondents were knowledgeable, interested in asthma and adhering to good practice. Additionally it is unknown whether the

children prescribed high-dose ICS had them commenced in primary or secondary care.

5 Summary

There is considerable variation in practice within the UK and Eire as to how the SST is employed. A commercially produced 1 mcg Synacthen formulation would avoid the different dilution strategies and doses reported in the national survey. The adherence to and knowledge of national recommendations for children with asthma on high-dose ICS is poor, more evident in junior hospital doctors than GPs. Research enabling risk stratification of children at risk of AI due to high-dose ICS would enable more uniform recommendations, national consensus guidelines and less variance in practice.

Chapter 3:

Validation of a Synacthen assay

To enable the pharmacokinetic analysis necessary to compare the bioavailability of nasal and i.v Synacthen an estimation of the plasma levels of Synacthen was required. Few previous researchers have measured Synacthen and there is no commercially available assay. Options to modify an existing ACTH assay were therefore investigated, an assay chosen and validation of that assay to measure plasma Synacthen performed. This chapter details the trial of numerous methodological variations and, despite acknowledged imperfections in the assay performance, eventual success when the assay was validated and considered fit for purpose.

1 History of measuring plasma Synacthen

Most of the studies to date looking at the optimal dose of Synacthen or alternative routes of administration have not measured plasma Synacthen directly, opting instead to compare a known quantity of intravenously injected Synacthen with the resultant cortisol response (Dickstein, Shechner et al. 1991, Dickstein, Spigel et al. 1997, Tordjman, Jaffe et al. 2000, Hiroi, Ichijo et al. 2002).

In the small number of studies where plasma Synacthen levels have been quantified almost all groups have employed ACTH radioimmunoassays (RIA) for their sensitivity but also their relative lack of specificity, as they detect only the N-terminus of the ACTH molecule, shared by both ACTH and Synacthen. Quantification has been by a number of different methods, employing different anti-sera and using extraction or non-extraction techniques. Some researchers

have chosen to measure ACTH assuming the majority of what is detected following Synacthen injection is ACTH(1-24) rather than endogenous ACTH(1-39) (Mayenknecht, Diederich et al. 1998). Others have used ACTH RIAs which are thought to 100% cross-react with Synacthen (Wade, Baid et al. 2010). Some have used dexamethasone to suppress endogenous ACTH secretion, with the inference that when endogenous ACTH is suppressed anything detected is plasma Synacthen (Wuthrich, Martenet et al. 1994).

The first reported measurement of Synacthen was in the 1970s when a number of groups employed the method first described by Orth *et al*, with Synacthen used to make up the assay kit standards and for the preparation of the radiolabelled iodinated hormone (ACTH ¹²⁵I) (Orth, Nicholson et al. 1973, Jeffcoate, Phenekos et al. 1977). In the 1980s rabbit anti-sera directed against the ACTH(5-18) portion of the molecule began to be used and some commercial production started (Nicholson, Davis et al. 1984, Dickstein, Shechner et al. 1991, Darmon, Dadoun et al. 1999, Nye, Grice et al. 1999). Later that decade a C18 Sep-pak extraction method was described to overcome the interference noted when using the rabbit antiserum, it was quoted as giving 80-90% recovery of the radiolabelled ACTH (Krishnan, Ritchie et al. 1988). A more recent publication described an extraction method and ACTH RIA with manufacturer-quoted 100% cross-reactivity with Synacthen. A chemiluminescent immunoassay measuring ACTH(1-39) was additionally employed and the endogenous ACTH assumed to be the difference between the two results. This group did not generate their own

control material, but did calculate coefficients of variation (CV) for ACTH(1-24) (Alia, Villabona et al. 2006).

In a departure from traditional RIA methods, two groups have reported the results of Synacthen detection using liquid chromatography/tandem mass spectrometry (LC-MS/MS) techniques. This has been investigated, both in urine and plasma, as a research tool for use in athletes misusing Synacthen as a performance-enhancing drug (Thevis, Bredehoft et al. 2006, Thomas, Kohler et al. 2009, Chaabo, Ceaurriz et al. 2011).

2 Immunoassays

Immunoassay describes any test in which the analyte of interest, usually a protein, is detected by use of a specific antibody. Once the antigen (analyte) and antibody have bound a method of detection (label) is attached. The label determines the type of immunoassay. A label may emit radiation (RIA); fluoresce under light (fluorescent immunoassay, FIA); cause a chemical reaction, which produces light (chemiluminescent immunoassay, CIA); be a DNA probe or emit light in response to an electrical current (electrochemiluminescence). Previously popular were enzyme labels which would chemiluminesce or cause a colour change or (enzyme-linked immunosorbent assays, ELISAs). Immunoassays may be competitive or non-competitive, one-site or two-site (sandwich); liquid or solid-phase and may have multiple steps in which reagents are added and

washed off (separation immunoassays) or be simple homogenous immunoassays (Christopoulos and Diamandis 1996).

Immunoassays began being used in analytical laboratory medicine in the 1960s with the development of RIAs to detect a number of compounds including hormones, immunoglobulins and drugs. The first RIA to measure ACTH was described by Berson and Yalow in 1968 (Berson and Yalow 1968). Yalow went on to win a Nobel Prize for their work in 1977. More recently automated ELISAs, CIAs and FIAs, none of which require the handling and disposal of radioactive materials, have largely superseded RIAs. However RIAs still have a role detecting analytes that exist in very small amounts, like hormones, with high levels of sensitivity or small antigens with space for only one antibody binding site.

2.1 Radioimmunoassays (RIAs)

RIAs require a compound to act as an antigen and bind to a specific and complimentary antibody. RIA is inherently sensitive and the affinity of the antibody for the antigen confers its specificity. RIA requires the preparation of a radiolabelled form of the antigen to be measured and most usually entail labelling the tyrosine residues of the protein of interest with radioactive iodine (^{125}I). Additionally RIA requires the substance to be measured to be available in pure form for antibody, tracer and calibrator production. RIA usually describes a competitive assay, where molecules compete for a single binding site on the antibody (Christopoulos and Diamandis 1996, Chaitoff 2008).

Double antibody methods (such as immunoradiometric assays, IRMA) are sandwich assays and require the antigen to have two (or more) binding sites conferring a superior measuring range to classical RIA. They have largely replaced classical RIAs in the measurement of ACTH due to greater sensitivity at the lower end. Additionally it is felt that single epitope detection, whilst identifying the intact ACTH(1-39) molecule, may additionally detect fragments of related molecules within the HPA-axis such as POMC, MSH, lipotropin, shorter ACTH analogues and endorphins (*figure 1.2*) (Talbot, Kane et al. 2003).

2.1.1 Competitive RIAs

The competitive technique entails adding known quantities of radioactive antigen (Ag^*) and antibody (Ab) with unknown quantities of unlabelled antigen (Ag), (which maybe patient serum or a known standard). The Ag^* and Ag compete to bind the antibody. The greater the quantity of unlabelled antigen that binds the more Ag^* is left unbound and therefore the lower the ratio of antibody-bound- Ag^* to free- Ag^* (*figure 3.1*) (Chaitoff 2008). The competitive RIA technique must recognise the labelled and unlabelled antigen with the same affinity. Additionally it is important that the ligand-binding capacity of the antibody is not compromised by the presence of excess tracer such that half the tracer is displaced if half of the antibody sites are saturated with antibody. To this end antibody and tracer concentrations are varied during assay development

to give optimal measuring range and sensitivity (Personal communication, Martin Loxley).

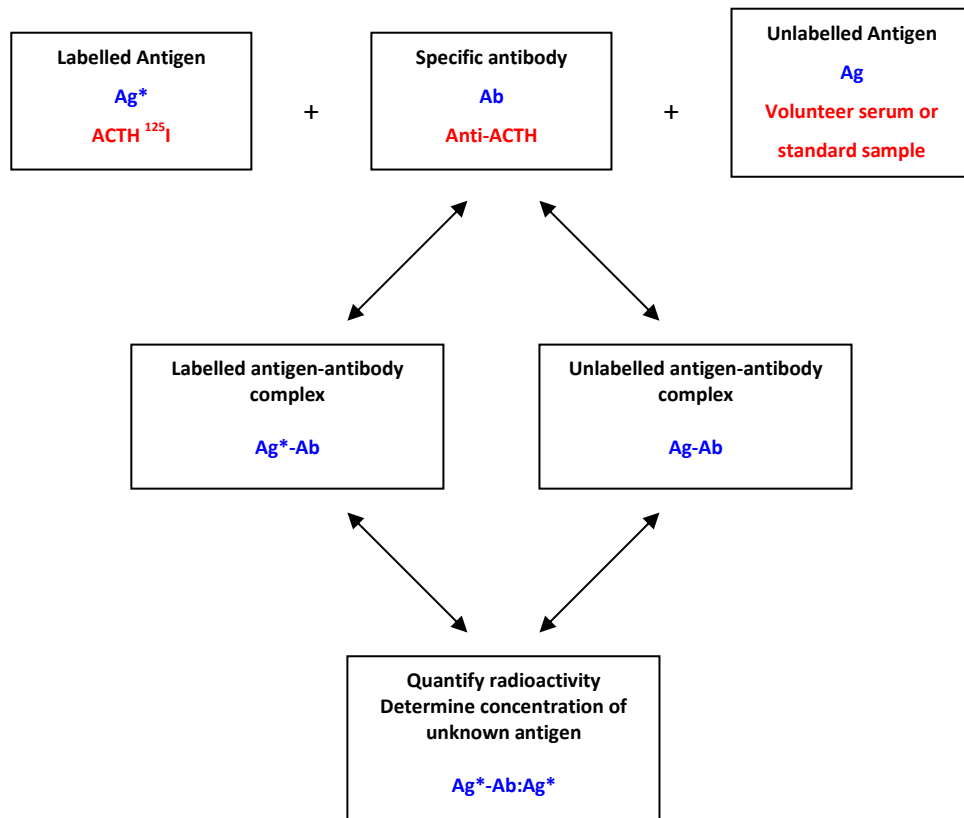


Figure 3.1 : Schematic diagram of a competitive radioimmunoassay. Features of the assay used in the current work are shown in red. *indicates radioactive labelling. Ag = antigen, AB = antibody.

The antigen-antibody complexes need to be separated from the free fraction prior to determining the radioactivity. The separation (of bound and free fractions) is usually achieved with the addition of a precipitant solution containing a second antibody, which binds to the antigen-antibody complex, leaving the free fraction in a solution. Following centrifugation the free fraction is

then removed either by decanting or aspiration. Other separation methods include filtration and the use of a solid phase second antibody. The radioactivity of the remaining antigen-antibody complexes is quantified and the concentration of the unknown sample determined by comparing the ratio of antibody-bound- Ag^* :free- Ag^* , with that produced by a series of standards containing known amounts of the substance (unlabelled antigen, Ag) tested at the same time and used to generate a standard calibration curve (*figure 3.2*).

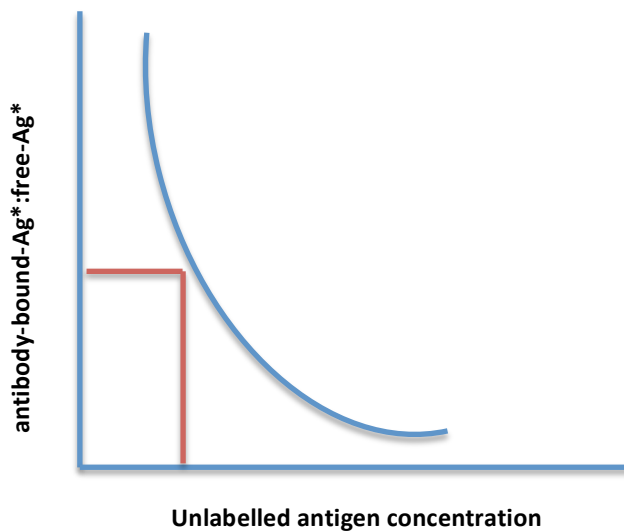


Figure 3.2: Standard calibration curve for a competitive RIA. The curve is used to determine the antigen concentration in a patient sample, example shown in red.

A typical RIA dose-response curve is sigmoidal as the addition of increasing amounts of antigen progressively displaces Ag^* from the antibody but with saturation at high concentrations. The sigmoidal curve is logit transformed such that the x-axis of the dose-response curve is expressed as a log, converting the concentration of double diluted standards to linear for simpler numerical

quantification (Personal communication, Martin Loxley)(Christopoulos and Diamandis 1996).

2.1.2 Sandwich RIAs

Sandwich RIAs are a type of IRMA, which are non-competitive in design and have enhanced sensitivity over classical RIA. In IRMA it is the antibody that is labelled so antigen and labelled antibody form complexes when incubated. The sandwich format is a two-site IRMA with two antibodies and detects two separate epitopes of the antigen of interest. It requires two different antibodies, one which captures and one which is radiolabelled (usually ^{125}I) and is used for detection. It is a solid phase method, in which a “capture” antibody is immobilised (e.g. bound to insoluble polystyrene beads or coated tubes) and binds the free fraction. The unbound free fraction is washed away. The labelled antibody is then added and binds at a separate site thus allowing detection and quantification of antigen. Monoclonal antibodies are often used as they enable specific, non-overlapping epitopes of the antigen to be targeted. As this is non-competitive, the amount of antigen determined by the amount of bound fraction directly correlates unlike in a competitive RIA where the amount of substance is inversely proportional to the detected radioactivity.

3 Selecting a Synacthen assay

The majority of ACTH assays currently in commercial use employ a “sandwich” analytical technique with monoclonal antibodies in a 2-site immunometric format, requiring the binding of two epitopes for detection (CIA or IRMA) (Pecori Giraldi, Sacconi et al. 2011). The two epitopes are generally found at, or near the N and C termini respectively. The sandwich technique results in a complete lack of detection of Synacthen because, although the N-terminus is shared by ACTH(1-39) and its synthetic analogue Synacthen, the C-terminus is truncated and therefore is not recognized by the assay’s antibody.

High Performance Liquid Chromatography (HPLC) was considered as a potential method of detection and quantification. Initially this method was explored locally but it was felt by laboratory colleagues to be excessively time-consuming and require considerable expertise to set up, especially in view of the need for a sensitive enough detector, with no guarantee of success. Additionally it was thought to be an inefficient method for the processing of hundreds of samples. A Synacthen HPLC is used by Archimedes Pharmaceuticals Ltd (Nottingham, UK), the pharmaceutical company whom manufactured the Synacthen doses for use in the second part of the project (NeSST2 study). They investigated whether it would be an appropriate technique for analysis of the volunteer plasma Synacthen samples in this work, however the lowest concentration detectable was found to be 0.5 mcg/ml, approximately 2000 times greater than the pg/ml plasma levels it would be required to detect.

Bachem (Bupendorf, Switzerland; the pharmaceutical company from whom the tetracosactide was purchased for manufacturing in NeSST2) and Alliance Pharmaceuticals (Wiltshire, UK; the company with the marketing authorisation for Synacthen in the UK) both use HPLC for the analytical work involved in quality controlling their products. Enquiries regarding whether their techniques were sensitive enough to be used in the analysis of our samples were made to both companies. The lower limit of detection of their HPLC methods was considerably higher than required. It was advised that to quantify down to the necessary concentration HPLC with detection by mass spectrometry would be needed.

The head of the German group (Professor Thevis) who have developed a LC-MS/MS method for the detection of Synacthen in doping tests was contacted for advice about adopting their method or for their assistance in processing the samples. The reply (June 2010) stated two major issues with Synacthen detection making the use of LC-MS/MS unviable. The German group had validated the LC-MS/MS assay for detection of depot formulations of 1mg Synacthen (1000 times greater than the doses administered in the NeSST study). At these doses the LC-MS/MS technique is already at the limit of its detection at the lower range. It was felt with considerably smaller doses, such as those following intranasal administration, the technique would not be adequately sensitive. The second concern was regarding the stability of Synacthen, recognising that it is not stable in plasma or urine and therefore rapid degradation may make detection even more challenging. They were unaware of any other laboratory doing analysis of this kind.

Mid-way through the study a paper from a French group was published describing improved sensitivity and limits of detection of Synacthen using cation exchange chromatography and solid-phase extraction and analysis by LC-MS/MS with positive-mode electrospray ionization using ACTH(7-38) as an internal standard. The recovery was quoted as 70% and lower limit of quantification as 15 pg/ml (Chaabo, Ceaurriz et al. 2011). When contacted the corresponding author (Dr Lasne) responded that the technique developed did not provide enough recovery of Synacthen after the preparation step to be useful, being more a test of Synacthen presence than accurately quantifying plasma levels.

On discussion with laboratory colleagues in Sheffield, it was felt that developing a sensitive enough LC-MS/MS technique may take years, if possible at all, and therefore the RIA, accepting its flaws, was chosen as the best assay available for the study. The single epitope RIAs used in the studies described above (*section 1*) are mostly out of production, having been superseded initially by more specific two-site IRMAs (Gibson, Pollock et al. 1989) and more recently by non-radioisotopic, fully-automated techniques (Talbot, Kane et al. 2003). The RIA selected was sourced after extensive searching by the senior biomedical scientist (BMS) in the local endocrine laboratory and was thought to be the only assay available that had an antibody capable of detecting an epitope within the Synacthen molecule. It was an endogenous ACTH(1-39) assay which manufacturers, MP Biomedicals (Santa Ana, CA, USA), quote as cross-reacting 100% with Synacthen and was later discovered to have been successfully

validated for plasma Synacthen detection by other researchers (Wade, Baid et al. 2010).

3.1 Selected assay characteristics

The MP Biomedicals human ACTH (hACTH) RIA is a competitive RIA in which the antibody recognises human ACTH(5-18). This assay was selected for its quoted cross-reactivity with Synacthen (*table 3.1*).

Table 3.1: Specificity of the antiserum of the MP Biomedicals hACTH RIA. Reproduced with permission from MP Biomedicals' directional insert for hACTH RIA kit.

Compound	% Cross-reaction
ACTH (1-39)	100
ACTH (1-24)	100
h β -Lipoprotein	0.8
h α -Lipoprotein	0.1
h β -Endorphin	<0.1
h α -MSH	<0.1
h β -MSH	<0.1

The company quotes the minimum detectable dose to be approximately 5.7 pg/ml. This had been ascertained by running 20 replicates of the zero standard and subtracting two standard deviations from the mean of the zero tubes, the standard calculation method for limit of detection (Rodbard 1978, MP Biomedicals 2012).

MP Biomedicals have calculated the intra (within batch) and inter-assay (between batch) variations for their assay using ten sets of data (*table 3.2*).

Table 3.2: Intra-assay and inter-assay variation of MP Biomedicals hACTH RIA.
 Reproduced with permission from MP Biomedical directional insert for hACTH RIA kit.
 Values are in pg/ml.

	Intra-assay variation (n=10)		Inter-assay variation (n=10)	
Mean	40.9	139	36.3	107.5
S.D	2.8	5.7	3.9	4.2
C.V	6.8%	4.1%	10.7%	3.9%

The kits are designed for human EDTA plasma samples and contain the following:

- Eight ACTH(1-39) lyophilised standards
 - At the following target concentrations: 0, 10, 25, 50, 100, 250, 500, 1000 pg/ml in human plasma
- Anti-ACTH
 - A purified porcine ACTH-conjugate is used to generate this antiserum in rabbits
 - Diluted in a phosphate buffer to give pH 7.6
 - The anti-serum binds 20-40% of hACTH ¹²⁵I (freshly iodinated) in the absence of nonradioactive hACTH
- ACTH controls
 - A high and low concentration of ACTH added to human plasma
- hACTH¹²⁵I
 - Synthetic hACTH(1-39)

- Diluted in a phosphate buffer to give pH 7.6
- This radioactive material contains less than 0.4 μCi (microcurie) per vial
- Precipitant solution
 - Mixture of goat anti-rabbit gamma globulins and PEG (polyethylene glycol) is contained in a 0.1M Tris buffer
 - 0.5 ml will immediately precipitate all the antibody bound antigen, eliminating the need for a second incubation.
- Polystyrene test tubes (uncoated) as it is advised not to use glass test tubes due to previous research showing adsorptive losses (Stouffer and Lipscomb 1963).

MP Biomedicals quote the performance characteristics of their kit constituents, which were tested against standardised WHO 74/555 ACTH preparations (*table 3.3*).

Table 3.3: Performance characteristics of MP Biomedical hACTH RIA. Adapted and reproduced with permission from MP Biomedicals' directional insert for hACTH RIA kit.

ACTH added (pg/ml)	ACTH expected (pg/ml)	ACTH obtained (pg/ml)	% recovery
Sample A = 40 pg/ml			
12.5	52.5	56	107
25	65	70	108
50	90	92	102
125	165	180	109
Sample B = 11 pg/ml			
12.5	23.5	23	98
25	36	37	103
50	61	64	105
125	136	145	107
Sample C = 20 pg/ml			
12.5	32.5	32	98
25	45	45	100
50	70	74	106
125	145	150	103
Sample D = 23 pg/ml			
12.5	35.5	37	104
25	48	52	108
50	73	76	104
125	148	160	108

The company additionally quote the response to serial dilution using four plasma samples diluted 1:2, 1:4, 1:6, 1:8 with the zero standard (table 3.4).

Table 3.4: Parallelism of MP Biomedicals hACTH RIA. Adapted and reproduced with permission from MP Biomedical directional insert for hACTH RIA kit. Values are in pg/ml with means in red. Dil = diluent

Sample	1:2 ACTH	1:4 ACTH	1:6 ACTH	1:8 ACTH	Mean
A (225)	120	58	39	28	
A x dil	240	232	234	224	232.5
B (370)	190	96	67	47	
B x dil	380	384	402	376	385.5
C (550)	290	140	100	70	
C x dil	580	560	600	560	575
D (760)	370	195	135	96	
D x dil	740	780	810	768	774.5

4 Validation experiments

The intention was to perform a series of dilution experiments prior to the analysis of volunteer samples, which would allow validation of the ACTH RIA, gain knowledge of its working characteristics and to ensure it reliably detected Synacthen. This proved considerably more difficult than anticipated, taking more than two years to successfully validate the assay. All experiments were undertaken in the manual endocrine laboratory at the Royal Hallamshire Hospital's Clinical Chemistry department using accurate, calibrated laboratory equipment.

As a clinician, with an ostensibly clinical project, much of the Synacthen assay validation work was considerably outwith my area of expertise. Close supervision was provided by Martin Loxley (ML), laboratory manager at the Department of

Clinical Chemistry, Royal Hallamshire Hospital, who has an extensive theoretical and working knowledge of RIA. ML and Victoria Moyse (VM) (senior BMS) provided training on the setting up and running of the assay, the laboratory equipment involved, appropriate handling of radioactive materials and safe laboratory practice. All the practical aspects of the laboratory work (except for validation experiment 1, during which ML trained me and experiment 11, which was carried out by VM to ensure that any inconsistency in results were not due to my relative laboratory inexperience) were performed by the author. However ML, with some input from VM, interpreted the results and suggested the methodology and theory behind each progressive attempt at assay validation. My contribution to this process increased over time as my understanding deepened.

Two preparations of Synacthen are commercially available in the UK (Alliance Pharmaceuticals, Chippenham, Wiltshire, UK). A 1 mg depot preparation and a 250 mcg/ml formulation used for all the assay validation experiments. The hACTH RIA detects ACTH in picomolar amounts and thus large dilutions were required to bring the Synacthen into a detectable range. Particular care was therefore taken to pipette accurately and mix solutions by vortexing thoroughly.

4.1 Set up and running of MP Biomedicals' hACTH RIA

- *Step 1:* The lyophilised reagents (hACTH standards, high and low quality controls, antiserum (Ab) and labelled antigen (hACTH-¹²⁵I)) were reconstituted with water and refrigerated for a minimum of 15 minutes.
- *Step 2:* Polystyrene test tubes were labelled (totals, blanks, standards 8 to 1, low and high quality controls and then sample tubes).
- *Step 3:* The diluent water, hACTH standards, controls, samples, antiserum and hACTH-¹²⁵I were added to the test tubes as illustrated in *figure 3.3*. A finnipipette 4540 (Thermo scientific, Waltham, MA, USA) with 2.5ml tip and wheel dial 1 was used to repeat pipette the antiserum and hACTH-¹²⁵I. All standards, controls and samples were run in duplicate unless otherwise stated.
- *Step 4:* The tubes were shaken for 30 seconds, then vortexed and incubated in the refrigerator until the following day (minimum 16 hours).
- *Step 5:* Following incubation, samples had precipitant solution by a finnipipette 4540 (Thermo scientific, Waltham, MA, USA) with 25.0 ml tip and wheel dial 1 and were again shaken for 30 seconds and then vortexed thoroughly, ensuring a homogenous colour throughout.
- *Step 6:* The samples were then centrifuged in a CellSep 6/720R centrifuge (Sanyo, Moriguchi, Osaka, Japan) at 3000 x g for 15 minutes at a temperature of 8°C.

- *Step 7:* The supernatant was aspirated and the remaining precipitate counted in a DPC gamma C12 counter (Berthold technologies, Bad Wildbad, Germany).
- *Step 8:* AssayZap 3.1 (Biosoft, Palo Alto, CA, USA), assay calculator software, was used to generate a standard curve and derive sample results.

All RIA kits were used within the 5-week expiry window and most within a fortnight of production, ensuring maximum sensitivity. Tracer decay reduces the sensitivity of the assay as the counts become lower and the curve becomes flatter resulting in less differentiation between values. In all experiments standards and samples were run in duplicate unless otherwise stated. When poor duplicates were obtained the likely erroneous result was excluded.

Tube No.	Description	Diluent Water (mL)	Standard/ Sample (mL)	Anti- ACTH (mL)	hACTH ¹²⁵ I (mL)	Precipitant Solution (mL)
1,2	Blank	0.1	0.1 (0 pg/mL)	0	0.1	0.5
3,4	0 pg/mL	0	0.1	0.1		
5,6	10 pg/mL					
7,8	25 pg/mL					
9,10	50 pg/mL					
11,12	100 pg/mL					
13,14	250 pg/mL					
15,16	500 pg/mL					
17,18	1000 pg/mL					
19,20	Low Control					
21,22	High Control					
23,24	Sample					
25,26	Sample					
27,28	Sample					
29,30	Sample					
31,32	Sample					
33,34	Sample					

VORTEX AND INCUBATE 16 HOURS AT 4°C ± 2°

CENTRIFUGE, ASPIRATE, AND COUNT

Figure 3.3: Protocol for MP Biomedical hACTH RIA. Instruction for use: ImmChem Double Antibody hACTH ¹²⁵I RIA kit (MP Biomedicals 2012). Reproduced with permission from MP Biomedicals.

4.1.1 Quality Controls (QCs) and Coefficient of Variations (CVs)

A high and low QC was supplied with the hACTH RIA kit. These help to give an estimate of inter-assay (batch-to-batch) variability with repeated analysis. As there is no independently sourced material containing Synacthen (Alliance Pharmaceuticals have the Marketing Authorisation and are the sole supplier of Synacthen in the UK) the kit ACTH QCs were used for experiments 1-14.

The QCs quoted for the kit were:

- Low 43 (33-53 pg/ml)

- High 121 (100-142 pg/ml)

These ranges enable the calculation of inter-assay coefficient of variations (CVs), an estimation of measurement error and therefore represent the acceptable range for the dispersion of results between assay batches. CVs are a ratio of the standard deviation (SD) to the mean and are expressed as a percentage.

Standard laboratory practice (Levey-Jennings method) is to accept an assay when QCs are within +/- 2 SDs. Results between 2-3 SDs acts as an alert that a laboratory should be aware of a potential flaw and if more than +/- 3 SDs the results would be rejected (personal communication, ML).

For the low QC, 33-53 pg/ml a range of 20 pg/ml is given, this represents 4 SDs (+/- 2) and thus 1 SD is 5 pg/ml. Similarly for the high QC, the range of 100-142 is 42 pg/ml, so that 1 SD is 10.5 pg/ml.

- *Low QC* - the inter-assay CV is therefore $5/43 = 11.6\%$.
- *High QC* - the inter-assay CV is therefore $14/121 = 8.7\%$.

Inter-assay CVs of around 10% are typical for a competitive RIA of this type (personal communication, ML). If one uses a cut-off of 3 SDs then acceptable results are 28-58 pg/ml for the low QC and 89.5-152.5 pg/ml for the high QC. These were the ranges used to assess the QC results obtained with each assay (*vide infra, table 3.23*).

No assessment of intra-assay variability (within batch), where the same samples are run multiple times on the same assay, was performed. Inter-assay variability is a more useful metric of assay performance and has typically higher values than intra-assay variability. It was felt that establishing intra-assay variability would take up a large number of tubes in an already expensive assay without adding significant meaningful information concerning the practical aspects of the assay.

The experiments undertaken to validate the assay for the quantification of Synacthen are described in detail below (*section 4.2 – 4.20*). A summary table of the experiments is included below (*table 3.5*).

Table 3.5. Summary of the Plasma Synacthen assay validation experiments

Expt number	Summary of theory behind experiment	Summary of methodology	Summary of results
1	Simple serial dilution experiment to find sensitive part of the RIA measuring curve.	Dilution of 250mcg/ml Synacthen in 0.9% saline to give 7 concentrations from 250,000 to 31.3 pg/ml.	All samples over the upper limit of assay detection.
2	Repeat of serial dilution experiment to find the most sensitive part of the RIA measuring curve.	Dilution of 250mcg/ml Synacthen in 0.9% saline to give 7 concentrations from 250 to 3.9 pg/ml.	No obvious relationship between observed and expected values, however some samples on scale and approximately 250 pg/ml appeared to be most sensitive part of the curve.
3	Synacthen is an inherently unstable protein therefore its degradation in room temperature was tested.	Three concentrations of Synacthen (25, 5 and 2.5 pg/ml) were diluted in 0.9% saline and left for between 0 minutes and 24 hours (8 time points) at room temperature.	All samples over the upper limit of assay detection.
4	Investigation of the matrix effect – using a more physiological matrix may enable Synacthen to retain its 3D configuration and be detected by the RIA.	Dilution of 250mcg/ml Synacthen in 0.9% saline and PBS BSA* to give 9 concentrations from 1000 to 3.9 pg/ml. Controlled temperature variation more closely (for all experiments thereafter).	Some linearity between observed and expected values for saline samples between 150-500 pg/ml. PBS BSA showed minimal detection of Synacthen.
5	Repeat of experiment 4 with controlled temperature conditions and saline as diluent.	Dilution of 250mcg/ml Synacthen in 0.9% saline to give 11 concentrations from 1000 to 31.3 pg/ml.	Linearity of experiment 4 not replicated. All samples much lower than expected values with no discernable relationship.
6	Matrix effect investigated again.	Dilution of 250mcg/ml Synacthen in 0.9% saline, PBS** and PBS BSA* to give 5 concentrations from 2500 to 125 pg/ml. Controlled temperature conditions.	Similar results obtained from saline and PBS samples, both lower than expected but some degree of linearity. Results from PBS BSA samples higher than expected.
7	Use of stock solution to remove the possibility of dilution errors.	Stock solutions of dilutions of 250mcg/ml Synacthen in 0.9% saline and PBS giving 6 concentrations from 750 to 125 pg/ml were made up and run on three different assays on three different days.	1 st and 3 rd runs showed consistency for saline and PBS and yet different from each other. The 2 nd run gave higher results for both and more similar to one another. Although limited interpretation possible PBS appeared the better matrix.
8	Matrix effect investigated again.	Dilution of 250mcg/ml Synacthen in ACTH deplete, pooled human EDTA plasma to give 7 concentrations from 750 to 94 pg/ml.	No obvious relationship between observed and expected values with samples much higher than anticipated. ACTH depletion not successful – high

			ACTH in pooled plasma.
9	Use of external quality assurance samples to test ability of RIA to detect ACTH.	Three external quality assurance samples run in RIA and compared to UKNEQAS [§] results	Results considerably higher than expected from UKNEQAS even when bias correction was applied.
10	Re-analysis of research samples to investigate inter-assay reliability.	Three volunteer samples from NeSST Study rerun with 11-month lag and additional freeze-thaw cycle.	Results were significantly higher on 2 nd run.
11	Use of an acidified buffer to replicate previously published work.	Dilution of 250mcg/ml Synacthen in PBS BSA acidified to pH to give 12 concentrations from 1000 to 23.5 pg/ml. Samples run in quadruplicate and assay set up and run by senior BMS.	Acidified buffer with no Synacthen recorded measurable ACTH. A relationship between observed and expected was seen whereby for each 1:1.5 dilution between 187.5-23 pg/ml a 1.4 fold concentration reduction was noted.
12	Use of external quality assurance samples to test ability of RIA to detect ACTH again.	Three external quality assurance samples run in quadruplicate and compared to UKNEQAS [§] results	Results considerably lower than expected from UKNEQAS even when bias correction was applied. Possible effect of freezing the NEQAS standards.
13	Matrix effect investigated using spiked human plasma.	ACTH depleted human blood was spiked with Synacthen to give 2 concentrations 250 and 124 pg/ml. Samples run in sextuplicate and assay set up and run by senior BMS.	Values higher than expected, with considerable variability between samples of the same concentration. Unspiked control had much higher ACTH than when run on Immulite.
14	Re-analysis of research samples to investigate inter-assay reliability.	Seven volunteer samples were re-run 17 months after the initial analysis and with an additional freeze-thaw cycle.	Results were 2.1-3 fold lower than those obtained previously.
15	Create standards using the same buffer as in the assay	Dilution of 250mcg/ml Synacthen in MP Biomedicals hACTH RIA zero standard to give 7 serial concentrations from 1000 to 15.6 pg/ml. Samples made up and frozen then run on 3 separate assays.	All three runs showed similar results with close relationship between observed and expected values.
16	Test of repeatability and reliability of assay using Synacthen standards	Synacthen standards made as part of experiment 15 were frozen and run with each assay.	Very good linearity and correlation between observed and expected.

*PBS BSA - Phosphate buffered saline with bovine serum albumin ** PBS - Phosphate buffered saline § - UKNEQAS – UK National External Quality Assessment Service BMS – Biomedical Scientist

4.2 Validation experiment 1

4.2.1 Aim

The aim was to validate the assay prior to the analysis of volunteer samples by performing serial dilution experiments. This would allow its working characteristics to be ascertained and to ensure it reliably detected Synacthen.

4.2.2 Method

The first experiment involved dilution of Synacthen (250 mcg/ml) in 0.9% saline to give the concentrations given in *table 3.6a*.

Table 3.6a: Dilution ratios, summarised method and resultant solutions of experiment 1 – serial Synacthen dilution in 0.9% saline. Final solutions assayed displayed in bold.

Dilution ratio	Dilution method	Resultant concentration
1:1000	0.1ml of 250 mcg/ml Synacthen in 99.9ml of 0.9% saline	250,000 pg/ml
1:25	0.4 ml of resultant solution in 9.6 ml of 0.9% saline	10,000 pg/ml
1:10	0.1 ml of resultant solution in 0.9 ml of 0.9% saline	1000 pg/ml
1:4	0.5 ml of resultant solution in 2 ml of 0.9% saline	250 pg/ml
1:2	1 ml of resultant solution in 1 ml of 0.9% saline	125 pg/ml
1:2	1 ml of resultant solution in 1 ml of 0.9% saline	62.5 pg/ml
1:2	1 ml of resultant solution in 1 ml of 0.9% saline	31.3 pg/ml

4.2.3 Results

Table 3.6b: Experiment 1 – serial Synacthen dilution in 0.9% saline. Raw data and mean of duplicate results are displayed. Standards and QCs shown.

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)
Standard 8	1098	> max*
Standard 7	535	513.5
Standard 6	255	246.2
Standard 5	86	90.3
Standard 4	39	37.0
Standard 3	19	21.1
Standard 2	8	< min**
Low Quality control (QC)	43 (28-58)	44.0
High Quality control (QC)	121 (89.5-152.5)	150.7
Sample 1	250,000	> max*
Sample 2	10,000	> max*
Sample 3	1000	> max*
Sample 4	250	> max*
Sample 5	125	> max*
Sample 6	62.5	> max*
Sample 7	31.3	> max*

* > max = more than maximum standard measurable by hACTH RIA (> 1098 pg/ml)

** <min = less than the minimum standard measurable by hACTH RIA (< 8 pg/ml)

Standard 1 = zero standard (used to calculate % binding and not plotted on a log scale therefore the result is not displayed)

4.2.4 Interpretation

Despite acceptable QCs and a reasonable fit standard curve all the dilution samples were above the measurable scale. This was unexpected as a large range had been tested (250,000 – 31.3 pg/ml) and the standard curve range indicated that the detectable range would be between those values.

Despite plotting expected (assumed Synacthen concentration following dilution) against observed (RIA results obtained) values the expectation was not to see identical or even similar values. It was acknowledged that Synacthen was likely to behave differently from ACTH(1-39) and would not cross-react in identical amounts. To validate the assay for the detection of Synacthen a consistent and interpretable relationship between observed and expected values was required.

4.3 Validation experiment 2

4.3.1 Method

Following the disappointing results of the first serial dilution experiment the range was extended down to 3.9 pg/ml although it was thought that the most sensitive part of the assay curve was likely to be around 250 pg/ml. The 250 mcg/ml Synacthen was again diluted in 0.9% saline to give the following concentrations.

Table 3.7a: Dilution ratios, summarised method and resultant solutions for experiment 2. The final solutions assayed displayed in bold.

Dilution ratio	Dilution method	Resultant concentration
1:100	0.1ml of 250mcg/ml Synacthen in 9.9 ml of 0.9% saline	2500 ng/ml
1:100	0.1ml of 2500ng/ml solution in 9.9 ml of 0.9% saline	25,000 pg/ml
1:100	0.1ml Of 25,000pg/ml solution in 9.9 ml of 0.9% saline	250 pg/ml
1:2	1 ml of resultant solution in 1 ml of 0.9% saline	125 pg/ml
1:2	1 ml of resultant solution in 1 ml of 0.9% saline	62.5 pg/ml
1:2	1 ml of resultant solution in 1 ml of 0.9% saline	31.3 pg/ml

1:2	1 ml of resultant solution in 1 ml of 0.9% saline	15.6 pg/ml
1:2	1 ml of resultant solution in 1 ml of 0.9% saline	7.8 pg/ml
1:2	1 ml of resultant solution in 1 ml of 0.9% saline	3.9 pg/ml

4.3.2 Results

Table 3.7b: Experiment 2: raw data and the mean of duplicate results. Standards and QCs shown.

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)
Standard 8	1098	> max
Standard 7	535	474.1
Standard 6	255	266
Standard 5	86	73.3
Standard 4	39	43.5
Standard 3	19	23.0
Standard 2	8	< min
Low Quality control (QC)	43 (28-58)	28.5
High Quality control (QC)	121 (89.5-152.5)	122.1
Sample 1	250	894.4
Sample 2	125	212.3
Sample 3	62.5	52.4
Sample 4	31.3	< min
Sample 5	15.6	< min
Sample 6	7.8	12.6
Sample 7	3.9	< min

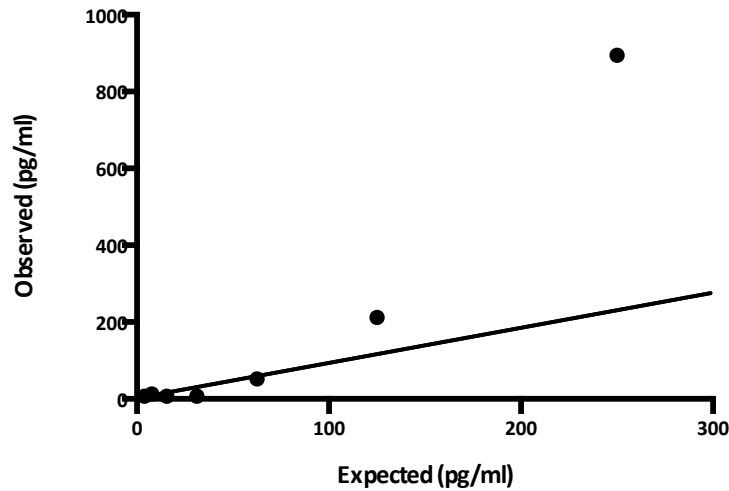


Figure 3.4: Validation experiment 2: Scatter graph depicting the relationship between observed and expected values when Synacthen is serially diluted in 0.9% saline. Values less than the minimum standard are plotted as 7 pg/ml. Line denotes perfect positive correlation (ideal fit).

4.3.3 Interpretation

The QCs were acceptable and the standard curve a reasonably good fit. This was the first assay set up and run by the author and the results showed poor correlation between the duplicate samples. The results reflected the prediction that the most sensitive part of the curve was around 250 pg/ml. The curve (*vide supra* figure 3.4) may appear logarithmic, or to be the bottom part of a sigmoidal curve, however there are too few data points, with the possible undue influence of outliers, to draw significant conclusions.

4.4 1 mcg dilution accuracy experiment (performed between validation experiments 2 and 3)

Having selected 250 pg/ml as the most sensitive part of the curve an experiment was carried out looking at the reliability and accuracy of diluting Synacthen to 1 mcg for use in the low-dose Short Synacthen Test (LDSST). The literature describing dilution strategies for the LDSST coupled with the recent British Society of Paediatric Endocrinology and Diabetes (BSPED) survey had revealed more than 14 different dilution methods (Elder, Sachdev et al. 2012).

In this experiment ten of the dilution strategies were chosen (based on popularity) and replicated five times, with three samples taken from each bag of diluent. These 150 samples were diluted in 0.9% saline to a presumed concentration of 250 pg/ml and run in duplicate on the hACTH RIA. The results were all below the lower limit of detection. Higher concentration solutions (from the same 1 mcg dilution accuracy experiment), which had been stored for six weeks and frozen at -20°C, were run. The 25,000, 10,000 and 5000 pg/ml samples analysed were all below the lower limit of detection.

It was thought that these results may have been due to the inherent instability of Synacthen, with rapid degradation at room temperature. The experiment making up the 1 mcg dilutions of Synacthen and sampling from them had been performed on a hot day in July 2010, with faulty air conditioning in the laboratory. Samples were left unrefrigerated for considerably longer than would

be recommended due to the large numbers and this is likely to have resulted in degradation of the Synacthen to undetectable levels.

4.5 Validation experiment 3 – Synacthen stability

It is widely recognised that ACTH and Synacthen rapidly degrade. Little exists in the literature about Synacthen stability. Alliance Pharmaceuticals were approached but were unable to provide stability data. They recommend that Synacthen be protected from light as it rapidly degrades and should be stored between 2-8°C and not be exposed to temperatures of more than 8°C for more than a few hours (personal communication, Alliance Pharmaceuticals, Wiltshire, UK). A group using the MP Biomedicals hACTH RIA reported no difference between results of Synacthen samples processed immediately and those left at room temperature for 60 minutes (Wade, Baid et al. 2010). They did however find, as others have before, that plastic tubing reduces recovery of Synacthen (22-59% loss), presumed to be by adsorptive losses (Murphy, Livesey et al. 1998, Wade, Baid et al. 2010). In view of the stability concerns following the 1 mcg dilution accuracy work an experiment was devised to look at Synacthen degradation over time (performed prior to the publication of Wade *et al's* paper).

4.5.1 Method

Synacthen stability was assessed by testing three different concentrations, diluted in 0.9% saline, and leaving them for increasing lengths of time at room

temperature prior to freezing. The final three Synacthen concentrations (25 pg/ml, 5 pg/ml and 2.5 pg/ml) were separated into eight aliquots of approximately 1 ml each. The first was frozen immediately, the others were left at room temperature for the following times: 15 minutes, 30 minutes, one hour, two hours, four hours, six hours and 24 hours. All samples were frozen at -20°C and were then thawed, vortexed and run six days later.

Table 3.8a: Dilution ratios, summarised method and resultant solutions of experiment 3. Final solutions assayed displayed in bold.

Dilution ratio	Dilution method	Resultant concentration
1:100	0.1ml of 250mcg/ml Synacthen in 9.9 ml of 0.9% saline	2500 ng/ml
1:100	0.1ml of 2500ng/ml solution in 9.9 ml of 0.9% saline	25,000 pg/ml
1:100	0.1ml Of 25,000pg/ml solution in 9.9 ml of 0.9% saline	250 pg/ml
1:10	1 ml of 250 pg/ml solution in 9 ml of 0.9% saline	25 pg/ml
1:50	0.2 ml of 250 pg/ml in 9.8 ml of 0.9% saline	5 pg/ml
1:100	0.1 ml of 250 pg/ml in 9.9 ml of 0.9% saline	2.5 pg/ml

4.5.2 Results

Table 3.8b: Standards and QCs shown for experiment 3. The mean of duplicate results displayed.

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)
Standard 8	1098	> max
Standard 7	535	229.9
Standard 6	255	129.3
Standard 5	86	86.1
Standard 4	39	37.2
Standard 3	19	26.9
Standard 2	8	< min
Low Quality control (QC)	43 (28-58)	61.5
High Quality control (QC)	121 (89.5-152.5)	80.5

All the samples from all three dilutions and at all time points gave results more than the maximum standard.

4.5.3 Interpretation

These results were very unexpected, especially in view of the low concentrations chosen to test, and were in stark contrast to those obtained previously. The standard curve and QCs, although poor, were not felt to be an adequate explanation. A dilution error may have occurred such that higher concentrations than thought were being tested. An alternative interpretation is that the disparity was due to a matrix effect.

The matrix describes all the components in the mixture other than the analyte (ACTH(1-24)). The effect the matrix rather than the analyte has on the results is known as the matrix effect. The 250 mcg/ml vial of Synacthen contains acetic acid, sodium acetate, sodium chloride and water (Alliance Pharmaceuticals 2011). It was considered that Synacthen may behave differently to ACTH in the 0.9% saline diluent, causing it to have a different 3-dimensional configuration and either increase or decrease binding in the assay. Analogues or related compounds may cross-react more or less than the analyte the assay is designed to detect as was found by Darmon *et al* who noted Synacthen cross-reacting five times more on a mass basis than ACTH(1-39) when using a similar RIA (Darmon, Dadoun et al. 1999). Why such disparate results were obtained when using the same matrix is difficult to explain, however it was felt important to investigate this as a cause of the inconsistent results.

4.6 Validation experiment 4

Despite not having satisfactorily validated the hACTH RIA for use detecting Synacthen considerable time constraints meant that study volunteer samples (EDTA plasma samples containing Synacthen following either i.v or intranasal (i.n) administration (*vide infra, chapter 4*) were processed prior to validation experiment 4. The results from the volunteer samples demonstrated considerable inter-individual variation in plasma Synacthen levels following administration with 1 mcg of i.v Synacthen (*table 3.9*)(mean peak plasma Synacthen level was 222.7 pg/ml (SD 87 pg/ml) (range 103.6 to 360.8 pg/ml) yet

detection of Synacthen in a consistent and interpretable manner in plasma (*vide infra*, chapter 4, figure 4.4a) giving credence to the matrix effect hypothesis.

Table 3.9 Ranges, means and standard deviations (SDs) (in brackets) of plasma Synacthen on hACTH RIA for 11 volunteers after administration of 1 mcg i.v Synacthen.

Time (mins)	-1	5	10	15	20
Plasma Synacthen range (mean and SD) (pg/ml)	22.4-168.7 (48.3 ± 39.2)	162.4-463.7 (261.7 ± 104.8)	56.2-273.2 (108.2 ± 54.4)	41.1-196.1 (71.5 ± 41.4)	32.7-192.7 (60.3 ± 43.2)
Time (mins)	30	40	50	60	75
Plasma Synacthen range, mean and SD (pg/ml)	31.5-189.1 (56.6 ± 43.5)	24.5-174.7 (51.6 ± 40.3)	25.1-173.9 (50.8 ± 40.2)	22.9-162.7 (48.3 ± 37.4)	21.8-162.8 (48.8 ± 36.7)
Time (mins)	90	120	150	180	
Plasma Synacthen range, mean and SD (pg/ml)	25.8-165.4 (46.4 ± 38.4)	21.8-166 (48.3 ± 38.7)	15.3-172.4 (46.2 ± 40.8)	11.3-155.5 (45.0 ± 36.2)	

4.6.1 Method

In order to investigate whether the inconsistent response of the assay was due to the matrix effect Synacthen was serially diluted in two different matrices: 0.9% saline and Phosphate buffered saline with bovine serum albumin (PBS BSA), also known as Midgley's Buffer (*table 3.10a*).

Phosphate buffered saline with bovine serum albumin is a more physiological buffer with a pH of 7.4 (pH of normal saline is approximately 5.5). It was chosen to more accurately replicate the pH and ionic strength found in human plasma. Plasma is essentially a phosphate buffer in saline with additional proteins. The 3-

dimensional structure of fragile peptides is known to unravel in solutions of low ionic strength and thus PBS BSA offers some protection. It is made up in the RHH laboratory and contains the following constituents: phosphate disodium hydrogen phosphate dihydrate 7.5g, sodium dihydrogen phosphate dihydrate 1.2g, EDTA 37.3g, sodium chloride 41.1g, bovine saline albumin 50g, sodium azide 5g in 5 litres of distilled water.

Table 3.10a: Dilution ratios, summarised method and resultant solutions of experiment

4. Final solutions assayed displayed in bold.

Dilution ratio	Dilution method	Resultant concentration
1:100	0.1ml of 250mcg/ml Synacthen in 9.9 mls of 0.9% saline and PBS BSA buffer	2500 ng/ml
1:100	0.1ml of 2500 ng/ml solution in 9.9 mls of 0.9% saline and PBS BSA buffer	25 ng/ml
1:25	0.1ml of 25 ng/ml solution in 2.4mls of 0.9% saline and PBS BSA buffer	1000 pg/ml
1:2	0.5ml of 1000 pg/ml solution in 0.5 ml of 0.9% saline and PBS BSA buffer	500 pg/ml
1:2	0.5ml of 500 pg/ml solution in 0.5 ml of 0.9% saline and PBS BSA buffer	250 pg/ml
1:2	0.5ml of 250 pg/ml solution in 0.5 ml of 0.9% saline and PBS BSA buffer	125 pg/ml
1:2	0.5ml of 125 pg/ml solution in 0.5 ml of 0.9% saline and PBS BSA buffer	62.5 pg/ml
1:2	0.5ml of 62.5 pg/ml solution in 0.5 ml of 0.9% saline and PBS BSA buffer	31.3 pg/ml
1:2	0.5ml of 31.3 pg/ml solution in 0.5 ml of 0.9% saline and PBS BSA buffer	15.6 pg/ml
1:2	0.5ml of 15.6 pg/ml solution in 0.5 ml of 0.9% saline and PBS BSA buffer	7.8 pg/ml
1:2	0.5ml of 7.8 pg/ml solution in 0.5 ml of 0.9% saline and PBS BSA buffer	3.9 pg/ml

Due to the assumed instability of Synacthen at room temperature the conditions were controlled as far as possible. The dilutions and the assay set up for all experiments from this point onwards were performed in an ice bath at approximately 4°C and the diluents and Synacthen were refrigerated up until the moment of use. Measures to improve the speed and efficiency were additionally taken to reduce the overall experiment time.

4.6.2 Results

Table 3.10b: Experiment 4 raw data. Standards and QCs shown

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)	
		<u>0.9% saline</u>	<u>PBS BSA</u>
Standard 8	1098		
Standard 7	535		
Standard 6	255		
Standard 5	86		
Standard 4	39		
Standard 3	19		
Standard 2	8		
Low Quality control (QC)	43 (28-58)		
High Quality control (QC)	121 (89.5-152.5)		
		<u>0.9% saline</u>	<u>PBS BSA</u>
Sample 1	1000	> max	120.3
Sample 2	500	490.3	59.5
Sample 3	250	315.7	39.5
Sample 4	125	94.7	20.3
Sample 5	62.5	23.4	14.5
Sample 6	31.3	15.1	< min
Sample 7	15.6	< min	< min
Sample 8	7.8	< min	10.2
Sample 9	3.9	< min	14.5

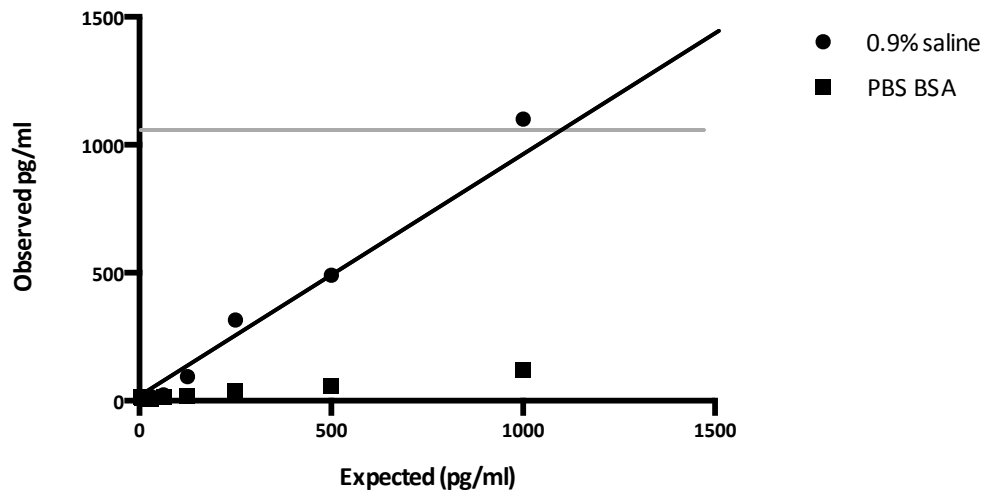


Figure 3.5: Validation experiment 4: Scatter graph depicting the relationship between observed and expected values when Synacthen is serially diluted in 0.9% saline and PBS BSA. Values less than the minimum standard are plotted as 7 pg/ml and those more than the maximum standard are plotted as 1100 pg/ml. The grey line denotes the maximum measureable value. The black line denotes perfect positive correlation.

4.6.3 Interpretation

The QCs and standards were satisfactory, perhaps indicating that with repeated assay set up the author's technical abilities were improving and causing less discrepant results. Unlike previous experiments the serial dilutions with saline appeared to show some linearity, with observed and expected values correlating well, especially between 150-500 pg/ml. Results with PBS BSA showed minimal detection of Synacthen. Saline was therefore assumed to be an appropriate diluent, with the controlled temperature conditions having prevented significant Synacthen degradation, and perhaps surprisingly a more suitable matrix compared with PBS BSA.

4.7 Validation experiment 5

In order to validate the use of the hACTH RIA for detecting Synacthen it was necessary to repeat experiment 4 in an attempt to reproduce the closely correlated results.

4.7.1 Method

Serial dilutions from 1000 to 31.25 pg/ml were made up as detailed in *table 3.11a*. Results from experiment 4 had indicated a lack of sensitivity when analysing the most dilute samples, these were removed and replaced with additional samples in the most sensitive (steepest) part of the curve.

Table 3.11a: Dilution ratios, summarised method and resultant solutions of experiment**5. Final solutions assayed displayed in bold.**

Dilution ratio	Dilution method	Resultant concentration
1:100	0.1ml of 250mcg/ml Synacthen in 9.9 mls of 0.9% saline	2500 ng/ml
1:100	0.1ml of 2500 ng/ml solution in 9.9 mls of 0.9% saline	25 ng/ml
1:25	0.1ml of 25 ng/ml solution in 2.4mls of 0.9% saline	1000 pg/ml
1:15	0.25ml of 1000pg/ml and 0.25ml of 500pg/ml solution	750 pg/ml
1:2	0.5ml of 1000 pg/ml solution in 0.5 ml of 0.9% saline	500 pg/ml
1:2	0.25ml of 750 pg/ml solution in 0.25 ml of 0.9% saline	375 pg/ml
1:2	0.5ml of 500 pg/ml solution in 0.5 ml of 0.9% saline	250 pg/ml
1:2	0.25ml of 375 pg/ml solution in 0.25 ml of 0.9% saline	187.5 pg/ml
1:2	0.5ml of 250 pg/ml solution in 0.5 ml of 0.9% saline	125 pg/ml
1:2	0.25ml of 187.5 pg/ml solution in 0.25 ml of 0.9% saline	94 pg/ml
1:2	0.5ml of 125 pg/ml solution in 0.5 ml of 0.9% saline	62.5 pg/ml
1:2	0.25ml of 94 pg/ml solution in 0.25 ml of 0.9% saline	47 pg/ml
1:2	0.5ml of 62.5 pg/ml solution in 0.5 ml of 0.9% saline	31.3 pg/ml

4.7.2 Results

Table 3.11b: Experiment 5 raw data. Standards and QCs shown

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)
Standard 8	1098	1063.3
Standard 7	535	479.4
Standard 6	255	302.2
Standard 5	86	67.5
Standard 4	39	47.1
Standard 3	19	20.8
Standard 2	8	10.5
Low Quality control (QC)	43 (28-58)	34.3
High Quality control (QC)	121 (89.5-152.5)	84.5
Sample 1	1000	411.9
Sample 2	750	72.5
Sample 3	500	94.9
Sample 4	375	9.87
Sample 5	250	21.2
Sample 6	187.5	< min
Sample 7	125	< min
Sample 8	94	< min
Sample 9	62.5	40.4
Sample 10	47	< min
Sample 11	31.3	< min

4.7.3 Interpretation

The standard curve was a good fit, although the high QC was poor. The results were disappointing with the linearity seen in experiment 4 not replicated. Once again the matrix effect was considered, whereby Synacthen with its different molecular weight and 3-D configuration, behaves differently in the non-physiological media compared with ACTH(1-39). Despite the unexpected results from experiment 4, where saline had appeared better than PBS BSA, it was considered reasonable to investigate this area further.

4.8 Validation experiment 6

To investigate the matrix effect further three different buffers were compared with serial dilutions of Synacthen.

4.8.1 Method

The three buffers chosen were 0.9% saline, phosphate buffer saline (PBS) and PBS BSA. The 0.9% saline is the least physiological and PBS BSA the most physiological diluent. PBS is made up to a pH of 7.4 but has a different ionic strength to plasma due to its lack of protein. *Table 3.12a* displays the dilutions performed with all three buffers.

Table 3.12a: Dilution ratios, summarised method and resultant solutions of experiment 6. Final solutions assayed displayed in bold. All dilution methods were done using the three diluents: 0.9% saline, PBS and PBS BSA.

Dilution ratio	Dilution method	Resultant concentration
1:100	0.1ml of 250mcg/ml Synacthen in 9.9 mls of the 3 diluents	2500 ng/ml
1:10	1ml of 2500 ng/ml solution in 9 mls of the 3 diluents	250 ng/ml
1:10	1ml of 250 ng/ml solution in 9 mls of the 3 diluents	25 ng/ml
1:10	1ml of 25 ng/ml solution in 9 mls of the 3 diluents	2500 pg/ml
1:25	0.1ml of 2500 pg/ml solution in 2.4 mls of the 3 diluents	1000 pg/ml
1:2	1ml of 1000 pg/ml solution in 1 ml of the 3 diluents	500 pg/ml
1:2	1ml of 500 pg/ml solution in 1 ml of the 3 diluents	250 pg/ml
1:2	1ml of 250 pg/ml solution in 1 ml of the 3 diluents	125 pg/ml

4.8.2 Results

Table 3.12b: Experiment 6 raw data. Standards and QCs shown

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)		
Standard 8	1098	> max		
Standard 7	535	526.6		
Standard 6	255	239.5		
Standard 5	86	97.7		
Standard 4	39	28.2		
Standard 3	19	19.4		
Standard 2	8	9.4		
Low Quality control (QC)	43 (28-58)	22.9		
High Quality control (QC)	121 (89.5-152.5)	122.8		
		0.9%	PBS	PBS BSA
Sample 1	2500	> max	> max	> max
Sample 2	1000	340.4	499.23	> max
Sample 3	500	42.2	115.5	> max
Sample 4	250	< min	102.6	571.3
Sample 5	125	< min	40.3	295.8

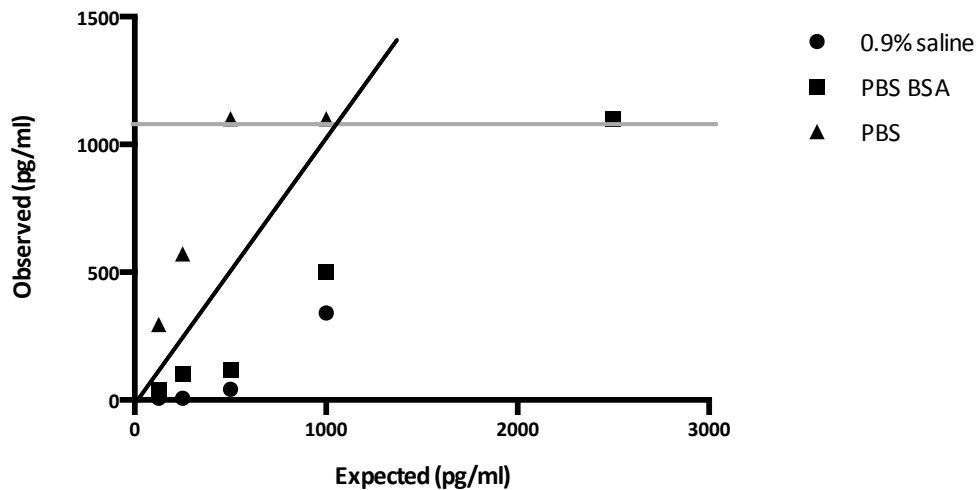


Figure 3.6: Validation experiment 6: Scatter graph depicting the relationship between observed and expected values when Synacthen is serially diluted in 0.9% saline, PBS BSA and PBS. Values less than the minimum standard are plotted as 7 pg/ml and those more than the maximum standard are plotted as 1100 pg/ml. The grey line denotes the maximum measurable value. The black line denotes the perfect positive correlation.

4.8.3 Interpretation

The standard curve was a reasonable fit. The high QC was good, the low QC poor. Some linearity was evident with each of the buffers, although with the small number of data points and some values reaching the limit of detection interpretation is difficult. The results demonstrated that Synacthen in PBS BSA behaved differently compared with 0.9% saline and PBS, possibly explained by matrix differences. There was a lack of consistency of results with the PBS BSA, results from experiments 4 and 6 differing considerably. It was becoming increasingly difficult to explain why the assay, which appears to function when using human plasma, does not produce consistent and reproducible results in a serial dilution experiment conducted in a more physiological matrix. The

possibility of an interfering molecule, variably cross-reacting and causing the Synacthen to bind inconsistently, was considered.

4.9 Validation experiment 7

In order to examine the issue of assay inconsistency and address the concerns that this was being caused by dilution errors an experiment was performed with stock solutions of Synacthen dilutions, run on three different assays on three different days.

4.9.1 Method

A stock solution of 1000 pg/ml was made up by a three-step dilution of 250 mcg/ml in both 0.9% saline and PBS. These were divided into five aliquots of 2 ml and frozen at -20°C. One week later one aliquot of each of the diluents was thawed and further dilutions made and run (*table 3.13a*). This was repeated on two further occasions 13 and 14 days later. The three assays were from the same manufacturing lot.

Table 3.13a: Dilution ratios, summarised method and resultant solutions of experiment

7. Final solutions assayed displayed in bold. All dilution methods were done using both 0.9% saline and PBS.

Dilution ratio	Dilution method	Resultant concentration
1:25	0.4ml of 250 mcg/ml Synacthen in 9.6 mls of both diluents	10 mcg/ml
1:100	0.1ml of 10 mcg/ml solution in 9.9 mls of both diluents	100 ng/ml
1:100	0.1ml of 100 ng/ml solution in 9.9 mls of both diluents	1000 pg/ml
1:1.5	0.5ml of 1000pg/ml and 0.5ml of 500pg/ml solution	750 pg/ml
1:2	0.5ml of 1000 pg/ml solution in 0.5 ml of both diluents	500 pg/ml
1:2	0.5ml of 750 pg/ml solution in 0.5 ml of both diluents	375 pg/ml
1:2	0.5ml of 500 pg/ml solution in 0.5 ml of both diluents	250 pg/ml
1:2	0.5ml of 375 pg/ml solution in 0.5 ml of both diluents	187.5 pg/ml
1:2	0.5ml of 250 pg/ml solution in 0.5 ml of both diluents	125 pg/ml

4.9.2 Results

Table 3.13b: Experiment 7a raw data. Standards and QCs shown

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)	
Standard 8	1098	> max	
Standard 7	535	491.8	
Standard 6	255	251.9	
Standard 5	86	93.6	
Standard 4	39	39.8	
Standard 3	19	19.2	
Standard 2	8	9.4	
Low Quality control (QC)	43 (28-58)	34.5	
High Quality control (QC)	121 (89.5-152.5)	120.0	
		0.9%	PBS
Sample 1	750	40.1	500.6
Sample 2	500	< min	328.4
Sample 3	375	< min	48.9
Sample 4	250	< min	70.8
Sample 5	187	< min	15.5
Sample 6	125	13.5	< min

Table 3.13c: Experiment 7b raw data. Standards and QCs shown

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)	
Standard 8	1098	> max	
Standard 7	535	576.3	
Standard 6	255	241.4	
Standard 5	86	91.8	
Standard 4	39	39.1	
Standard 3	19	21.9	
Standard 2	8	< min	
Low Quality control (QC)	43 (28-58)	48.7	
High Quality control (QC)	121 (89.5-152.5)	123.7	
		<u>0.9%</u>	<u>PBS</u>
Sample 1	750	> max	1025.7
Sample 2	500	853.3	674.6
Sample 3	375	543.1	530.7
Sample 4	250	79.4	255.8
Sample 5	187	106.7	72.9
Sample 6	125	40.9	50.1

Table 3.13d: Experiment 7c raw data. Standards and QCs shown

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)	
Standard 8	1098	> max	
Standard 7	535	491.8	
Standard 6	255	251.9	
Standard 5	86	93.6	
Standard 4	39	39.8	
Standard 3	19	19.2	
Standard 2	8	9.4	
Low Quality control (QC)	43 (28-58)	34.5	
High Quality control (QC)	121 (89.5-152.5)	120.0	
		0.9%	PBS
Sample 1	750	< min	593.1
Sample 2	500	< min	396.5
Sample 3	375	< min	63.6
Sample 4	250	< min	16.1
Sample 5	187	< min	< min
Sample 6	125	11.3	17.8

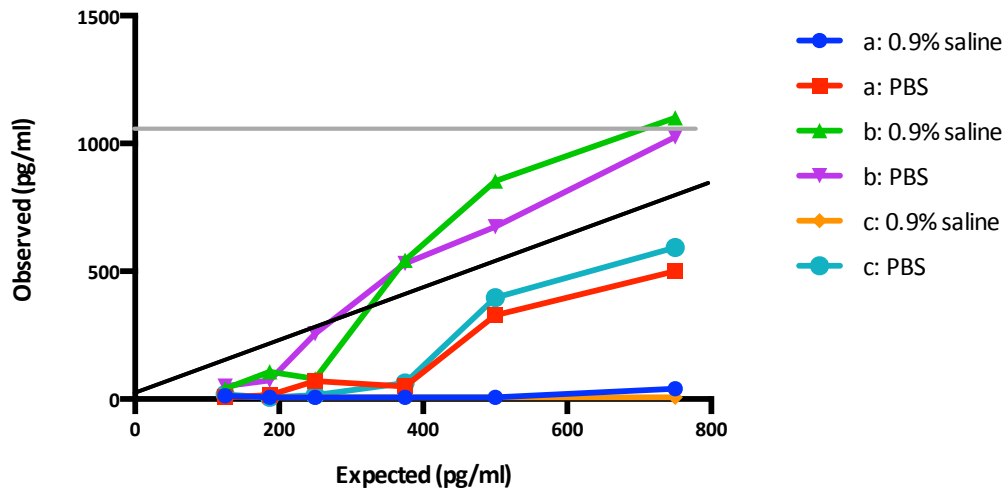


Figure 3.7: Validation experiment 7a-c: Line graph depicting the relationship between observed and expected values when Synacthen is serially diluted in 0.9% saline and PBS and samples are run on three different occasions. Values less than the minimum standard are plotted as 7 pg/ml and those more than the maximum standard are plotted as 1100 pg/ml. a, b and c denote the three experiments run on different days. The grey line denotes the maximum measurable value. The black line denotes the perfect positive correlation.

4.9.3 Interpretation

Once again the results were inconsistent and difficult to interpret. There was no discernable consistency between the three assays, 7a and 7c being similar yet markedly different to 7b. Both saline and PBS results were similarly low for 7a and 7c but very high in 7b. A dilution error may explain the higher results in 7b, however the progression of fall off (curve) does not entirely support this; the curve is a different shape, not just shifted left.

In contrast to previous results at the same dilutions, the saline results were unexpectedly low in 7a and 7c and unexpectedly high in 7b. The results with PBS

were more expected, with results lower than the diluted amount, as would be anticipated in a competitive RIA, especially one designed for the detection of a related but different molecule. However in both 7a and 7c the drop off, after initially promising results, at about 375 pg/ml, was precipitate and again difficult to explain. Although only limited interpretation is possible PBS looked to be the superior diluent.

4.10 Validation experiment 8

Having obtained interpretable results when Synacthen was assayed in human plasma, an attempt was made to further ameliorate the presumed matrix effect. This assumes the 3-dimensional configuration of Synacthen is different in non-physiological matrices and therefore a dilution experiment was performed using diluted pooled human plasma.

4.10.1 Method

Pooled human EDTA plasma was used as the diluent. Locally, ACTH samples are collected in EDTA, booked in at clinical chemistry where they are spun, separated and frozen until weekly batch analysis. Residual EDTA plasma following patient sample analysis was pooled, left at room temperature overnight to allow the endogenous ACTH to degrade and refrozen.

The pooled EDTA plasma was thawed and vortexed before serial dilutions were undertaken (*table 3.14a*). To ensure the ACTH in the pooled plasma was

sufficiently degraded a sample was analysed on the Immulite 2000 assay (Siemens).

EDTA (ethylenediaminetetraacetic acid) is a polyamino carboxylic acid with a hexagonal structure, which in part confers its property as a chelating agent. If the enzymes that degrade a molecule are calcium or magnesium dependent the EDTA removes them by chelation thus preventing significant loss by degradation before analysis. ACTH (and Synacthen) samples are collected in EDTA so that endopeptidases are chelated and prevented from breaking down the ACTH.

Table 3.14a: Dilution ratios, summarised method and resultant solutions of experiment 8. Final solutions assayed displayed in bold.

Dilution ratio	Dilution method	Resultant concentration
1:25	0.2 ml of 250mcg/ml Synacthen in 4.8 mls of EDTA plasma	10 mcg/ml
1:100	0.1 ml of 10 mcg/ml solution in 9.9 mls of EDTA plasma	100 ng/ml
1:100	0.1 ml of 100 ng/ml solution in 9.9 mls of both diluents	1000 pg/ml
1:1.5	0.5 ml of 1000pg/ml and 0.5ml of 500pg/ml solutions	750 pg/ml
1:2	1 ml of 1000pg/ml and 1ml EDTA plasma	500 pg/ml
1:2	0.5 ml of 750 pg/ml solution and 0.5ml of EDTA plasma	375 pg/ml
1:2	1 ml of 500 pg/ml solution in 1 ml EDTA plasma	250 pg/ml
1:2	0.5 ml of 375 pg/ml solution and 0.5ml of EDTA plasma	187.5 pg/ml
1:2	1 ml of 250 pg/ml solution in 1 ml EDTA plasma	125 pg/ml
1:2	0.5 ml of 375 pg/ml solution in 0.5 ml of both diluents	94 pg/ml

4.10.2 Results

Table 3.14b: Experiment 8 raw data. Standards and QCs shown

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)
Standard 8	1098	1058.1
Standard 7	535	508.7
Standard 6	255	259.6
Standard 5	86	83
Standard 4	39	33.3
Standard 3	19	19.5
Standard 2	8	13.3
Low Quality control (QC)	43 (28-58)	43.4
High Quality control (QC)	121 (89.5-152.5)	125.5
Sample 1	750	> max
Sample 2	500	> max
Sample 3	375	> max
Sample 4	250	1058.2
Sample 5	187	1015
Sample 6	125	871.8
Sample 7	94	812.1

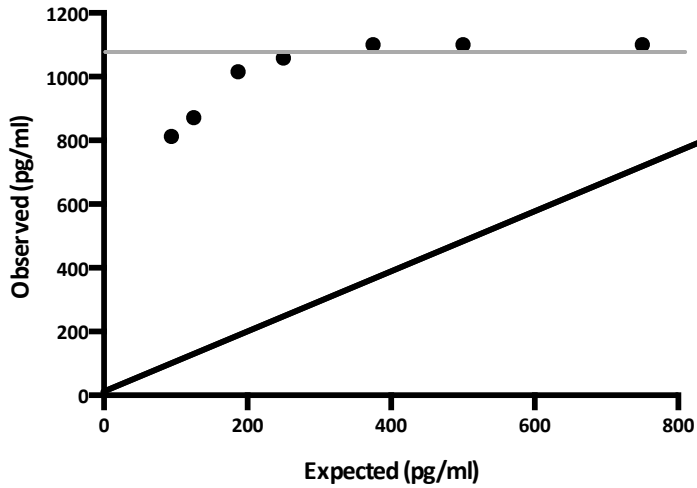


Figure 3.8: Validation experiment 8: Scatter graph depicting the relationship between observed and expected values when Synacthen is serially diluted in EDTA plasma. Values more than the maximum standard are plotted as 1100 pg/ml. The grey line denotes the maximum measurable value. The black line denotes the perfect positive correlation.

The pooled plasma was analysed on the ACTH Immulite 2000 assay (Siemens) and results given below (table 3.14c).

Table 3.14c: Experiment 8 pooled plasma ACTH results from Siemens Immulite 2000.

Date of addition to pool and freezing	ACTH result (pg/ml)
22/9/11	14.9
29/11/11	45
6/10/11	66.4
14/10/11	159
Pooled	76.4
Normal range: 9am <46 pg/ml; Midnight <15 pg/ml	

4.10.3 Interpretation

In order to avoid the potential deleterious effects of a further freeze-thaw cycle the Immulite ACTH results were not known prior to the Synacthen dilution experiment. Unfortunately the ACTH in the pooled plasma had not sufficiently degraded and therefore contained a considerably higher concentration of ACTH than anticipated. It is likely that the high background ACTH, detectable by the RIA, contributed to the high values obtained, although should not have accounted for the lack of correlation between observed and expected values.

4.11 Validation experiment 9

With considerable doubt being cast over the ability of the RIA to reliably detect Synacthen, another experiment was planned to establish whether it could accurately detect ACTH.

4.11.1 Method

The three external quality assurance samples for ACTH, provided to the lab by UK NEQAS (UK National External Quality Assessment Service) for quality control purposes and to allow comparison with other laboratories and other methods, were run on the hACTH RIA (October 2011). These were run in duplicate on the same assay used in experiment 8.

4.11.2 Results

Table 3.15: Experiment 9: UK NEQAS samples (performed on same assay as used in experiment 8). All results in pg/ml.

	Base pool EDTA plasma	Base pool EDTA plasma with synthetic ACTH 153.6	Base pool EDTA plasma with synthetic ACTH 307.2
MP Biomedicals hACTH RIA	56.5	218.4	643.3
UKNEQAS results	7.2	160.5	339.3
NEQAS results with + bias of 27.1%	9.2	204.0	431.2

The UK NEQAS results shown are the target ACTH values. The UK NEQAS data did not display the same method as the MP Biomedicals RIA for direct comparison. The most similar method was the BRAHMS RIA but only bias data were given, showing a positive bias of 27.6% (N = 1). The MP Biomedicals results obtained were accordingly adjusted for this positive bias as shown in *table 3.15*.

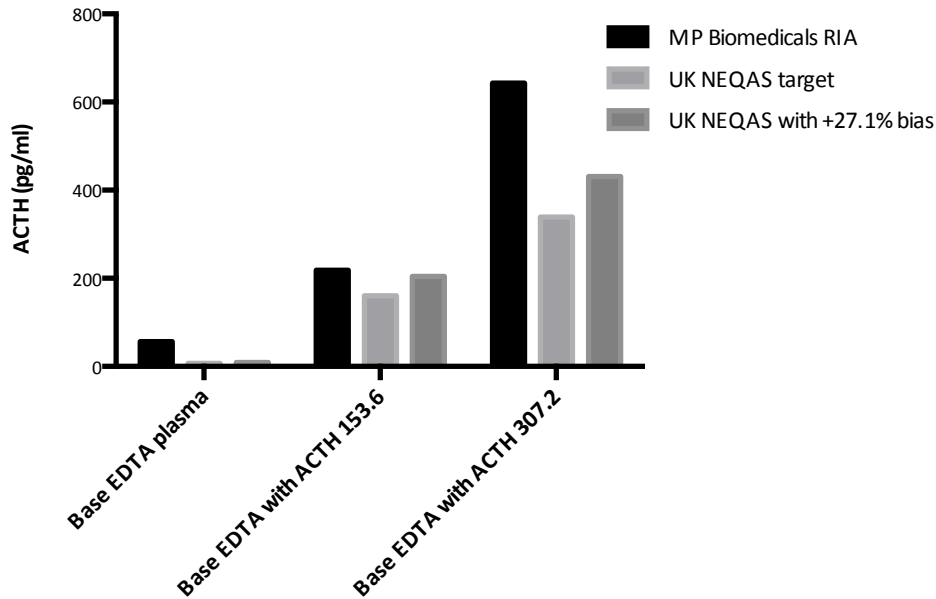


Figure 3.9: Validation experiment 9: Bar chart depicting the ACTH values obtained when UK NEQAS reference samples were run on MP Biomedicals' RIA. Adjustment for the positive bias seen with similar method is also shown.

4.11.3 Interpretation

Correction for the positive bias seen with the similar method improved the significant disparity between the expected (UK NEQAS target value) and the observed results. However the RIA results, particularly for the baseline value and higher concentration pool, were disappointing. Using an hACTH assay to detect Synacthen has obvious limitations but the NEQAS samples are synthetic hACTH(1-39) and should have been detected relatively accurately. This led to the supposition that there was something fundamentally wrong with ability of the RIA to detect ACTH in addition to Synacthen.

4.12 Validation experiment 10

As validation of the assay was proving difficult it was decided to further test its reliability.

4.12.1 Methods

Re-analysis of three research volunteer samples (*vide infra*, chapter 4) was undertaken and the results from the two runs compared. There was an eleven-month gap between the first and the second analysis, during which the samples were kept at -80°C. In order to be reanalysed they had undergone an additional freeze-thaw cycle. The samples were run on the assay used in both experiments 8 and 9.

4.12.2 Results

Table 3.16: Experiment 10 NeSST study volunteer sample repeats (performed on same assay as used in experiment 8).

	Volunteer sample 1 (25mcg nasal 0 mins)	Volunteer sample 2 (25mcg nasal 15 min)	Volunteer sample 3 (25mcg nasal 50 mins)
1 st Analysis November 2010	12.7	13.8	11.8
2 nd Analysis October 2011	42.6	53.7	49.3

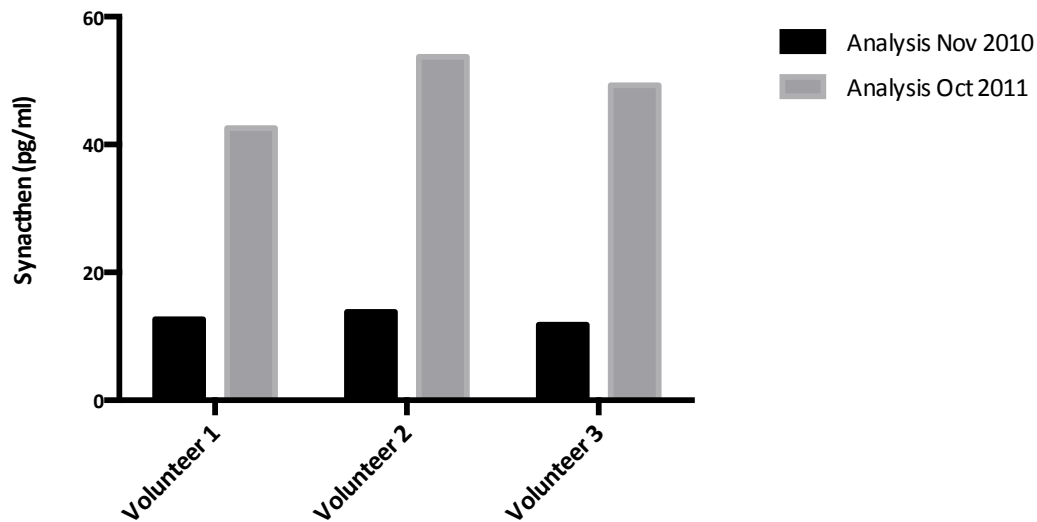


Figure 3.10: Validation experiment 10: Bar chart depicting the Synacthen values obtained when three volunteer samples run in November 2010 and re-run in October 2011 were compared.

4.12.3 Interpretation

Unexpectedly rather than lower values, caused by additional degradation associated with an extra freeze-thaw cycle, the results were higher. The cross-reactivity of the Synacthen epitope and the tracer antibody seems to vary, yielding inconsistent results. Whether the additional freeze-thaw cycle exacerbated this effect can only be postulated. The epitope recognised by the RIA, although structurally the same in 2-dimensions, may be presented differently in ACTH and Synacthen in 3-dimensions. This may cause times when little is detected and others when more than 100% cross-reactivity is seen.

4.13 Assay meeting and manufacturer contact

Following 17 months of work attempting to validate the MP Biomedicals hACTH RIA to reliably detect plasma Synacthen the results of the various experiments continued to be contradictory and difficult to explain. There were two problems: the inability to validate the RIA for use measuring Synacthen, with erratic results during serial dilution experiments, and high baseline plasma ACTH values in dexamethasone suppressed research volunteers. Only discussion of the first problem is covered in this chapter (*vide infra chapter 4, section 5.1* for discussion of assay interference).

A meeting with the following attendees:

- PhD supervisor (Dr N Wright)
- Supervisor of laboratory work (Mr M Loxley)
- Senior Biomedical Scientist (BMS) in endocrine laboratory (Ms V Moyse)
- Associate Specialist in Chemical Pathology (Dr K Page).

All three of the laboratory personnel have a particular interest and expertise in RIA and endocrine laboratory medicine and had all been involved to a greater or lesser extent advising on the laboratory methodology of the project.

Data pertaining to the assay, the author presented the methodology and results of each one of the experiments to date with pertinent literature from the few other groups who have measured Synacthen. The main discussion points of the

meeting are presented below and the plan for further validation work follows (*section 4.13.1*)

1. There was no significant concern about my assay preparation technique. Although the QCs presented (*table 3.23*) revealed some inconsistencies it was felt they were not adequate to explain the lack of interpretable results. The low QC was less than the high QC and thus the trend was adequate even if individual results were poor.
2. The possibility of conformational change in the Synacthen on freezing was raised, although most of the experiments were performed with fresh samples. No information was available from Alliance Pharmaceuticals about the impact of freezing, although it is not advised with the depot preparation (which was not used in any of the experiments). ACTH is stable when frozen and on reviewing the constituents of the Synacthen vial it seemed unlikely that freezing would have a significant impact.
3. The ambient laboratory temperature may have caused Synacthen to degrade, although after steps were taken to ameliorate this the results continued to show considerable inconsistency, incompatible with degradation.
4. Some molecules have a greater affinity for RIA than others, with reports of 5 times greater binding of related molecules (Darmon, Dadoun et al. 1999). It was postulated that the antibody binds more easily to ACTH(1-24) compared with ACTH(1-39) however the results are inconsistent not just high, it was suggested that the binding of Synacthen may be variable and at times all or nothing.

5. Dr Page examined the molecular structure of Synacthen, commenting on its basic N-terminus and acidic C-terminus, causing attraction and conformational folding. He postulated that it may be a molecule that is particularly sensitive to changes in pH. I presented the methodology from two papers in which extraction techniques had been used and both had acidified down to a low pH (Krishnan, Ritchie et al. 1988, Alia, Villabona et al. 2006).

4.13.1 Plan for on-going validation work

- Perform an experiment in which the matrix is acidified, as described by Krishnan *et al* (Krishnan, Ritchie et al. 1988)
- Analyse the next batch of UK NEQAS ACTH samples
- Re-analyse more research volunteer samples from the NeSST study
- Perform an experiment in which whole blood is used as the matrix. The assay appeared to work more reliably in the volunteer samples and therefore it was postulated that more reliable results may be obtained when Synacthen is directly added to and serially diluted in whole blood (an *in vitro* version of the Synacthen Test).
- Produce Synacthen standards in order to calibrate the assay

4.13.2 Correspondence with MP Biomedicals

The manufacturer of the hACTH RIA was contacted to raise concerns about the assay and request advice about the use of their assay for our purposes. The data were shared with regard to the high baseline values following dexamethasone suppression (*chapter 4, section 4.3.1*), inconsistent results with serial Synacthen dilution experiments, UK NEQAS comparison and different results when re-analysing the research volunteer samples. Although not immediately forthcoming a response was forthcoming.

Despite quoting 100% cross-reactivity in the hACTH RIA directional insert, MP Biomedicals have never validated the assay for Synacthen. It was an assumption made based on the fact that the assay recognises the 5-18 portion of the molecule, shared by both ACTH and Synacthen. During further correspondence they were unable to offer an explanation for our inability to obtain consistent results with ACTH or Synacthen.

4.14 Validation experiment 11

Following the assay meeting an experiment was performed as discussed above with an acidified buffer in an attempt to replicate the results of other studies (Krishnan, Ritchie et al. 1988, Alia, Villabona et al. 2006).

4.14.1 Methods

PBS BSA was acidified down to a pH of 3 with hydrochloric acid (HCL). A vial of 250 mcg/ml Synacthen was diluted in the acidified buffer to give the concentrations below (*table 3.17a*). All samples were run in quadruplicate in an attempt to increase the accuracy of results. VM (senior BMS) made up the acidified buffer, performed the dilutions and assay set-up, with the author present, in order to examine whether the previous results were partly attributable relative laboratory inexperience.

Table 3.17a: Dilution ratios, summarised method and resultant solutions of experiment**11. Final solutions assayed displayed in bold.**

Dilution ratio	Dilution method	Resultant concentration
1:1000	50 mcl of 250mcg/ml Synacthen in 50 mls acidified buffer	250 ng/ml
1:250	200 mcl of 250 ng/ml solution in 50 mls of acidified buffer	1000 pg/ml
1:1.5	0.5 ml of 1000pg/ml and 0.5ml of 500pg/ml solutions	750 pg/ml
1:2	0.5 ml of 1000 pg/ml solution in 0.5 mls of acidified buffer	500 pg/ml
1:2	0.5 ml of 750 pg/ml solution in 0.5 mls of acidified buffer	375 pg/ml
1:2	0.5 ml of 500 pg/ml solution in 0.5 mls of acidified buffer	250 pg/ml
1:2	0.5 ml of 375 pg/ml solution in 0.5 mls of acidified buffer	187.5 pg/ml
1:2	0.5 ml of 250 pg/ml solution in 0.5 mls of acidified buffer	125 pg/ml
1:2	0.5 ml of 187.5 pg/ml solution in 0.5mls of acidified buffer	94 pg/ml
1:2	0.5 ml of 125 pg/ml solution in 0.5 mls of acidified buffer	62.5 pg/ml
1:2	0.5 ml of 94 pg/ml solution in 0.5mls of acidified buffer	47 pg/ml
1:2	0.5 ml of 62.5 pg/ml solution in 0.5mls of acidified buffer	31.3 pg/ml
1:2	0.5 ml of 47 pg/ml solution in 0.5mls of acidified buffer	23.5 pg/ml

4.14.2 Results

Table 3.17b: Experiment 11 raw data. Standards and QCs shown

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)
Standard 8	1098	> max
Standard 7	535	470.9
Standard 6	255	244.4
Standard 5	86	94
Standard 4	39	33.3
Standard 3	19	20.9
Standard 2	8	< min
Low Quality control (QC)	43 (28-58)	23.0
High Quality control (QC)	121 (89.5-152.5)	83.4
Sample 1	1000	> max
Sample 2	750	> max
Sample 3	500	> max
Sample 4	375	> max
Sample 5	250	973.3
Sample 6	187.5	530.1
Sample 7	125	371.7
Sample 8	94	237.2
Sample 9	62.5	145.7
Sample 10	47	114
Sample 11	31.3	77.1
Sample 12	23	63.2
Sample 13 (unspiked acidified buffer)	<min	24.4

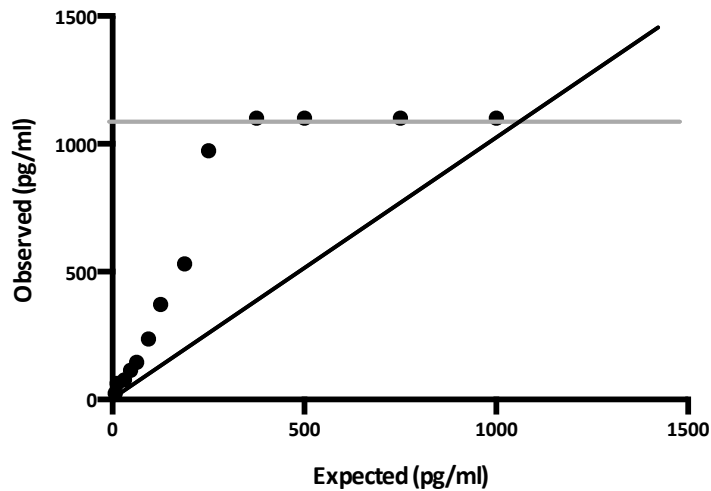


Figure 3.11: Validation experiment 11: Scatter graph depicting the relationship between observed and expected values when Synacthen is serially diluted in acidified PBS BSA. Values more than the maximum standard are plotted as 1100 pg/ml. The grey line denotes the maximum measurable value. The black line denotes the perfect positive correlation.

4.14.3 Interpretation

Despite an experienced BMS performing the dilutions and assay set-up the standard curve was a less good fit than in most previous experiments and the QCs were both more than 3 SDs below the acceptable range (but within 4 SDs). This may have been due to VM's lack of familiarity with this particular RIA. There appeared to be greater sensitivity at the lower concentrations, not always seen previously.

The unspiked (no Synacthen added) acidified buffer was analysed as having 24.4 pg/ml of ACTH, more than the lowest standard (8 pg/ml). Once again the values were considerably higher than expected, indicating either a greater binding affinity of Synacthen compared with ACTH(1-39) or an interfering molecule. A

relational change was seen whereby for each 1:1.5 dilution from 187.5 pg/ml down to 23 pg/ml there was an approximately 1.4 times concentration reduction. The expectation of these serial dilution experiments is not a perfect correlation between expected and observed values but a reproducible and consistent relationship between the two. Acidification of the buffer did appear to result in some linearity and therefore improved the reliable and accurate detection of Synacthen.

4.15 Validation experiment 12

The next UK NEQAS samples were run on the MP Biomedicals hACTH RIA and compared to results from other similar assays nationally.

4.15.1 Method

The three UK NEQAS samples were run in quadruplicate on the same assay as used in experiment 11, set up by the endocrine laboratory's senior BMS and under the same controlled conditions. The UK NEQAS materials are supplied lyophilised and made up prior to use. Due to the timing of the assay kit delivery from USA the reconstituted samples were frozen at -20°C then thawed and analysed the following week. As before the results were later compared to national data (N= 92) and data derived from centres using similar methods (N=1).

Additionally the low and high QCs from the Immulite 2000 (Siemens), the sandwich immunoassay used locally for ACTH quantification, were run on the

RIA. These were run in triplicate, under controlled conditions and set up by the same senior BMS.

Table 3.18a: Experiment 12a: UK NEQAS samples (performed on same assay as in expt 11).

	Base pool EDTA plasma	Base pool EDTA plasma with synthetic ACTH 219 ng/l	Base pool EDTA plasma with synthetic ACTH 878 ng/l
MP Biomedicals hACTH RIA	19.5	17.7	66.3
UKNEQAS results	19.8	311.8	1149.6
NEQAS results with + bias of 24.7%	24.6	388.8	1433.6

The UK NEQAS results shown are the target ACTH values. The UK NEQAS data did not display the same method as the MP Biomedicals RIA for direct comparison. The most similar method was the CIS ELISA IRMA but only bias data were given, showing a positive bias of 24.7% (N = 1). The MP Biomedicals results obtained were accordingly adjusted for this positive bias as shown in *table 3.18a*.

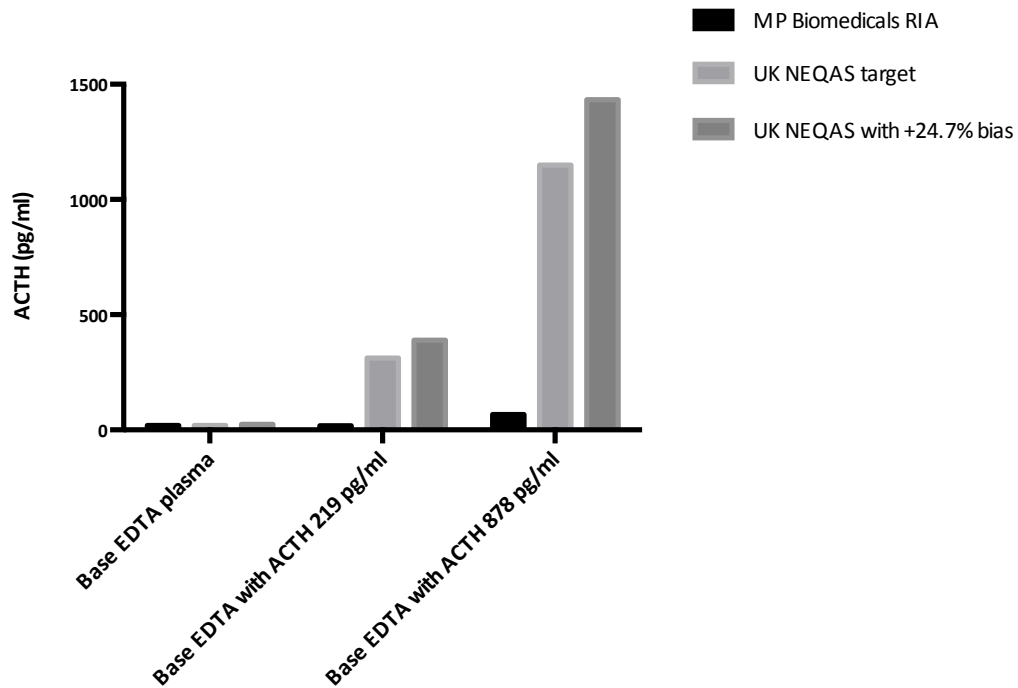


Figure 3.12: Validation experiment 12: Bar chart depicting the ACTH values obtained when UK NEQAS reference samples were run on MP Biomedicals' RIA. Adjustment for the positive bias seen with similar method is also shown.

Table 3.18b: Experiment 12b: Siemens' Immulite 2000 hACTH high and low QCs (performed on same assay as in experiment 11). Results in pg/ml

	High QC	Low QC
MP Biomedicals hACTH RIA	> max	262
Immulite 2000	434	33

4.15.2 Interpretation

In experiment 12 the hACTH RIA was being used to detect ACTH not Synacthen. This was to test the theory that it was the conformational change of the epitope seen in Synacthen as different to ACTH that was causing a lack of reliable

detection. It was concerning that both the UK NEQAS samples and the Immulite QCs showed very poor correlation with the values expected of an ACTH assay.

So few laboratories use RIA to measure ACTH that the most similar method from the UK NEQAS data for comparison, used by one centre only, was the CIS ELISA IRMA. It had a 24.7% positive bias from target on its submitted results, making the values obtained in experiment 12 even more disparate. The results from the two samples spiked with synthetic ACTH(1-39) are so much lower than expected as to make any possible variance caused by different assay methods irrelevant. The explanations for these results are an interfering molecule, a significant problem with the ability of the RIA to detect ACTH and Synacthen or that the freeze-thaw process in some way damaged the molecule, changing its 3-D configuration. It may be a combination as the results from experiment 9, whilst poor in comparison with the UK NEQAS targets, were considerably closer than those in experiment 11 and the difference may have been the freezing of the NEQAS samples.

4.16 Validation experiment 13

Concerns over a possible matrix effect had been tested with a number of different buffers and matrices in an attempt to emulate the interpretable results seen when the volunteer samples were analysed. To further investigate the ability of the RIA to detect Synacthen reliably in human plasma an experiment was conducted using Synacthen spiked human blood.

4.16.1 Method

On the same day as experiment 12 a further RIA was set up with samples of Synacthen spiked human plasma. The author had 30 mls of blood taken and stored in EDTA. The blood was left at room temperature for six hours to allow the endogenous ACTH to degrade. The whole blood was then spun and separated. The remaining 15 mls of plasma was spiked with Synacthen as displayed in *table 3.19a*. The two concentrations tested were chosen as those most likely to be on the steepest (most sensitive) part of the curve. The unspiked plasma was also run, both on the RIA and on the Siemens' Immulite 2000 sandwich assay for comparison. Samples were run in sextuplicate. Senior BMS, Victoria Moyse, performed the dilutions and set up the assay with the author.

Table 3.19a: Dilution ratios, summarised method and resultant solutions of experiment 13. Final solutions assayed displayed in bold.

Dilution ratio	Dilution method	Resultant concentration
1:1000	0.01ml of 250mcg/ml Synacthen in 9.99 mls of CJE's plasma	250 ng/ml
1:500	0.02ml of 250ng/ml Synacthen in 9.98 mls of CJE's plasma	500 pg/ml
1:2	1 ml of 500pg/ml and 1 ml of CJE's plasma	250 pg/ml
1:2	1 ml of 1000 pg/ml solution in 1 mls of CJE's plasma	125 pg/ml

4.16.2 Results

Table 3.19b: Experiment 13 raw data. The means of each duplicate and the sextuplicate results are displayed. Standards and QCs shown, SDs given in brackets after mean.

Sample/Standard	Expected (pg/ml)	Obtained (pg/ml)
Standard 8	1098	> max
Standard 7	535	468
Standard 6	255	278
Standard 5	86	75.7
Standard 4	39	42.2
Standard 3	19	23.2
Standard 2	8	< min
Low Quality control (QC)	43 (28-58)	18.5
High Quality control (QC)	121 (89.5-152.5)	88.9
Sample 1	250	600.8
Sample 2	250	1078.0
Sample 3	250	986.7
		Mean = 888.5 (253.3)
Sample 4	125	363.3
Sample 5	125	341.6
Sample 6	125	354.6
		Mean = 353.2 (10.9)
Sample 7 (unspiked plasma)	<8	21.4
Sample 8 (unspiked plasma)	<8	19.7
Sample 9 (unspiked plasma)	<8	21.1
		Mean = 20.7 (0.9)
Unspiked plasma results on Immulite = 5.8 and 7.2 pg/ml (normal <46 pg/ml)		

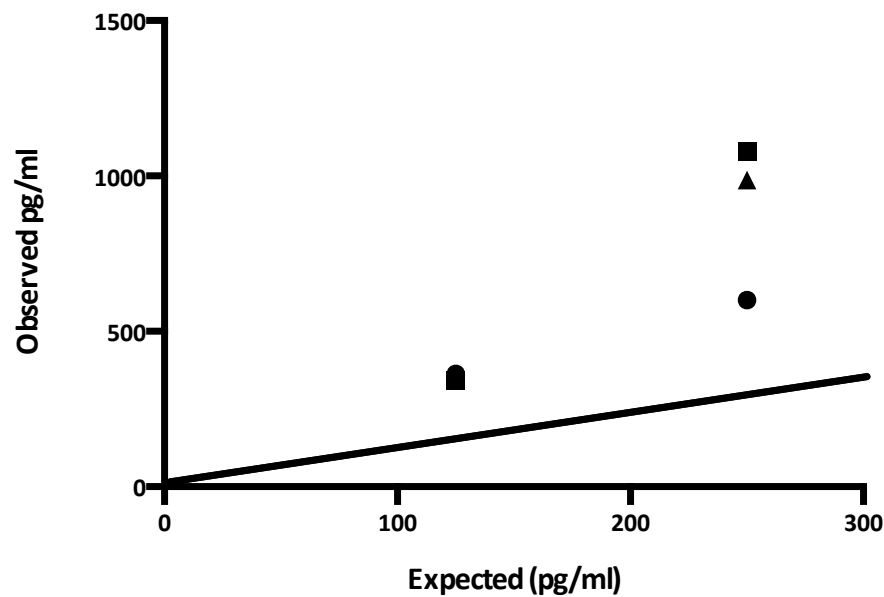


Figure 3.13: Validation experiment 13: Graph depicting the relationship between observed and expected values when human plasma is spiked with two different concentrations of Synacthen– 250 pg/ml and 125 pg/ml. The black line denotes the perfect positive correlation.

4.16.3 Interpretation

As seen in experiment 12 the QCs were both low and the standard curve not as good as previously. This may be a result of inter-operator variability, despite VM's experience. The low QCs were not so abnormal as to invalidate the results.

The results of the Synacthen spiked plasma were disappointing as it was hoped that eliminating the matrix effect would provide more reliable, repeatable results. The values were significantly higher than expected and showed considerable variability at the 250 pg/ml concentration. It is unlikely that dilution

error would account for this variance. The unspiked plasma values were over three times higher using the RIA compared with the Immulite.

Human error has to be considered as an explanation for the unexpected results in all the experiments however the assay is not especially complex to set up and run and it is unlikely that errors could account for the inconsistency of results both within and between assays. These results led to serious concern about the credibility of the RIA to detect ACTH and in particular, Synacthen.

4.17 Validation experiment 14

Part of the attempt to validate this assay was to test its reliability through repeatability. To this end a selection of the volunteer samples from winter 2010 were re-run.

4.17.1 Method

A random selection of seven volunteer samples was chosen. They ranged from a baseline sample to one at 50 minutes and all were from one or other of the nasal Synacthen visits (*vide infra chapter 4*). The samples had previously been thawed and then re-frozen after the initial analysis 17 months before. The samples were run on the same assay as used in experiment 13 and therefore the same controlled conditions and set up by a senior BMS. The samples were run in duplicate.

4.17.2 Results

Table 3.20: Experiment 14 raw data. The mean of duplicate results displayed.

Standards and QCs shown

Sample	Expected (pg/ml) (results from 2010 analysis)	Obtained (pg/ml) (results from 2011 analysis)	Factor difference from 2010 analysis
N1a03 v3*, -1 min**	188	80.9	X 2.3
N1a03 v3*, 10 min**	187.6	88.8	X 2.1
N1a03 v3*, 40 min**	187.0	88.8	X 2.1
N1a12 v2*, 15 min**	56.3	18.7	X 3.0
N1a12 v2*, 20 min**	59.5	22.8	X 2.6
N1a12 v2*, 40 min**	46.6	19.4	X 2.4
N1a12 v2*, 50 min**	44.5	17.6	X 2.5

*v2 = Visit 2, 100 mcg nasal Synacthen, v3 = visit 3, 25 mcg nasal Synacthen administered

**Time in minutes after Synacthen administered, where time 0 minutes is drug administration.

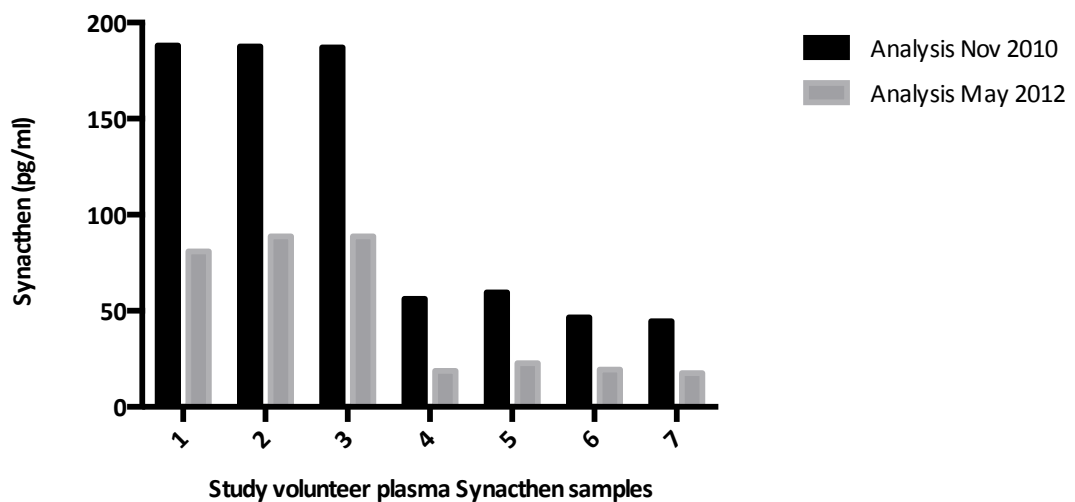


Figure 3.14: Validation experiment 14: Bar chart depicting comparison of seven volunteer samples run in November 2010 and re-run in May 2012.

4.17.3 Interpretation

All the results are considerably lower, between 2.1 and 3 times, when re-analysed 17 months after the first analysis. There is consistency in relative loss of Synacthen between the two analyses, especially for the higher value samples. This suggests the change may have been due to degradation of Synacthen or the effect of the second freeze-thaw cycle rather than inconsistency within the assay. This loss of Synacthen is in strict contrast to the results of experiment 10 (*section 4.12.2*) where values had increased on re-analysis.

On reflection re-analysing a wider range of samples including those from visit 1, where 1 mcg of Synacthen was administered intravenously, would have been preferable. This may have served to further test the degradation theory, as the range of Synacthen values would have been greater. Additionally, when samples were originally spun after being taken from the volunteers they were split into two aliquots to allow for further analysis at a later date if necessary. At the time of experiment 14, with continued failure to validate the RIA, the sourcing of a new assay was being actively pursued and therefore there was a possibility of re-running all the study samples. The second aliquot was kept for that purpose and experiment 14 performed on samples that had already undergone a freeze-thaw cycle. Running the second aliquot samples may have provided different results.

4.18 Validation experiment 15

The RIA chosen did not appear fit for purpose. The RIA antibody appeared at times to be cross-reacting with higher affinity for Synacthen compared with ACTH and at other times to barely bind it. Synacthen, like ACTH, is an unstable molecule but measures were taken to prevent excess degradation and the results were not all consistent with Synacthen loss. Additionally the matrix effect had been extensively examined and did not appear to improve results.

A number of sources had suggested that using an ACTH standard curve may be the cause of the inconsistency as ACTH and Synacthen are molecules of different molecular weights and are thus likely to behave differently in the RIA. It was recommended that to overcome this the standards should be incubated in the same buffer as the samples.

At this time a paper came to light which reported a validated method using the MP Biomedicals RIA to measure Synacthen (Wade, Baid et al. 2010). The corresponding author of the paper, Dr Lynette Nieman (NIH, USA), was contacted for advice. The author responsible for the RIA work on the study, Professor Hershel Raff, responded that in his experience the RIA did cross-react 100% with Synacthen. He suggested using the MP Biomedicals zero standard as the diluent for Synacthen standards, to reduce matrix effects, and to consider plasma extraction procedures. However he also pointed out that they had only used the RIA for their *in vitro* work, not to measure plasma Synacthen levels on their research participants. Finally he only knew of one other ACTH RIA that

would measure Synacthen but reported it was “much more labour intensive and gives similar results”.

It was decided to repeat a serial dilution experiment using Synacthen standards and the MP Biomedicals zero standard as the diluent.

4.18.1 Method

Frozen zero standard was purchased from MP Biomedicals, thawed on the day of use and used as a diluent for the dilutions outlined in *table 3.21a*. Due to financial constraints and the expense of the zero standard large dilutions were required to make up the standards and therefore a positive displacement pipette was used to improve accuracy. The pipette was internally calibrated and a CV for 10 ml calculated by ten serial weights on laboratory weighing scales. The CV was 1.01%.

A high concentration stock (250,000 pg/ml) was created after the first dilution step, split into seven aliquots and six were frozen at -20°C. Following the second dilution step a standard stock was formed (1000 pg/ml) which was similarly split and frozen. One aliquot of the standard stock was used to dilute to the concentrations shown in *table 3.21a* and the unused volume of the final dilutions (500, 250, 125, 62.5 pg/ml) was divided into two aliquots and frozen. Experiment 15a involved running 1000, 500, 250, 125 and 62.5 pg/ml in triplicate.

The results obtained supported continuation using Synacthen standards made up in zero standard and therefore experiment 15b, performed one month later, used thawed samples made up at the time of experiment 15a with the addition of a zero standard sample (unspiked) and a 31.3 pg/ml sample diluted using the zero standard provided with the assay kit. Samples in experiment 15b were run in triplicate.

Finally experiment 15c, carried out three weeks after 15b, again utilised the frozen dilutions from experiment 15a this time with the addition of a 15.6 pg/ml sample and a zero standard sample made up that day from the lyophilised sample in the RIA kit. Samples for all experiment 15c were run in duplicate.

Table 3.21a: Dilution ratios, summarised method and resultant solutions of experiment 15. Final solutions assayed displayed in bold.

Dilution ratio	Dilution method	Resultant concentration
1:1000	10 mcl of 250mcg/ml Synacthen in 10mls of zero standard	250 ng/ml
1:250	20 mcl of 250 ng/ml solution in 5 mls of zero standard	1000 pg/ml (15a+b)
1:2	0.5 ml of 1000 pg/ml solution in 0.5 mls of zero standard	500 pg/ml
1:2	0.5 ml of 500 pg/ml solution in 0.5 mls of zero standard	250 pg/ml
1:2	0.5 ml of 250 pg/ml solution in 0.5 mls of zero standard	125 pg/ml
1:2	0.5 ml of 125 pg/ml solution in 0.5 mls of zero standard	62.5 pg/ml
1:2	0.5 ml of 62.5 pg/ml solution in 0.5 mls of zero standard	31.3 pg/ml (15 b+c)
1:2	0.5 ml of 31.3 pg/ml solution in 0.5 mls of zero standard	15.6 pg/ml (15c)

4.18.2 Results

Table 3.21b: Experiment 15 raw data. Results displayed are the mean of duplicate (15c) and triplicate (15a+b).

Standard (pg/ml)	Experiment 15a (pg/ml)	Experiment 15b (pg/ml)	Experiment 15c (pg/ml)
Synacthen Standard - 1000	900.6	960.8	-
Synacthen Standard - 500	560.7	530.6	482.4
Synacthen Standard - 250	240.2	241.1	230.1
Synacthen Standard - 125	130.1	129.8	123.2
Synacthen Standard - 62.5	< min	< min	64.7
Synacthen Standard - 31.3	-	< min	27.6
Synacthen Standard - 15.6	-	-	< min
Synacthen Standard - 0	-	-	-

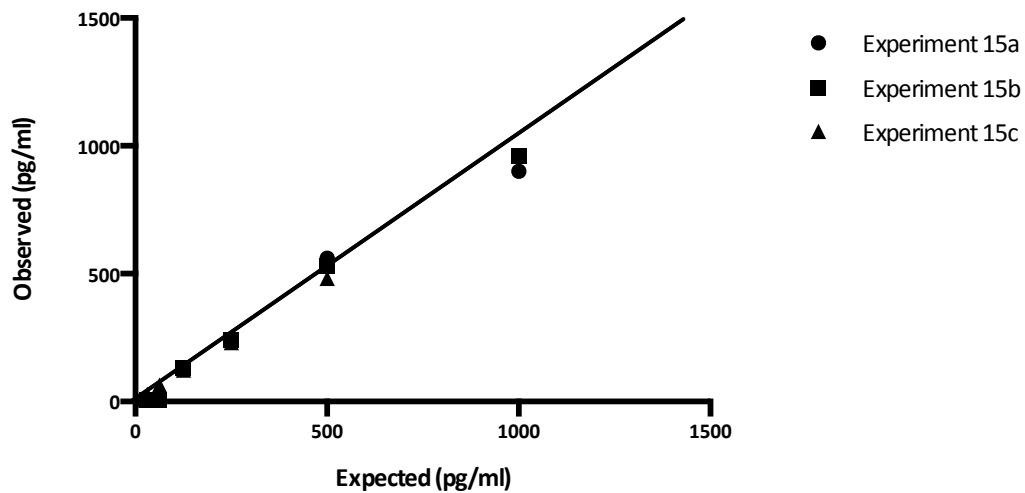


Figure 3.15: Validation experiment 15a-c: Scatter graph depicting the relationship between observed and expected values when Synacthen standards were run on three different occasions. Values less than the minimum standard are plotted as 7 pg/ml. a, b and c denote the three experiments run on different days. The black line denotes the perfect positive correlation.

4.18.3 Interpretation

The results of experiment 15 finally showed consistency, interpretability and repeatability indicating that the incubation of standards in a different buffer to the samples was the cause of the inconsistency of results. It was thought that with the use of Synacthen standards, made up with the kit zero standard, the RIA had been validated and was reliable enough to be used to analyse the volunteer samples from the NeSST2 study. However it is acknowledged that because the antibody is raised against ACTH and not Synacthen the calibration standards cannot be truly validated.

The Synacthen standards, made up from the frozen high concentration stock (250,000 pg/ml), were divided into aliquots and frozen to allow standards made up from the same batch to be used for each assay when NeSST2 Study volunteer samples were analysed.

4.19 Further Validation

Ideally, to complete the assay validation work, an experiment would have been carried out in which the measurement of Synacthen in a biological matrix (blood/plasma) consisting of the zero diluent was demonstrated e.g. diluting spiked samples of ACTH-deplete EDTA plasma in zero calibrator. The Synacthen assay validation had used up considerable and unanticipated funds and thus, given the financial restrictions, a surrogate of clinical samples was used. This was considered a reasonable compromise as it had not been possible to validate the assay before analysis of the original NeSST clinical study samples and

interpretable results had been obtained. The analysis of the NeSST2 clinical study samples (*vide infra chapter 5*), especially those diluted in zero diluent, presented the opportunity to validate the assay in a biological matrix. The results of the Synacthen standards run with each assay are tabulated below (*table 3.22*).

Table 3.22: NeSST2 Study Synacthen standards

		Assay run								
		1	2	3	4	5	6	7	8	9
Concentration of Synacthen standards (pg/ml)	500	> max	483.7	492.9	> max	488.0	> max	466.9	> max	454.5
	250	228.3	237.2	227.7	204.0	235.8	234.5	245.0	196.9	261.3
	125	132.4	121.7	130.6	127.1	125.6	122.1	115.2	129.0	123.1
	62.5	69.4	64.9	60.2	72.9	78.0	91.0	84.0	56.5	56.6
	31.3	36.6	39.6	32.2	28.6	24.1	26.8	45.1	30.7	39.3
	15.6	< min	< min	21.9	18.8	22.6	24.1	28.8	25.9	10.1

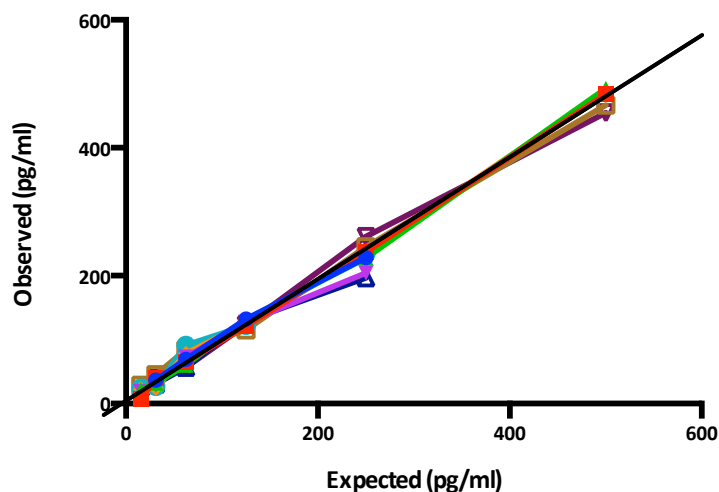


Figure 3.16: Line graph depicting the relationship between observed and expected values for the Synacthen standards run on 9 hACTH assays over 9 months. Each coloured line depicts a different assay run. The black line denotes the perfect positive correlation.

The reproducibility over nine different assays run over nine months, the linearity and the close relationship to expected values were immensely reassuring.

Despite the difficulties validating the Synacthen assay there had always been the sense, after processing the NeSST Study samples, that it reliably detected Synacthen *in vivo*. Further credence was given to this when similar results were obtained for the 1 mcg i.v formulation (the only dose to be repeated over the two studies) during the analysis of the NeSST2 Study when the Synacthen standards were employed (*table 3.23*).

Table 3.23: Comparison of the mean, SD and range of peak plasma Synacthen obtained after 1 mcg i.v administration during the NeSST and NeSST2 Studies. Range in parenthesis.

	NeSST Study1	NeSST2 Study
Mean, SD and range of peak plasma Synacthen (pg/ml)	222.7 +/- 87 (103-360.8)	258 +/- 102.6 (92.2-393.4)

4.20 Coefficient of Variations (CV)

QCs enable assessment of inter-assay (batch-to-batch) variability. The kit ACTH QCs were used for experiments 1-14 and a table of the results is shown below (*table 3.24*). The low and high QCs obtained on the 12 assay runs using the hACTH RIA were excessively high reflecting the problems i.e. molecular instability and sensitivity to temperature, encountered when using this assay to detect and quantify Synacthen. Inter-assay CVs were calculated (ratio of the SD to the mean)

and displayed in *table 3.23*. Inter-assay CVs are always higher than intra-assay CVs and ideally inter-assay CVs of competitive RIA should not be significantly above 10%.

Table 3.24: Quality Control data for all experiments with mean, SD and CV for experiments 1-14 (those which utilised the hACTH RIA kit QCs). High values (>3SD) in red, low values (<3SD) in blue.

	Experiment number												Mean (SD)	CV %
	1	2	3	4	5	6	7a	7b	7c	8	11	13		
Low QC	44.0	28.5	61.5	49.4	34.3	22.9	34.5	48.7	34.5	43.4	23.0	18.5	36.9 (12.8)	34.7
High QC	150.7	122.1	80.5	124.8	84.5	122.8	120.0	123.7	120.0	125.5	83.4	88.9	122.2 (22.2)	19.8
Low QC (SD) = 43 (28-58) high QC (3SD) = 121 (89.5-152.5)														

5 Discussion

It was not anticipated that the work validating a Synacthen assay would be so challenging, protracted or expensive. It was performed over more than two years, with significant periods of time elapsing between some experiments. On reflection this has, in parts, given a lack of cohesiveness and a progressive narrative to the work.

RIAs have been largely superseded by sandwich format ACTH assays, which do not detect Synacthen. With no commercially available Synacthen assay and limited choice of ACTH assays using a method of detection suitable for Synacthen the only viable option was to attempt validation of an hACTH RIA. The MP Biomedicals RIA was thought to be the only assay available that met the specifications and the manufacturer quoted 100% cross-reactivity with Synacthen was mistakenly assumed to represent laboratory validation.

It was demonstrated over a number of experiments that diluted Synacthen did not behave like ACTH standards despite controlling conditions to prevent degradation of Synacthen at room temperature, eliminating the matrix effect, acidifying buffers and investigating the influence of laboratory experience. In retrospect the different molecular weights of ACTH (4538 g/mol) and Synacthen (2932 g/mol) meant that using the standard curve derived from one molecule to measure the concentration of the other was unlikely to be successful. Researchers have been criticised for this before with authors reporting that the

standard curves derived from ACTH(1-24) and ACTH(1-39) were not parallel and therefore counselling against using ACTH(1-39) standards to measure Synacthen (Grino, Darmon et al. 1999). In defence of this strategy the critiqued study authors reported they found the displacement curves to be almost identical at concentrations up to 125 pg/ml but at higher concentrations the ACTH(1-24) displaced the ACTH(1-39) tracer more than ACTH(1-39). This was attributed to its lower molecular weight, but the difference was found to be no greater than their molecular weights ratio (0.65) (Oelkers 1999).

The major on-going concern with the assay is that there remains a disparity between the tracer (ACTH) and the standards/samples (Synacthen). This contravenes the basic premise of competitive RIA - that the antibody and samples should compete as identical molecules. As biological matter with different 3-dimensional configurations and molecular weights it is probable that Synacthen and the ACTH antibodies (antiserum) within the assay do not bind with the same affinity and therefore are not recognised on a one to one molar basis. Previously the antiserum from a similar RIA was found to cross-react with the Synacthen five times more readily than with ACTH(1-39) and the authors counsel caution when using a RIA designed for one peptide to measure another highly structurally similar peptide (Grino, Darmon et al. 1999). The only way to overcome this would be to manufacture a specific Synacthen antiserum and tracer, not felt to be feasible for this study. It was hoped that, whilst acknowledging binding would occur with different affinity, the two would relate in molar terms and therefore a reproducible and reliable relationship would be

demonstrated. It has been seen that, although ACTH antiserum is being used to detect Synacthen, the use of accurately made up Synacthen specific standards has meant a uniformity of samples and standards and yielded reliable and repeatable results with an interpretable curve from which to derive Synacthen values for the study.

An assessment of assay variability was not performed as part of the experiments, in part due to lack of foresight and additionally the timescale over which the experiments took place. Ideally a test plasma volunteer sample should have been taken, divided into multiple aliquots, frozen and then one thawed and run with each assay allowing assessment of assay “drift”. This would have been superior to the kit QCs, and latterly the Synacthen standards, which were used as a surrogate to the same volunteer sample tested on each assay run.

One of the concerns throughout the validation attempts had been the possible variability of Synacthen in each vial, although this would not be a plausible explanation for the inconsistent results. The certificate of analysis provided by Alliance pharmaceuticals (Wiltshire, UK) stated that 95.0-105.0% of the declared content is present (as detected by HPLC). That equates to 237.5-262.5 mcg, a wide range of potential concentrations. This degree of variability may have exaggerated some of the inconsistencies seen when large dilutions were required. The Synacthen, marketed by Alliance (the sole UK distributor), is diagnostic grade material and is not designed nor intended for use as a calibrator, which would require much more accurate measurement of content.

The use of accurately made up frozen standards all from one batch (and a single Synacthen vial) for experiment 15 and analysis of all the NeSST2 study volunteer samples will have helped ameliorate possible inconsistency.

The approximate measuring range of the assay is between 30 and 480 pg/ml, approximately from the top standard to the lowest limit of detection. The most sensitive measuring range (steepest part of the curve) lies between these values. There is a lack of sensitivity of the assay at the lower end.

6 Conclusion

The ACTH RIA with Synacthen standards, whilst accepting some compromise of scientific integrity, is felt to be “good enough”. Plasma Synacthen can be measured to allow relative, if not absolute, comparison between samples, enabling the adequate quantification of Synacthen for the pharmacokinetic modelling required of the study. Plasma Synacthen level is not a clinically useful measurement, cortisol is the clinical endpoint of interest in adrenal insufficiency, but this assay has been adequately validated for use as a research tool.

Chapter 4: NeSST study

*The first clinical study
in the development of
a non-invasive Short
Synacthen Test*

1 Introduction

The NeSST study (Non-invasive Short Synacthen Test) formed part of a body of work looking to simplify the way children undergo assessment of their adrenocortical function, particularly in relation to detecting suppression in those with asthma on long term, high dose inhaled corticosteroids (ICS). The department in which this work was undertaken had previously explored alternative methods for assessing adrenal reserve and possible adrenal suppression in children with asthma.

Cortisol can be readily measured in saliva (*vide supra chapter 1, section 6.2*). Previous work, conducted in 2007, validated a salivary cortisol enzyme immunoassay (Salimetrics, LLC, PA, USA) against plasma cortisol and conducted interference studies to ensure that ICS did not cross-react with the assay. This method was then used to compare fasting early morning cortisol in children with asthma (N=55) to a healthy cohort (N=152) to assess whether the technique could be used as a useful screening test for adrenal insufficiency (AI). Results showed that fasting morning cortisol levels were inversely related to the dose of ICS ($r=0.36$, $p=0.007$) such that the higher the dose of ICS, the lower the cortisol. While significantly more children on ICS had low levels of salivary cortisol (18.2% compared to 7.9%, ($p<0.05$) at a salivary cortisol cut off of <2.0 nmol/L (approximates to a plasma cortisol of <150 nmol/L)) the sensitivity and specificity of the test were not sufficient to discriminate those who may have AI from the general population (Nickson, Wilson et al. 2008).

It was hypothesised that the sensitivity and specificity of fasting morning salivary cortisol testing for AI may be significantly enhanced by repeated sampling e.g. on three consecutive mornings or sampling according to the Cortisol Awakening Response (CAR), a sharp increase in cortisol, maximal 30 minutes after waking (Pruessner, Wolf et al. 1997). However, a recent study on 269 non-fasted school-aged children on ICS has shown this not to be the case, although employing a cortisol cut off of <350 nmol/L in response to low-dose Synacthen shows promise (Blair, Lancaster et al. 2013).

The standard, although not gold standard, diagnostic tool for AI is the Short Synacthen Test (SST) (Davies and Howlett 1996, Elder, Sachdev et al. 2012) and thus the next logical step in this programme of work was to devise a simpler version to enable testing on large numbers of children. A study to evaluate whether Synacthen can be administered nasally rather than intravenously, using salivary cortisol to measure adrenal response, was devised.

It is known that some absorption of Synacthen is possible via the nasal mucosa. The historical studies cited (*vide supra chapter 1, section 5.3.1*) examined the potential of nasally administered ACTH analogues as a replacement for depot i.m. ACTH (which at that time was used as a regular therapy, as an alternative to corticosteroid treatment in a variety of inflammatory conditions). These studies looked at the use of i.n. ACTH from the perspective of discovering an analogue with the pharmacological properties to allow its long-term use in chronic conditions. These are not the same properties required for the SST, where only a

single dose is needed. There are no studies to date looking at the use of nasal Synacthen either in the childhood population or as a one-off diagnostic test.

A pharmacokinetic study in rats suggested that the bioavailability via the nasal route was approximately 4.4% of the i.v route (Wuthrich, Martenet et al. 1994). There are no other studies to date examining the pharmacokinetics of Synacthen delivery via this route. The previous research, though limited, has shown nasal Synacthen to be well tolerated and there have been no significant safety concerns reported. Whilst previous studies concluded that nasally administered ACTH was impractical for sustained therapeutic use, as a single dose, utilised for diagnostic purposes, the nasal route of administration holds considerable promise.

2 Aims and Objectives

2.1 Aim:

To develop a non-invasive alternative to the 1-microgram (low-dose) intravenous Short Synacthen Test (LDSST).

2.2 Objectives:

- To establish the first bioavailability and pharmacokinetic data of nasal Synacthen in humans.

- To compare the bioavailability of two different formulations of nasal Synacthen with the 1 mcg i.v SST.
- To establish the inter-individual variability of cortisol response to nasal Synacthen in humans.

3 Materials and Methods

3.1 Study Design

This was a pharmacological bioavailability study using an open label, triple arm, crossover design (*figure 4.1*). The crossover design, where between occasion variables are minimized (e.g. fasting or fed conditions, time of day, concomitant medication), is the recommended methodology for generating bioequivalence data (European Medicines Agency 2010). Expert advice (Dr Johnson, pharmacokinetic pharmacist and co-collaborator on NeSST Study) was that the study design be based on that recommended for bioequivalence studies, although this was a bioavailability study (*vide infra definitions section 3.5.1*). It was considered unnecessary to demonstrate that the two formulations (i.v and i.n) are the same (bioequivalence) but instead show that an adequate amount of Synacthen is absorbed to produce equivalence in the resultant cortisol response (bioavailability).

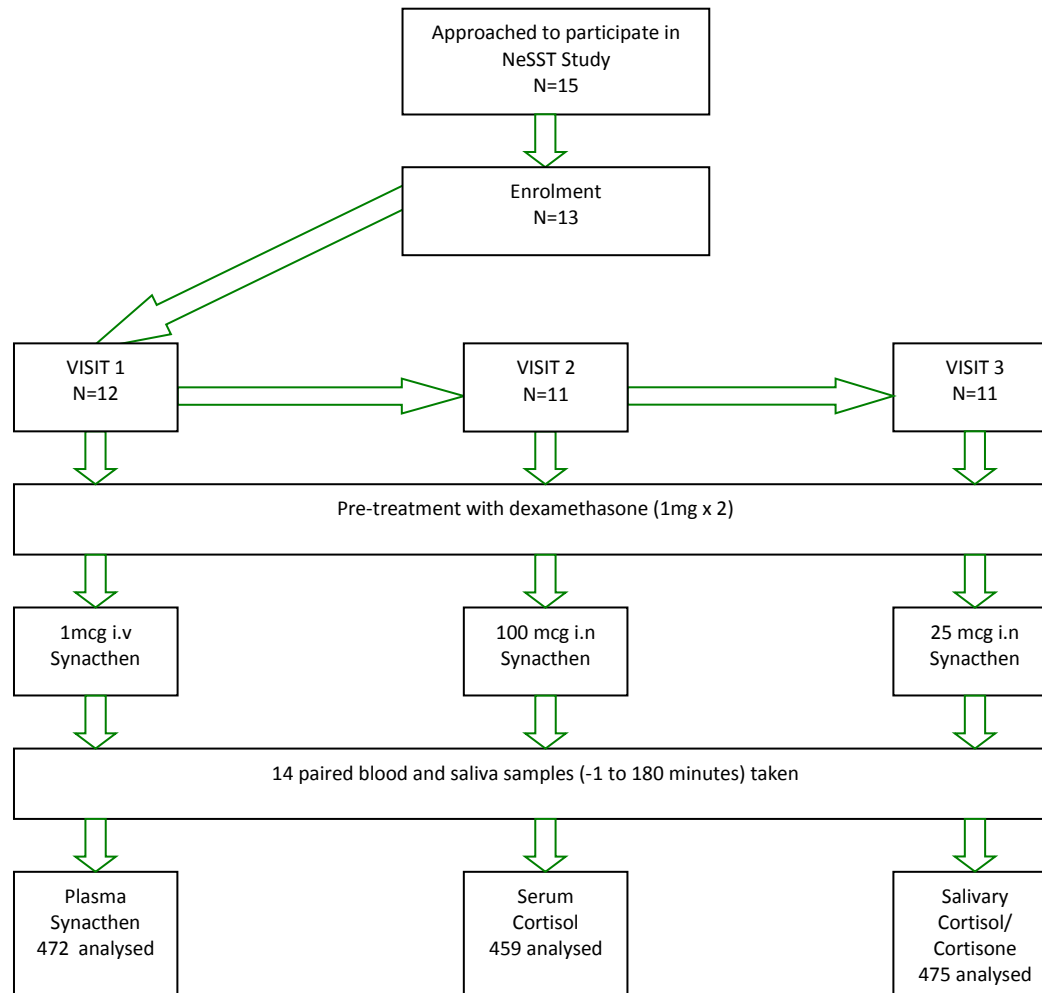


Figure 4.1: Recruitment flow chart and volunteer pathway for NeSST Study

In keeping with pharmacokinetic trials of this kind the subjects were neither randomized nor blinded and did not receive a placebo. It was a single-centre study conducted from Sheffield Children's NHS Foundation Trust (SCH), Sheffield, UK.

3.2 Subjects

The participants for the study were healthy, male volunteers aged between 18 and 64 years of age. European Medicines Agency (EMA) guidelines were followed and only adult, male volunteers were enrolled due to the possible adverse effects of the drug on a fetus or child and to reduce variability not attributable to the difference between routes of administration (European Medicines Agency 2010, Guimaraes Morais and Lobato 2010). EMA guidance states that "this model, *in vivo* healthy volunteers, is regarded as adequate in most instances to detect formulation differences and to allow extrapolation of the results to populations for which the reference medicinal product is approved (the elderly, children, patients with renal or liver impairment, etc.)" (European Medicines Agency 2010).

3.2.1 Number of subjects

European Medicines Agency guidance was followed and 12 subjects were selected as the minimum number for a bioavailability study (European Medicines Agency 2010). It was not felt to be ethically or financially viable to recruit or test

more than 12 subjects. The intention was to replace withdrawn subjects, as their omission would jeopardise the statistical validity of the results.

3.2.2 Inclusion criteria

1. Aged between 18 and 64 years of age
2. Male
3. Body Mass Index (BMI) between 18.5 and 30 kg/m²
4. Healthy (see exclusion criteria)
5. Non-smoker

3.2.3 Exclusion criteria

1. Past or present history of an endocrinopathy
2. Past or present history of asthma
3. Past or present history of allergic rhinitis
4. Past or present history of peptic ulcer disease/gastrointestinal bleed/significant dyspepsia
5. Past history of intra-cranial or renal/adrenal pathology
6. Presently on any medication
7. Presently, or within the last 3 months, been prescribed any type of corticosteroid (oral, inhaled, nasal, rectal, i.v, i.m, intra-articular, intra-ocular, topical)
8. Ever been prescribed a prolonged course of oral corticosteroids (more than 1 month)

9. Previous adverse reaction (including mild hypersensitivity) to ACTH or Synacthen
10. Previous severe allergic reaction or anaphylaxis
11. Coryzal symptoms within the last week (and will be asked to report any new symptoms occurring within 24 hours of the test)
12. Currently anaemic

3.2.4 Recruitment

All regulatory approvals (MHRA (Medicines and Healthcare products Regulatory Authority), REC (Research Ethics Committee) and R+D (Research and Development)) were in place before volunteer enrolment into the study. Recruitment was predominantly by word of mouth. The first volunteers were recruited when the study was discussed during a teaching session for medical students at Sheffield University. The students themselves then disseminated the participant information sheets (PIL) amongst friends, who contacted the principal investigator (PI, author). Ethical approval for the use of posters, emails and a short talk before lectures had been obtained but these methods were not required. In order to avoid costly translation services and the risk of volunteers misunderstanding important information only English speakers were recruited.

A detailed PIL describing the background to and aims of the study, the methodology, inclusion and exclusion criteria, was given to those who expressed an interest in participating. A minimum time of 24 hours was stipulated before

enrolment to allow volunteers time to consider participation. Those who felt themselves to be eligible contacted the PI who discussed the study in more depth and answered queries during a telephone conversation.

Informed consent was taken over the telephone and confirmed in writing at the first visit. The author performed all the recruitment and obtained consent from all the volunteers. Consent was additionally requested for subjects' General Practitioner to be informed of their participation in the study.

3.2.5 Subject payment

Volunteers were informed that they would be paid £75 per three and a half hour visit for participation in the study (£225 in total for the three visits). There were no additional payments for travel expenses and lost earnings and this amount was felt to suitably recognise the time and dedication that participation in the study required.

3.2.6 Subject data

Basic demographic data was collected on all volunteers at visit one. This included age and ethnic group (performed by self selecting from a standard ethnic categories form used in SCH). Volunteers had their height (Harpden Stadiometer, Holtain Ltd, Dyfed, UK) and weight (Marsden MS4202 Adult scales, Oxfordshire, UK) measured. This allowed calculation of body mass index (BMI)

and body surface area (BSA) to enable volume of distribution calculations for Synacthen.

At each visit subjects were asked to report coryzal symptoms beginning within 24 hours of the test (which may impact on nasal absorption due to changes in the mucosa) and this was additionally checked on all subsequent visits.

Paired samples of blood and saliva were taken throughout each visit (14 samples over a three hour time period). These were analysed for plasma Synacthen and serum cortisol and salivary cortisol and cortisone levels.

3.3 Subject visits

Following recruitment, subjects were asked to attend the Children's Clinical Research Facility (CCRF) at SCH on three occasions, each separated by a minimum of a fortnight. This allows for a sufficient washout period between administrations, at least 5-half lives of the drug, although one week is typical (European Medicines Agency 2010). When administering Synacthen on multiple occasions most researchers have left between two days and two weeks between visits (Dickstein, Shechner et al. 1991, Crowley, Hindmarsh et al. 1993, Bridges, Hindmarsh et al. 1998, Nye, Grice et al. 1999, Alia, Villabona et al. 2006), although no significant differences in adrenal responses were shown in one study when large ACTH doses were given two to five days apart (Leclercq, Bruno et al. 1972).

Prior to each visit volunteers were asked to abstain from alcohol and recreational drugs for 24 hours and to take 1 mg of dexamethasone (500mcg tablets, Essential Generics, Surrey, UK) on retiring the night before the visit and a second 1 mg dose after breakfast on the morning of the visit. There are no commercially available Synacthen assays, or any for research purposes, and therefore it was necessary to temporarily suppress the volunteers' endogenous production of ACTH to enable use of an ACTH assay, with the inference that anything detected was Synacthen. The use of 1 mg dexamethasone the night before and in the morning on the day of the test will adequately suppress endogenous ACTH and cortisol production in almost all adults (Krieger, Allen et al. 1971).

Each visit commenced between 08.30 and 09.30. Volunteers were not required to fast. On arrival the inclusion and exclusion criteria were checked, written informed consent taken and basic auxological measurements performed, as described above. An 18-gauge i.v cannula (Jelco, Smiths Medical International, Lancashire, UK) was sited with an i.v connector (Alaris Products, Rolle, Switzerland) and the volunteer was asked to rest, lying down, for 30 minutes to recover from the physiological stress of cannulation. Ten minutes before the first samples were taken the subject was asked to rinse their mouth thoroughly with water to minimise contamination that may compromise salivary cortisol assay performance. Additionally it was requested that they refrain from eating or drinking, other than water, during the visit. The volunteers were asked to remain supine for the duration of the test.

3.3.1 Subject safety

Synacthen has a short half-life and is not known to cause any long-term adverse effects. Generally nasal drugs are well tolerated (Davis and Illum 2003) and there are no reports of major side effects following the administration of nasal ACTH analogues. Minor side effects include nasal irritation, although this has generally only been seen with prolonged use.

Synacthen can rarely cause serious allergic reactions and is potentially anaphylactogenic. An emergency Standard Operating Procedure (SOP) (*appendix 3*) was written and approved by a Paediatric Intensive Care consultant and a resuscitation officer. Medical and nursing staff working on the study, were asked to sign that they had read and understood the emergency SOP. An individualised resuscitation form was completed for each subject. Resuscitation equipment and laminated copies of the most current Adult Basic Life Support, Advanced Life Support and anaphylaxis algorithm (issued by the Resuscitation Council, 2010) and the emergency SOP were readily available. In addition staff carrying out the Synacthen administration and subsequent monitoring had undergone specific resuscitation training with a resuscitation officer and been certified as competent. In the event of a serious allergic reaction there was a 24-hour emergency paediatric team (including an anaesthetist) available.

The dexamethasone taken by subjects prior to each visit, has potential side effects, mostly gastrointestinal. Precautions were taken to minimise this risk including the exclusion of volunteers with a history of dyspepsia, peptic ulcer

disease or a gastrointestinal bleed and advice to avoid taking the drug on an empty stomach was given (verbally, in PIL and detailed on the drug bottle label). In addition the letter sent to volunteers' GPs warned of these potential side effects. Each subject was given a copy of the letter as a hand held record to present to medical services if they became unwell. They also had 24-hour contact details of the PI for advice.

3.3.2 Synacthen administration

After the rest period of 30 minutes blood and saliva samples were taken for basal ACTH and cortisol levels (-1 minute) and immediately afterwards the Synacthen (250mcg/ml, Alliance Pharmaceuticals Ltd, Wiltshire, UK) was administered. At the first visit this entailed 1 mcg of i.v Synacthen given as a bolus. This was made up using the SCH protocol for the routine clinical use of the LDSST, which instructs diluting 0.5 ml of 250 mcg/ml solution in 500 ml of 0.9% saline, mixing thoroughly (ten slow inversions) and then administering 4 ml of the solution to the subject.

At the second visit 100 mcg of i.n Synacthen was administered by atomiser syringe (Mucosal atomizer device™, Wolfe Tory Medical Inc. Utah, USA). The Mucosal atomizer device™ (MAD) atomises liquids to 30-100 microns allowing rapid absorption into the bloodstream and cerebrospinal fluid (Wolfe Tory Medical Inc. 2009) (*figure 4.2*) and was recommended by paediatric pharmacists and felt to be appropriate by nasal drug experts when consulted. Two sprays of

0.1 ml Synacthen (250mcg/ml) were given up each nostril, thereby administering 100 mcg.

At the third visit 25mcg of i.n Synacthen was administered (a single dose of 0.1ml of 250mcg/ml solution atomised using MAD to one nostril). Doses were selected assuming a 4% bioavailability compared with the i.v route (Wuthrich, Martenet et al. 1994, Davis and Illum 2003). A dose of 25 mcg nasally would approximate to 1 mcg i.v. The dose of 100 mcg was selected as 4-5 mcg i.v is thought to produce a maximal physiological cortisol response (Oelkers, Boelke et al. 1988).

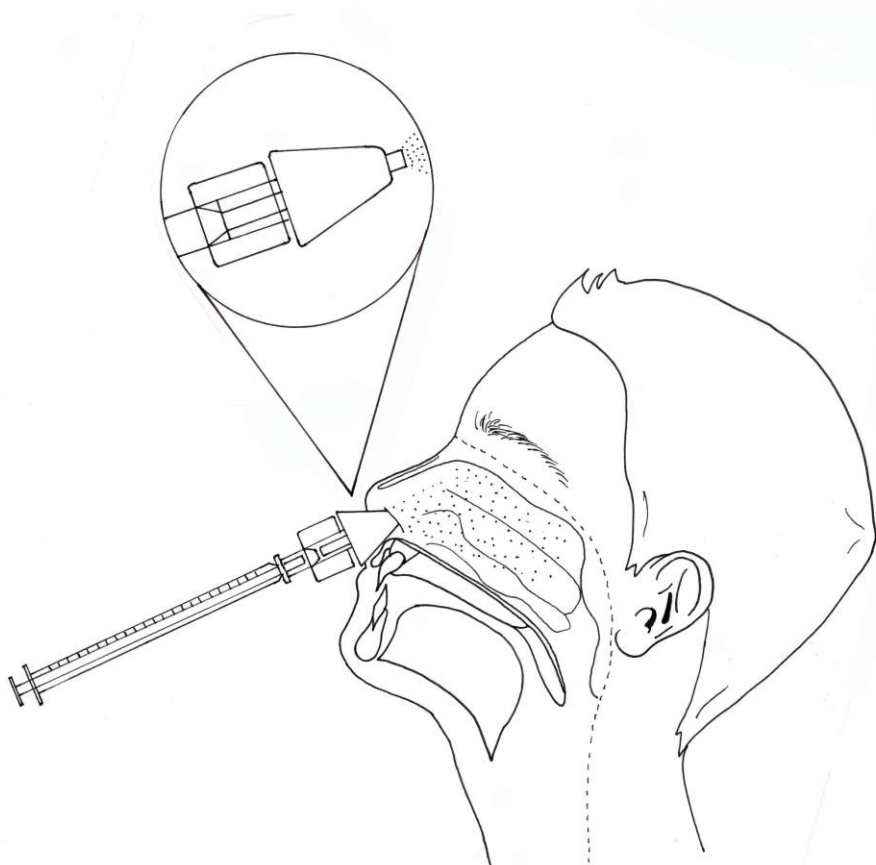


Figure 4.2: Nasal administration of Synacthen with a Mucosal atomizer device™ (Wolfe Tory Medical Inc. Utah, USA) and 1 ml syringe attached. Kindly drawn on instruction of the author for inclusion in this thesis by Kelly-Marie Nelson, research healthcare assistant, SCH.

3.3.3 Blood and saliva sampling

Paired blood and saliva samples were taken at -1, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 150 and 180 minutes (where Synacthen administration was 0 minutes). Approximately 1.8 mls of venous blood was taken from the cannula at each sampling (a total of 25 mls per visit). Following each extraction the 5 ml discard volume was replaced to avoid excess blood loss over the course of the visits and the cannula was flushed with 0.9% saline to prevent blockage by blood clot formation. Heparin was not used on the advice of the hACTH assay manufacturers (MP Biomedicals, Santa Ana, CA, USA).

3.3.3.1 Plasma Synacthen

At each sampling 1 ml of blood was taken into an appropriately labelled EDTA container and the container gently inverted. The samples were immediately placed in an ice bath and centrifuged within 60 minutes (due to the short half life of ACTH/Synacthen in whole blood). To enable long-term storage at -80°C , Trasylol (1000KIU/ml, Bayer Healthcare Pharma, Berkshire, UK) was added to the plasma as per assay manufacturer instructions. Trasylol is aprotinin, a bovine pancreatic trypsin inhibitor, which limits protein degradation of samples by the inhibition of trypsin and related proteolytic enzymes, and has been shown to slow the disappearance of both bioactive and immunoactive ACTH (Besser, Orth et al. 1971). All samples were stored in the -80°C freezer in the CCRF at SCH. Samples were transported (five minute walk) and batch analysed in the

Department of Clinical Chemistry at the Royal Hallamshire Hospital, Sheffield, by the author, between November and December 2010).

3.3.3.2 Serum Cortisol

A serum separator tube was filled with 0.8 mls of blood and appropriately labelled. Following the completion of the subject visit the samples were centrifuged and separated and the residual serum stored at -80°C in the freezer in CCRF until batch analysis in the department of Clinical Chemistry at SCH, between December 2010 and February 2011.

3.3.3.3 Salivary cortisol and cortisone

A minimum of 1 ml of saliva was collected at each time point using the drool technique (found to be the most effective technique in the department's preliminary studies (Nickson, Wilson et al. 2008)), which involves individuals spitting or drooling down a straw into a specialised polypropylene collection tube (Salicaps, IBL international, Hamburg, Germany). Following collection of saliva all samples were inspected for any obvious blood contamination, had this been discovered these samples would have been discarded. The freshly collected samples were frozen at -80°C and stored in the freezers in the CCRF until completion of all the visits. Salivary samples were sent frozen in a single transfer on dry ice to the Department of Biochemistry at University Hospital of South Manchester (UHSM), Wythenshawe, UK, for batch analysis in February 2011.

3.3.4 End of study questionnaire

At the end of each volunteer's involvement in the study they were asked to complete a five-question anonymous questionnaire (*appendix 4*) seeking their perception of the experience of participating in the study and the acceptability of the non-invasive test compared with the i.v LDSST.

3.4 Assays

3.4.1 Plasma Synacthen

The sourcing, characteristics, method and problems validating the hACTH RIA used to detect plasma Synacthen have been extensively discussed in *chapter 3*. A standard curve was established with each assay.

3.4.2 Serum Cortisol

Serum samples were batch analysed in order to minimise variation, using the Abbott Architect chemiluminescent microparticle immunoassay method (Abbott Diagnostics Ltd, Berkshire UK). Abbott Diagnostics quoted functional sensitivity (with applied 95% confidence interval) as 28 nmol/L, a linear range of 28-1650 nmol/L, and <10% total CV for serum samples in the ≥ 83 to ≤ 966 nmol/l range. Quoted limit of detection (LoD) is 22 nmol/L; typical daily Quality Control precision (CV) at the Sheffield Children's Hospital was 2-3% at 100, 500 and 800 nmol/l levels (personal communication, Matthew Jordinson, senior BMS, SCH).

Quoted cross-reactivity with dexamethasone was 0.0%.

3.4.3 Salivary Cortisol

Since the work done to validate a salivary cortisol enzyme immunoassay mass spectrometry analysis has rapidly evolved and is the preferred method for quantifying salivary steroids. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Waters Xevo TQ MS, Kent, UK) was used. UHSM derived assay characteristics showed the assay to be linear up to 3393 nmol/L, with lower limits of quantitation of 0.75 nmol/L and intra- and inter assay imprecision of <8.9% over three levels of internal QC, with recovery and accuracy within acceptable limits. Additionally interference studies conducted within the laboratory at UHSM demonstrated high specificity (Jones, Owen et al. 2012). The cortisol assay was unaffected by the presence of dexamethasone.

3.4.4 Salivary cortisone

Significant amounts of cortisol are converted to the more inert cortisone in saliva and it is thought that cortisone may have a closer relationship with, and be a better marker of, serum free cortisol compared with salivary cortisol (Perogamvros, Keevil et al. 2010). Additionally it is thought that cortisone may be partly responsible for the interference observed with salivary cortisol immunoassays (Jones, Owen et al. 2012). At the time of the study this was a research tool only and required no additional saliva. Salivary cortisol and

cortisone were simultaneously analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Waters Xevo TQ MS, Kent, UK). UHSM derived assay characteristics showed the assay to be linear up to 3676 nmol/L, with lower limits of quantitation of 0.50 nmol/L and intra- and inter assay imprecision of <6.5% over three levels of internal QC, with recovery and accuracy within acceptable limits (Jones, Owen et al. 2012). The cortisone assay was unaffected by the presence of dexamethasone.

3.5 Statistical analysis

The aim of the study was to determine the bioavailability of intranasal Synacthen and ascertain if equivalence (for the purposes of an adrenal suppression test) is possible via the nasal route.

The generation of bioavailability and pharmacokinetic (PK) data from plasma Synacthen and serum cortisol values requires specialist knowledge, expertise and use of pharmacokinetic modelling software. This was provided by Dr Trevor Johnson (co-investigator, Principal Scientist for Systems biology at Simcyp Limited, Sheffield UK and honorary senior pharmacist at SCH).

The analysis was performed using WinNonLin 6.1 (Pharsight, Missouri, USA). This is the industry standard for PK, pharmacodynamic (PD) and noncompartmental analysis.

3.5.1 Pharmacokinetic definitions

The standard PK parameters to measure bioavailability, as recommended by the EMA are given below (Rowland and Tozer 1995, Guimaraes Morais and Lobato 2010).

- **$AUC_{(0-t)}$** is the area under the concentration time curve from time zero until the last quantifiable time point i.e. 180 minutes.
- **$AUC_{(0-\infty)}$** area under the concentration time curve from time zero until infinity.
- **Bioavailability (F)** is concerned with both the amount of drug present in the systemic circulation and the rate of systemic absorption. Bioavailability is often measured by comparing administration via a non-intravenous route with the i.v route. Relative or absolute bioavailability is dependent on the dose and the area under the concentration-time profiles (AUC) following administration via the route of interest and i.v route (*figure 4.3*). Two different formulations of the same drug may have the same bioavailability as measured by the AUC but one formulation may release drug more quickly compared to the other, resulting in a higher initial concentration (C_{max}) at an earlier time (T_{max}), therefore the formulations would not be bioequivalent. In this study bioequivalence is not necessary but enough of the intranasal dose must be absorbed and rapidly enough to produce an equivalent cortisol response to the 1 mcg i.v SST.

$$F = \frac{AUC_{ev}}{AUC_{iv}} \times \frac{DOSE_{iv}}{DOSE_{ev}}$$

Figure 4.3 : Relative Bioavailability equation where F = bioavailability, AUC = area under the curve (concentration-time), iv = iv route, and ev = non-intravenous route i.e nasal

- **C_{max}** is the maximum plasma concentration achieved
- **T_{max}** is the time to maximum plasma concentration
- **Pharmacodynamics (PD)** - the effect of the drug on the body
- **Pharmacokinetics (PK)** - the effect of the body on the drug
- **Terminal $t_{1/2}$** - the time taken to halve the plasma concentration after achieving pseudo-equilibrium. If plasma absorption is not a limiting factor then terminal $t_{1/2}$ essentially reflects plasma clearance and distribution. However if plasma absorption is a limiting factor then terminal $t_{1/2}$ is a marker of the rate and degree of absorption. Terminal $t_{1/2}$ is an important parameter in multiple dosing regimes as it determines the extent of drug accumulation.

3.5.2 Pharmacokinetic data preparation

Prior to analysis the concentration-time data for i.v and nasal Synacthen were visually inspected by plotting the individual profiles in Microsoft® Excel. Any baseline effects (from endogenous substances i.e. ACTH or an interfering substrate detected in the assay) should be removed from the PK profile prior to analysis and thus(BTS/SIGN)(BTS/SIGN)(BTS/SIGN)(BTS/SIGN)(BTS/SIGN) the -1 minute Synacthen values were subtracted from all concentration time points. If

necessary the values were fixed after the concentration fell to zero, as PK analysis cannot compute negative numbers. All plasma concentrations reported as missing or below the lower limit of quantification were excluded from the analysis. Data was then arranged (subject, time, concentration, dose and route) for import into the pharmacokinetic (PK) software Phoenix WinNonLin 6.1 (Pharsight, Missouri, USA).

3.5.3 Pharmacokinetic data analysis

The standard PK parameters for bioavailability, time to maximum plasma concentration (Tmax), maximum plasma concentration (Cmax), area under the concentration time curve from time zero until the last quantifiable time point (AUC_{0-t}), area under the concentration time curve from time zero until infinity ($AUC_{0-\infty}$) and terminal half-life (terminal $t_{1/2}$) were calculated for each individual using standard methods within the Phoenix WinNonLin 6.1 non compartmental analysis software. These are the standard PK parameters to measure bioequivalence as recommended by EMA (European Medicines Agency 2010, Guimaraes Morais and Lobato 2010) and have been adopted as best practice in the NeSST Study to assess the bioavailability of the intranasal doses and formulations of Synacthen. For calculating the terminal $t_{1/2}$ a minimum of three of the last data points were used.

Descriptive statistics for the PK parameters were obtained for the i.v and i.n formulations prior to analysis. Bioavailability was calculated using the equation given in *figure 4.4*.

$$F = \frac{AUC_{in}}{AUC_{iv}} \times \frac{DOSE_{iv}}{DOSE_{in}}$$

Figure 4.4: Bioavailability equation for NeSST Study. *F* is bioavailability, AUC_{in} and AUC_{iv} are the AUC_{0-t} for the intranasal and intravenous formulations respectively and $Dose_{in}$ and $Dose_{iv}$ are the doses for the two routes.

Bioavailability of the i.n formulation to the i.v formulation were assessed on the basis of C_{max} and AUC_{0-t} ($AUC_{0-\infty}$ was not used as the Synacthen was eliminated very rapidly with plasma concentrations being virtually zero by time t).

The concentration-response data in terms of the C_{max} for the Synacthen and cortisol data was collated manually in Microsoft® Excel. The concentration-response data were analysed using a number of known models (E_{max} , sigmoidal E_{max} , linear and power function), models were fitted using non-linear regression. Weighted residuals were calculated to determine the difference between the observed and model predicted cortisol values at each Synacthen concentration, these were weighted based on the concentration of Synacthen to allow for potential analytical errors. The sum of the squares of the weighted residual values was calculated and the solver function in Excel used to fit model parameters to minimise this value. The best-fit model was determined using the Akaike information criteria (*figure 4.5*). All plasma concentrations reported as

missing or below the lower limit of quantification were excluded from the analysis.

$$AIC = N_{obs} \times \ln(WRSS) + 2 \cdot N_{par}$$

Figure 4.5: Akaike Information Criteria equation. N_{obs} is the number of observations, $\ln(WRSS)$ is the weighted residual sum of squares and N_{par} is the number of parameters in the model.

3.5.4 Non-PK data analysis

In addition to the pharmacokinetic modelling mean cortisol and Synacthen at the various time points were compared by paired t-tests with a Bonferroni correction applied. The timing of the peak cortisol response, the dose-response relationship between nasal Synacthen and cortisol production and the inter-individual variability of nasal Synacthen have all been deduced.

4 Results

4.1 Recruitment data

Full planned recruitment was achieved within five weeks, well within the three months allocated for the purpose. Due to the method of recruitment (word of mouth) it is unknown how many individuals were approached to participate in the study. All 12 of the healthy, adult males who contacted the PI, interested in enrolling, were eligible for inclusion (*figure 4.1*). When the study was fully recruited a further three interested candidates had their contact details recorded but only one was subsequently enrolled when an individual failed to attend his

first visit, became uncontactable, and after several weeks was deemed to have withdrawn. Another individual attended for his first visit and then failed to attend visits, despite multiple rearrangements. The data from his visit have been included in the analysis. No one was recruited in his place as it had become clear from the results that his further involvement would not alter the overall outcome and it was therefore deemed unethical.

Table 4.1: NeSST Study recruitment data

	Enrolled	Failed to attend first visit	Attended visit 1	Attended visit 2	Attended visit 3
Number	13	1	12	11	11

4.2 Demographic and anthropometric data

Basic data were collected from all volunteers at the first visit: age, ethnic origin, height and weight (both measured), from which body mass index (BMI) and body surface area (BSA) were calculated. *Table 4.2* summarises the 12 volunteers' demographic and auxological data with ranges, means and standard deviations given.

Table 4.2: NeSST Study subjects' demographic and anthropometric data

	Number of volunteers	Range	Mean	Standard deviation +/-
Age (years)	12	19-44	24	7
Height (m)	12	1.748-1.985	1.838	0.067
Weight (kg)	12	63.6-89.8	77.5	6.8
BMI (kg/m ²)	12	20-29.4	23.1	2.2
BSA (m ²)*	12	1.8-2.22	2.0	0.1
Ethnic origin	12	10 = white British, 2= white Irish		

* Dubois Formula: Body Surface area (m²) = 0.007184 × (height in cm)^{0.725} × (weight in kg)^{0.425}

4.3 Data derived from patient specimen sampling

Paired blood and saliva sampling was performed at 14 time points from -1 to 180 minutes. If all samples on all volunteers had been successfully obtained there would have been 1428 data points, however 22 were missing (*table 4.3*).

Table 4.3: NeSST Study samples collected for each subject at each visit with missing data points and reasons given. "Complete" indicates all 14 samples of plasma Synacthen, serum cortisol and salivary cortisol/cortisone (N=42) were successfully collected. (Pl=plasma, S=Synacthen C=cortisol)

Subject Number	Visit 1	Visit 2	Visit 3
1	Complete	No serum C samples*	No 5 min blood (S+C) [§]
2	Complete	Complete	No 120 min pl S [§]
3	Complete	Complete	Complete
4	Complete	Complete	Complete
5	No 5 min serum C [§]	Did not attend visit	Did not attend visit
6	Complete	Complete	Complete
7	Complete	Complete	Complete
8	Complete	Complete	Complete
9	Complete	Complete	Complete
10	Complete	No 50 min pl S [§] No 180 min salivary [£]	No 20 min blood (S+C) [§]
11	Complete	Complete	Complete
12	Complete	Complete	Complete

* Samples frozen prior to centrifugation and separation therefore haemolysed

§ Problem with cannula bleeding back

£ Research nurse forgot to ask for sample

4.3.1 Proof of dexamethasone suppression

The *in vitro* assay validation experiments detailed in chapter 2 were not complete when the NeSST Study Synacthen samples were analysed. In order to validate its use for measurement of Synacthen in study volunteers it was necessary to demonstrate that dexamethasone had successfully suppressed the subjects' ACTH and corticosteroid production. This allowed the inference that any "ACTH" detected by the assay was Synacthen. *Table 4.4* displays the plasma ACTH and cortisol for all volunteers at -1 minute (prior to Synacthen administration).

Table 4.4: Plasma ACTH and serum cortisol values at -1 minute for 12 NeSST Study volunteers. ACTH in pg/ml, normal range <10pg/ml. Cortisol in nmol/L, normal range <50 nmol/L. High values shown in red.

	Visit 1 (n=12)		Visit 2 (n=11)		Visit 3 (n=11)	
	ACTH	Cortisol	ACTH	Cortisol	ACTH	Cortisol
1	22.4	26.1	14.6	No sample	12.7	<22
2	38.3	<22	39.4	<22	42.3	<22
3	168.7	<22	184.1	<22	188	<22
4	40.6	<22	42.1	<22	40.1	<22
5	32.6	<22	No sample	No sample	No sample	No sample
6	52.9	25.1	45.3	<22	39.7	<22
7	37.9	<22	33.7	44	37.3	22.8
8	42.3	<22	44.2	<22	46.2	<22
9	30.1	<22	30.4	26.1	28.2	<22
10	56.5	33.2	58	24.3	45.9	<22
11	25.1	52	35.7	93.8	36.6	124.8
12	32.1	27.9	64.4	<22	31.5	24.5

The low cortisol values in *table 4.4* show that at all the visits, in all but one of the volunteers, adequate dexamethasone suppression was demonstrated. Subject 11, on all three visits, had partially or unsuppressed cortisol values. He was reportedly healthy and did not have a Cushingoid habitus. It is likely that he was either non-adherent with the request to take dexamethasone or that he had another, undisclosed, exogenous source of corticosteroid. His data were excluded from the relevant analyses. For all volunteers the ACTH values were higher than would be expected suggesting lack of suppression. They show marked inter-individual variability but intra-individual consistency. The -1 minute samples were reanalysed using an immunochemiluminometric (sandwich) ACTH

assay (Immulite 2000, Siemens Healthcare Diagnostics, Munich, Germany) and all had undetectable ACTH values (<5 IU/l).

4.3.1.1 Potential interference with Trasylol

The high baseline values raised the possibility of an interfering molecule. Two samples were tested with and without the addition of trasylol to assess whether its presence was affecting the results (*table 4.5*). The samples were treated the same in all other respects and were analysed at the same time on the same assay.

Table 4.5: Plasma Synacthen results for paired samples with and without the addition of trasylol (aprotinin).

Subject sample	Plasma Synacthen (without Trasylol) (pg/ml)	Plasma Synacthen (with Trasylol) (pg/ml)	Absolute and relative difference in values (pg/ml and %)
Subject 3 1 mcg i.v Synacthen 20 minute sample	104.5	86.2	18.3 (17.5%)
Subject 5 1 mcg i.v Synacthen 30 minute sample	198.7	175.8	22.9 (11.5%)

4.3.2 Comparison of intravenous and nasal administration routes

The individual plasma Synacthen responses to each of the three doses of Synacthen are shown in *figures 4.6a-c*. High baseline and individual variation can be seen. It is clear that a single individual (subject 3, depicted in light green) had a significantly higher baseline in all three tests, compared with the other

subjects, and had greater overall variability. The mean plasma Synacthen (*figure 4.7a*) and mean serum cortisol (*figure 4.7b*) over time following administration with each of the three doses of Synacthen is shown.

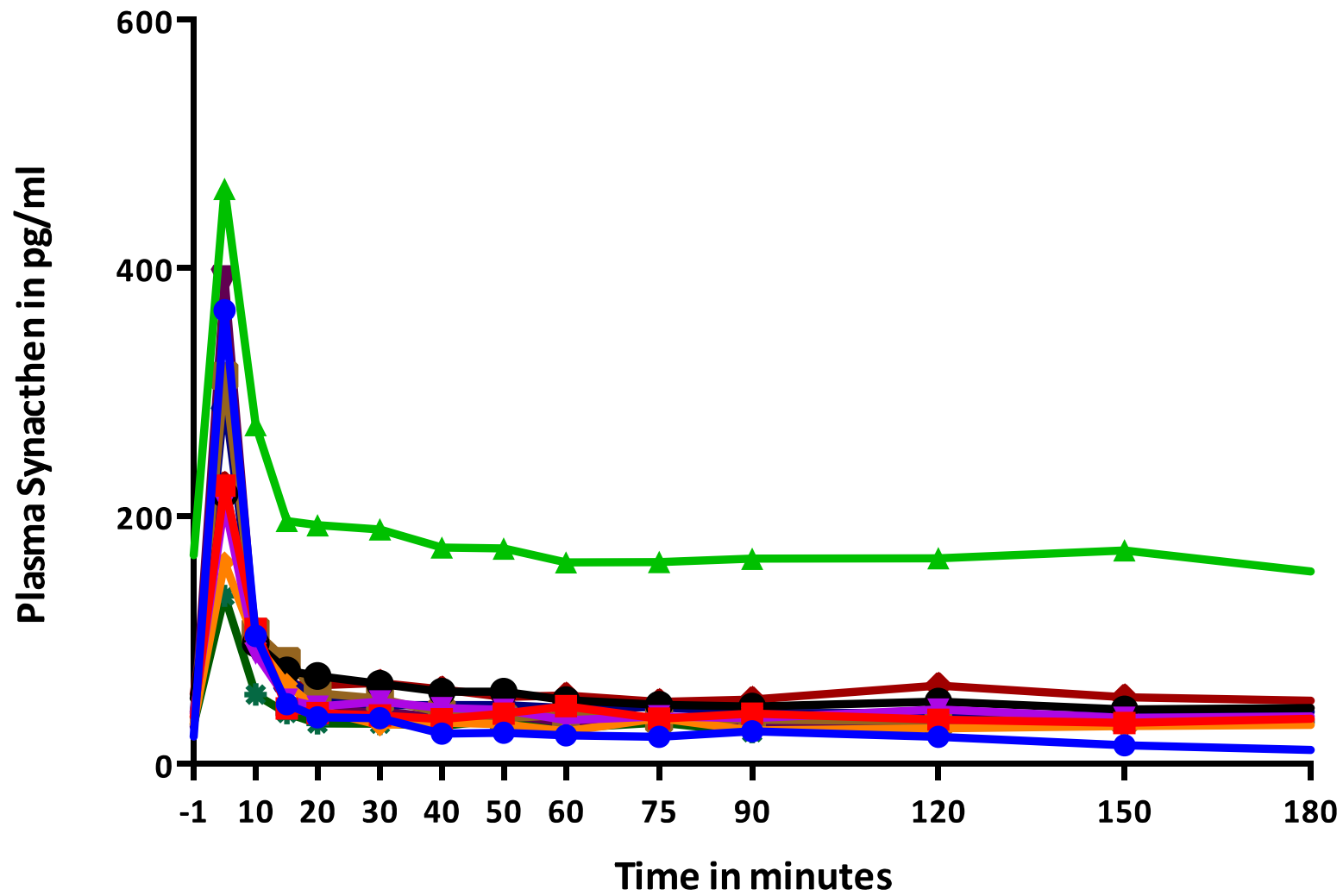


Figure 4.6a: Graph of the individual plasma Synacthen responses to i.v administration with 1 mcg Synacthen (baseline not subtracted). Each line depicts an individual study subject.

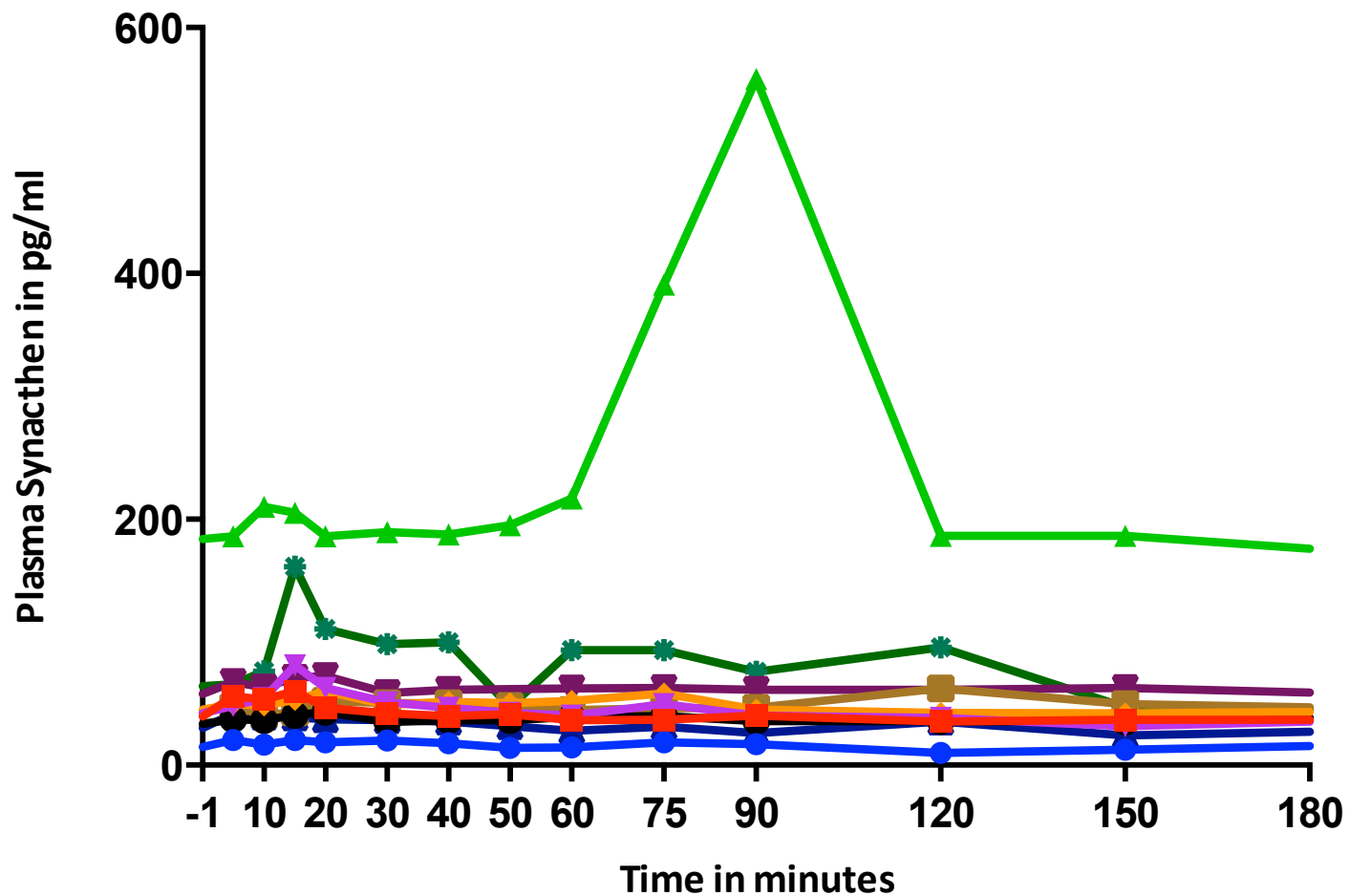


Figure 4.6b: Graph of the individual plasma Synacthen responses to i.n administration with 100 mcg Synacthen (baseline not subtracted). Each coloured line depicts a study subject.

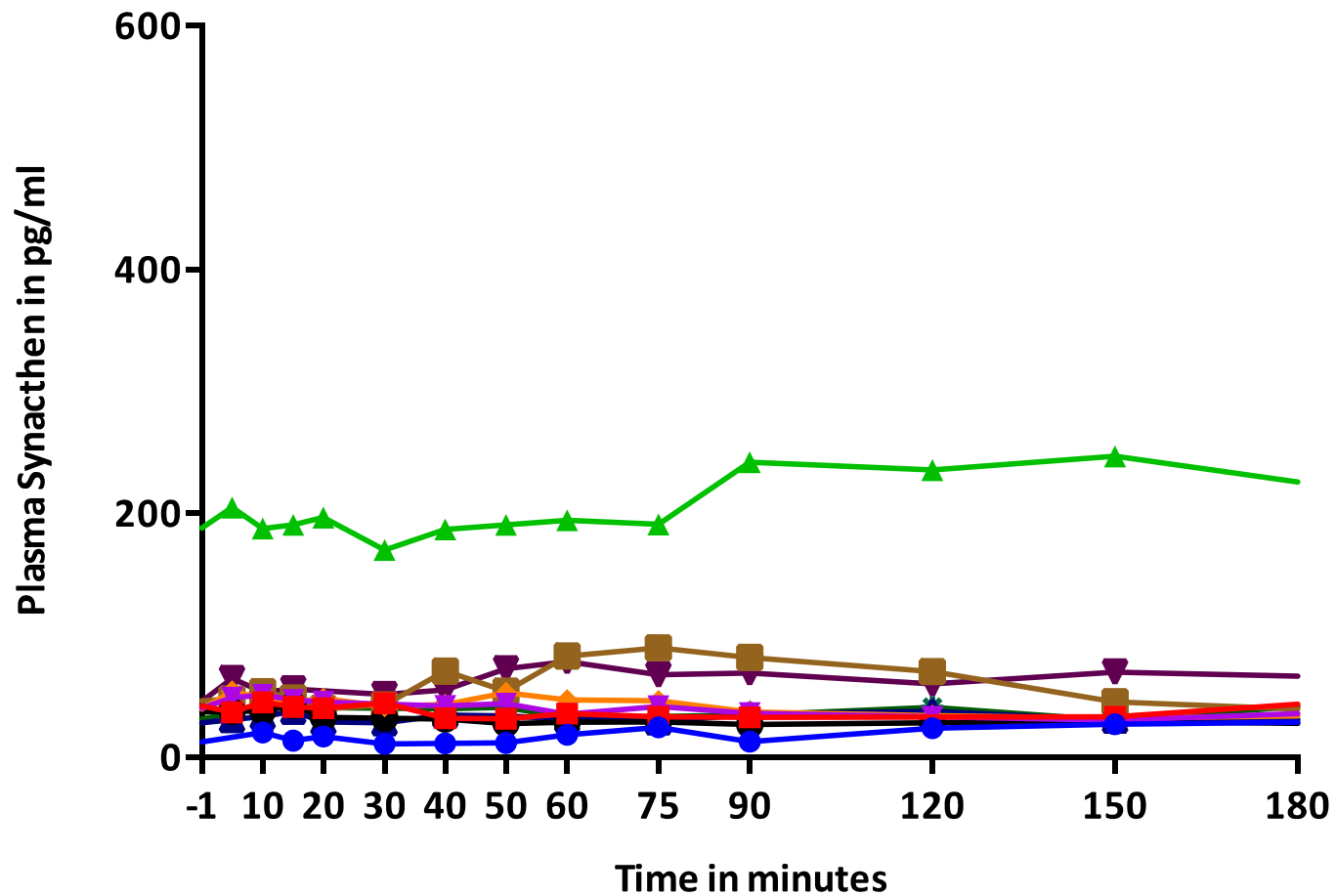


Figure 4.6c: Graph of the individual plasma Synacthen responses to i.n administration with 25 mcg Synacthen (baseline not subtracted). Each coloured line depicts a study subject.

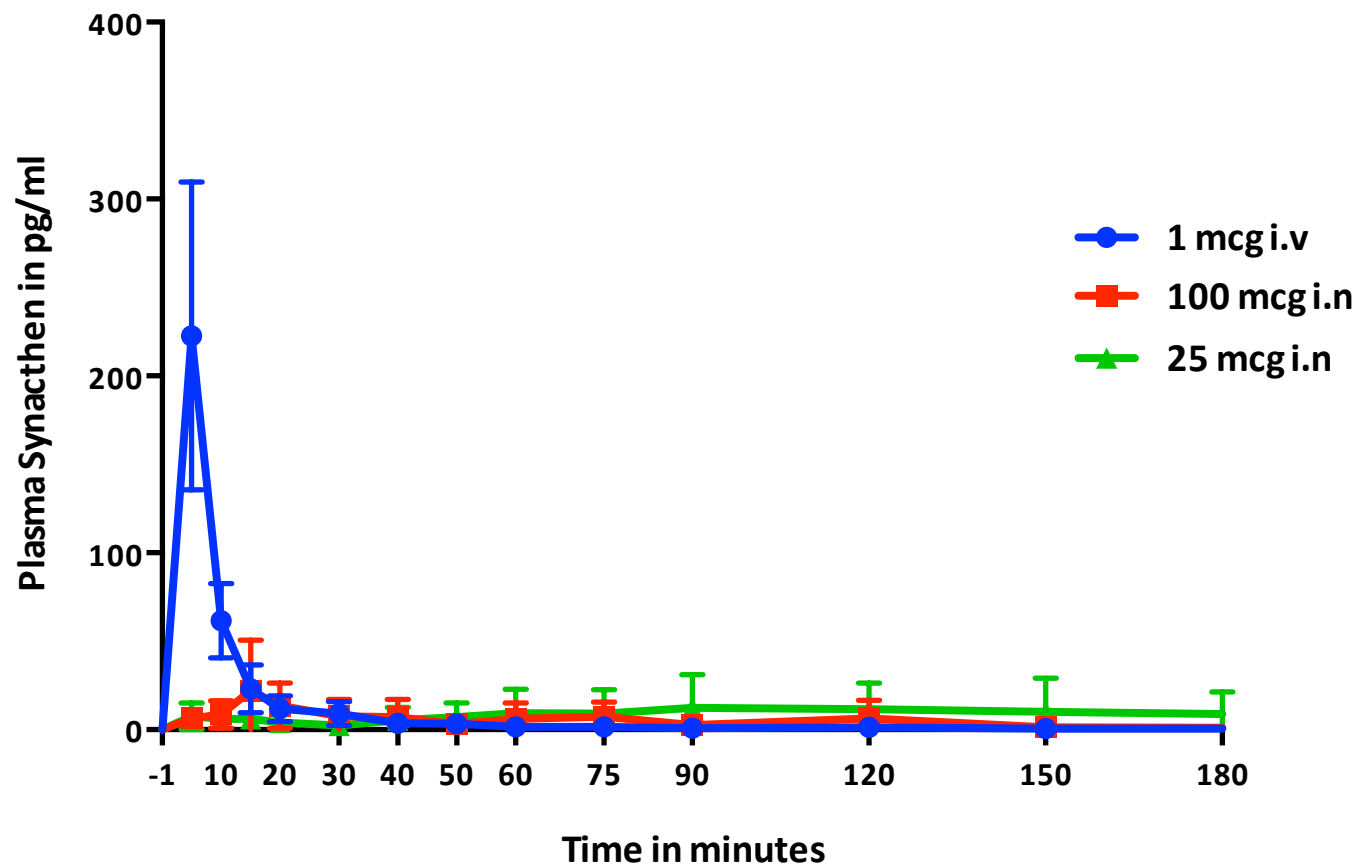


Figure 4.7a: Graph of the mean rise of plasma Synacthen from baseline in subjects following administration with 1 mcg i.v, 100 mcg i.n and 25 mcg i.n Synacthen. Standard deviations shown as error bars.

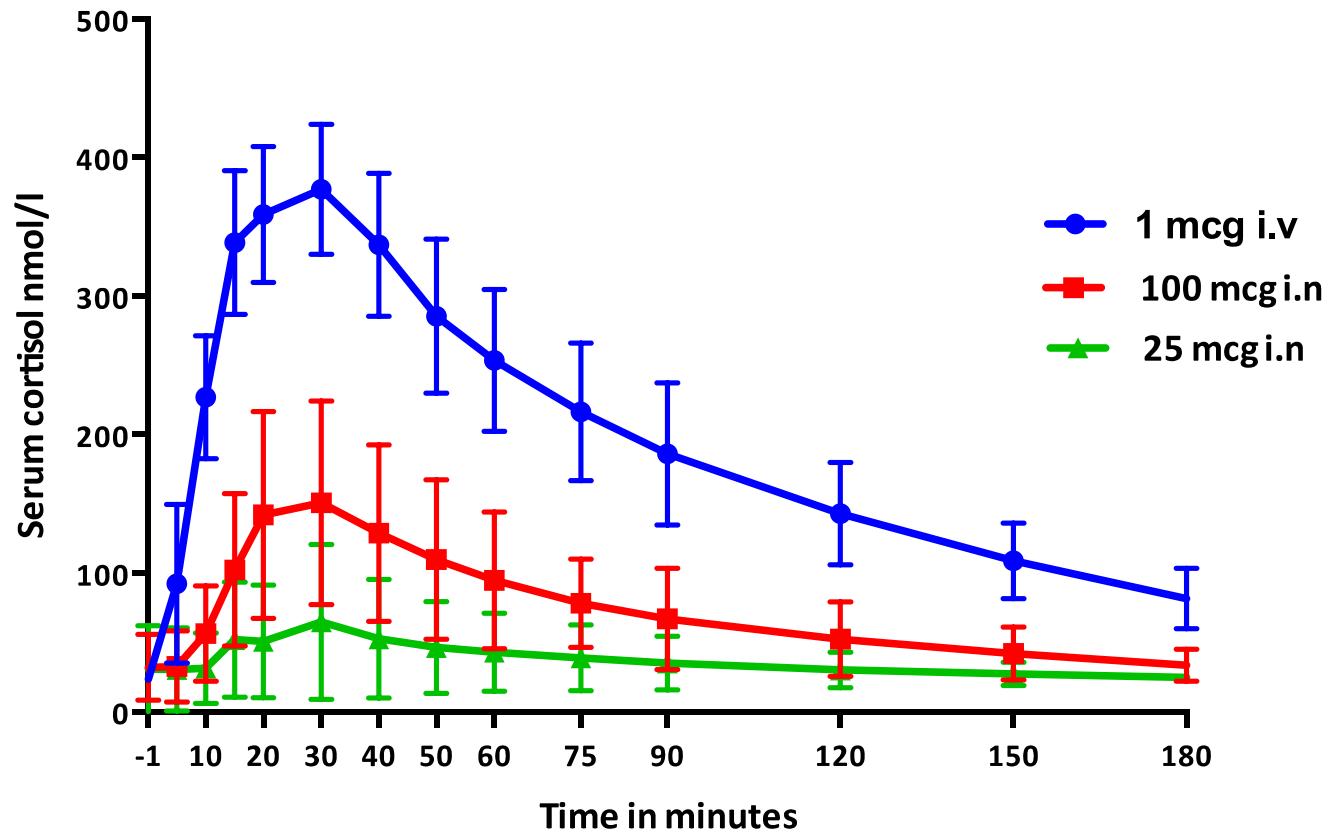


Figure 4.7b: Graph of the mean rise of serum cortisol from a suppressed baseline in subjects following administration with 1 mcg i.v, 100 mcg i.n and 25 mcg i.n Synacthen. Standard deviations shown as error bars.

In order to compare the efficacy of the two routes the subjects' mean plasma Synacthen and serum cortisol levels were compared at each time point (*table 4.6* summarises the data for all three tests and *tables 4.7a-4.7f* display the statistical analysis when each of the three doses were compared to one another).

Table 4.6: Mean plasma Synacthen and serum cortisol at each time point for subjects undergoing the three NeSST study visits. SDs shown in parentheses.

	1 mcg i.v visit				100 mcg i.n visit				25 mcg i.n visit			
	Synacthen pg/ml			Cortisol nmol/L N = 11	Synacthen pg/ml			Cortisol nmol/L N = 10	Synacthen pg/ml			Cortisol nmol/L N = 10
	Mins	N = 11	No S3* N = 10		Minus baseline N = 11	N = 10	No S3* N = 9		Minus baseline N = 10	N = 10	No S3* N = 9	
-1	50.4 (40.4)	38.6 (0.0)	0.0 (0.0)	23.6 (4.1)	55.6 (47.2)	41.3 (14.7)	0.0 (0.0)	32.0 (23.8)	51.2 (49.1)	36.0 (10.6)	0.0 (0.0)	30.9 (31.6)
5	273.1 (101.8)	254.0 (215.5)	222.7 (87.0)	92.2 (57.2)	62.0 (45.7)	48.3 (14.5)	6.4 (4.9)	32.5 (25.7)	62.5 (54.5)	44.7 (11.2)	7.6 (7.4)	30.5 (30.0)
10	112.0 (55.4)	95.9 (57.6)	61.6 (21.1)	226.9 (44.5)	63.9 (53.9)	47.7 (17.1)	8.6 (7.8)	56.4 (34.5)	57.3 (46.9)	42.9 (10.9)	6.2 (3.8)	31.4 (25.2)
15	73.5 (42.8)	61.2 (22.6)	23.1 (13.5)	338.7 (52.0)	77.5 (59.2)	63.3 (41.1)	22.0 (28.6)	102.2 (55.0)	57.1 (48.3)	42.2 (11.8)	6.0 (4.9)	52.0 (41.6)
20	62.2 (44.8)	49.1 (10.5)	11.8 (7.4)	359.0 (49.3)	69.1 (47.9)	56.1 (25.9)	13.5 (12.8)	142.1 (74.8)	54.5 (54.1)	36.7 (10.1)	4.1 (4.0)	50.7 (40.7)
30	59.3 (44.5)	46.4 (7.9)	9.1 (6.8)	377.1 (46.9)	63.1 (48.9)	49.1 (21.7)	7.5 (9.6)	150.9 (73.7)	50.6 (43.5)	37.3 (12.0)	2.3 (3.0)	64.9 (55.9)
40	53.6 (41.7)	41.5 (3.3)	3.5 (2.5)	337.0 (51.5)	62.7 (48.9)	48.8 (22.8)	7.1 (10.1)	128.9 (63.6)	54.8 (49.0)	40.1 (16.5)	5.4 (7.6)	52.7 (43.0)
50	53.5 (41.0)	41.5 (3.3)	3.4 (3.0)	285.4 (55.7)	56.4 (53.2)	39.1 (11.9)	3.1 (3.5)	109.7 (57.7)	56.1 (50.1)	41.1 (17.6)	7.0 (8.2)	46.4 (33.1)
60	49.9 (38.8)	38.6 (1.7)	1.5 (2.6)	253.5 (51.4)	62.9 (58.1)	45.8 (22.6)	6.0 (9.2)	94.7 (49.6)	58.5 (52.2)	43.4 (22.5)	9.3 (13.7)	42.9 (28.3)
75	49.8 (38.2)	38.5 (1.7)	1.5 (2.5)	216.4 (49.7)	82.7 (110.1)	48.5 (21.7)	7.4 (8.5)	78.2 (32.0)	58.6 (50.8)	43.9 (21.6)	9.2 (13.9)	39.0 (23.9)
90	48.2 (39.8)	36.4 (0.9)	0.8 (1.4)	186.0 (51.4)	94.6 (163.7)	43.2 (17.6)	2.7 (3.4)	66.9 (36.6)	61.0 (66.7)	40.8 (21.2)	12.4 (19.0)	35.0 (19.6)
120	50.2 (40.0)	38.7 (1.5)	1.4 (2.2)	142.9 (37.0)	60.4 (49.9)	46.4 (24.1)	6.3 (10.4)	52.4 (26.8)	62.7 (66.5)	41.1 (16.0)	11.6 (14.9)	30.1 (12.9)
150	47.9 (42.3)	35.5 (0.3)	0.6 (1.2)	108.8 (27.3)	52.9 (48.9)	38.1 (14.7)	1.4 (2.3)	42.1 (19.1)	57.6 (67.8)	36.6 (13.6)	10.0 (19.1)	27.2 (8.7)
180	46.9 (37.4)	36.0 (0.6)	0.5 (1.4)	81.8 (22.1)	52.4 (44.9)	38.7 (12.4)	1.0 (1.6)	33.7 (11.7)	57.4 (60.2)	38.7 (11.8)	8.9 (12.7)	24.8 (5.2)

*No S3 – data with subject 3 removed (high baseline Synacthen in all three visits)

Table 4.7a: Mean, Standard deviation (SD), Standard error of the mean (SEM) and p value (paired t-test with Bonferroni correction) results of plasma Synacthen levels comparing 1 mcg i.v and 100 mcg i.n Synacthen at 14 time points (N=10). Statistical significance defined as p value <0.004 and denoted in red.

Time (mins)		5	10	15	20	30	40	50	60	75	90	120	150	180
Mean	1 mcg i.v	232.0	61.3	22.2	11.8	10.0	3.8	4.2	1.7	1.2	0.9	1.5	0.6	0.6
	100 mcg i.n	6.4	8.6	22.0	13.5	7.5	7.1	3.1	6.0	7.4	2.7	6.3	1.4	1.0
SD	1 mcg i.v	85.8	22.2	13.8	7.8	6.4	2.4	2.7	2.7	2.5	1.5	2.3	1.2	1.5
	100 mcg i.n	4.9	7.8	28.6	12.8	9.6	10.1	3.5	9.2	8.5	3.4	10.4	2.1	1.6
SEM	1 mcg i.v	27.1	7.0	4.4	2.5	2.0	0.8	0.9	0.9	0.8	0.5	0.7	0.4	0.5
	100 mcg i.n	1.5	2.5	9.0	4.1	3.0	3.2	1.2	2.9	2.7	1.1	3.3	0.7	0.5
P value		<0.0001	<0.0001	NS (0.99)	NS (0.78)	NS (0.59)	NS (0.38)	NS (0.45)	NS (0.23)	NS (0.07)	NS (0.20)	NS (0.19)	NS (0.34)	NS (0.63)

Table 4.7b: Mean, Standard deviation (SD), Standard error of the mean (SEM) and p value (paired t-test with Bonferroni correction) results of plasma Synacthen levels comparing 1 mcg i.v and 25 mcg i.n Synacthen at 14 time points (N=10). Statistical significance defined as p value <0.004 and denoted in red.

Time (mins)		5	10	15	20	30	40	50	60	75	90	120	150	180
Mean	1 mcg i.v	219.6	61.3	22.2	12.3	10.0	3.8	3.8	1.7	1.2	0.9	1.5	0.6	0.6
	25 mcg i.n	7.6	6.2	6.0	4.1	2.3	5.4	7.0	9.3	9.2	12.4	11.6	10.0	8.9
SD	1 mcg i.v	81.0	22.2	13.8	8.1	6.4	2.4	2.9	2.7	2.5	1.5	2.3	1.2	1.5
	25 mcg i.n	7.4	3.8	4.9	4.0	3.0	7.6	8.2	13.8	13.9	19.0	14.9	19.1	12.7
SEM	1 mcg i.v	27.0	7.0	4.4	2.7	2.0	0.8	0.9	0.9	0.8	0.5	0.7	0.4	0.5
	25 mcg i.n	2.5	1.2	1.6	1.3	1.0	2.4	2.6	4.4	4.4	6.0	4.7	6.0	4.0
P value		<0.0001	<0.0001	NS (0.009)	NS (0.022)	NS (0.020)	NS (0.500)	NS (0.292)	NS (0.124)	NS (0.101)	NS (0.097)	NS (0.067)	NS (0.138)	NS (0.074)

Table 4.7c: Means, Standard deviations (SD), Standard error of the mean (SEM) and p values (paired t-test with Bonferroni correction) results of plasma Synacthen levels comparing 25 mcg and 100 mcg i.n Synacthen at 14 time points (N=10). Statistical significance defined as p value <0.004 and denoted in red.

Time (mins)		5	10	15	20	30	40	50	60	75	90	120	150	180
Mean	25 mcg i.n	7.6	6.2	6.0	4.1	2.5	5.8	5.2	9.3	9.1	12.1	11.4	9.9	8.9
	100 mcg i.n	6.5	8.6	22.0	13.4	7.2	9.5	6.2	8.5	9.8	5.5	8.8	4.4	4.0
SD	25 mcg i.n	7.4	3.8	4.9	4.0	2.9	7.3	4.7	13.1	13.2	18.1	14.2	18.1	12.0
	100 mcg i.n	5.2	7.8	28.6	13.6	9.2	12.6	10.3	12.1	11.4	10.0	12.9	10.0	10.0
SEM	25 mcg i.n	2.7	1.2	1.6	1.3	0.9	2.2	1.5	3.9	4.0	5.4	4.3	5.5	3.6
	100 mcg i.n	1.7	2.5	9.0	4.5	2.8	3.8	3.2	3.7	3.4	3.0	3.9	3.0	3.0
P value		NS (0.751)	NS (0.458)	NS (0.076)	NS (0.052)	NS (0.065)	NS (0.351)	NS (0.776)	NS (0.891)	NS (0.913)	NS (0.317)	NS (0.647)	NS (0.387)	NS (0.329)

Table 4.7d: Means, Standard deviations (SD), Standard error of the mean (SEM) and p values (paired t-test with Bonferroni correction) results comparing the serum cortisol response to 1 mcg i.v and 100 mcg i.n Synacthen at 14 time points (N=9). Statistical significance defined as p value <0.004 and denoted in red.

Time (mins)		5	10	15	20	30	40	50	60	75	90	120	150	180
Mean	1 mcg i.v	85.5	227.0	335.5	350.1	369.0	335.2	285.6	254.4	217.6	187.4	145.8	112.5	83.8
	100 mcg i.n	25.0	52.4	102.1	144.6	154.0	130.3	110.2	95.0	77.8	67.3	52.9	41.6	33.6
SD	1 mcg i.v	45.0	47.2	56.3	47.9	48.0	57.2	61.4	56.9	55.3	57.4	39.6	28.4	22.7
	100 mcg i.n	6.4	34.9	70.5	93.1	96.1	90.5	80.8	69.2	53.2	48.1	32.4	23.8	15.2
SEM	1 mcg i.v	15.0	15.7	18.8	16.0	16.0	19.1	20.5	19.0	18.4	19.1	13.2	9.5	7.8
	100 mcg i.n	2.1	11.6	23.5	31.0	32.0	30.2	26.9	23.1	17.7	16.0	10.8	7.9	5.1
P value		0.005	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table 4.7e: Means, Standard deviations (SD), Standard error of the mean (SEM) and p values (paired t-test with Bonferroni correction) results comparing the serum cortisol response to 1 mcg i.v and 25 mcg i.n Synacthen at 14 time points (N=10). Statistical significance defined as p value <0.004 and denoted in red.

Time (mins)		5	10	15	20	30	40	50	60	75	90	120	150	180
Mean	1 mcg i.v	85.5	226.9	334.8	350.8	372.9	335.2	287.5	255.5	217.8	186.9	146.1	111.7	84.4
	25 mcg i.n	21.0	24.0	46.7	45.1	61.5	50.4	43.6	39.2	36.1	32.3	27.7	26.4	23.7
SD	1 mcg i.v	45.0	44.5	53.1	48.2	47.1	54.0	58.3	53.7	52.2	54.1	37.3	26.9	21.5
	25 mcg i.n	0.0	5.3	39.8	39.0	57.7	44.5	33.4	26.8	22.9	18.4	10.8	8.8	3.9
SEM	1 mcg i.v	15.0	14.1	16.8	16.1	14.9	17.1	18.4	17.0	16.5	17.1	11.8	8.5	6.8
	25 mcg i.n	0.0	1.7	12.6	13.0	18.2	14.1	10.6	8.5	7.3	5.8	3.4	2.8	1.2
P value		0.003	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table 4.7f: Means, Standard deviations (SD), Standard error of the mean (SEM) and p values (paired t-test with Bonferroni correction) results comparing the serum cortisol response to 25 mcg and 100 mcg i.n Synacthen at 14 time points (N=9). Statistical significance defined as p value <0.004 and denoted in red.

Time (mins)		5	10	15	20	30	40	50	60	75	90	120	150	180
Mean	25 mcg i.n	21.0	24.3	49.5	48.2	66.0	53.6	46.1	41.2	37.7	33.5	28.4	27.0	24.0
	100 mcg i.n	25.0	52.4	102.1	128.7	154.0	130.3	110.2	95.0	77.8	67.3	52.9	41.6	33.6
SD	25 mcg i.n	0.0	5.5	41.1	40.5	59.3	46.0	34.5	27.6	23.7	19.1	11.2	9.1	4.0
	100 mcg i.n	6.4	34.9	70.5	85.4	96.1	90.5	80.8	69.2	53.1	48.1	32.4	23.8	15.2
SEM	25 mcg i.n	0.0	1.8	13.7	14.3	19.8	15.3	11.5	9.2	7.9	6.4	3.7	3.0	1.3
	100 mcg i.n	2.1	11.6	23.5	30.2	32.0	30.2	26.9	23.1	17.7	16.0	10.8	7.9	5.1
P value		0.097	0.049	0.025	0.009	0.001	0.003	0.006	0.008	0.006	0.015	0.012	0.055	0.056

The data demonstrate lower plasma Synacthen levels following nasal administration compared with the i.v route (more marked after the 25 mcg dose) with significant differences at 5 and 10 minutes in both nasal doses. There was no statistical difference between plasma Synacthen levels at any time point between the two nasal doses. The small rise in plasma Synacthen seen with 100 mcg is delayed to 15 minutes post administration compared with that following the i.v dose. The standard deviations displayed in *table 4.6* and error bars at the 5 minute i.v peak in *figure 4.7a* indicate considerable inter-individual variability at most time points and for all three tests, some of this variation is explained by skewing caused by individual data.

The cortisol responses from each of the three tests, shown in *figure 4.7b*, although present, are clearly reduced, in a dose-dependent manner, following nasal administration. The reduction in serum cortisol levels is highly statistically significant at all time points after 10 minutes when comparing 100 mcg i.n Synacthen and i.v and at all time points when comparing 25 mcg i.n to i.v. The cortisol response is significantly higher at 30 and 40 minutes when 100 mcg i.n Synacthen is administered compared to 25 mcg i.n. (*tables 4.7d-f*).

4.3.3 Dose-response relationship of Synacthen and cortisol

When looking at the dose of Synacthen that produces a maximal cortisol effect the relationship between peak plasma Synacthen, using the two different routes of administration, and peak serum cortisol was studied. Dr Johnson performed this analysis using a number of different models. The sigmoidal Emax gave the

best-fit for the concentration-response data. The concentration of plasma Synacthen to produce half maximum response was calculated to be 44 pg/ml as 10/11 Synacthen concentrations following a 1 mcg i.v dose were more than twice this concentration (88 pg/ml), indicating that for all the adult subjects studied a 1 mcg dose will give a near maximal effect (*figure 4.8*). Correction of values for differences in body size (using BMI and BSA) and volume of distribution (Vd) did not impact on the relationship.

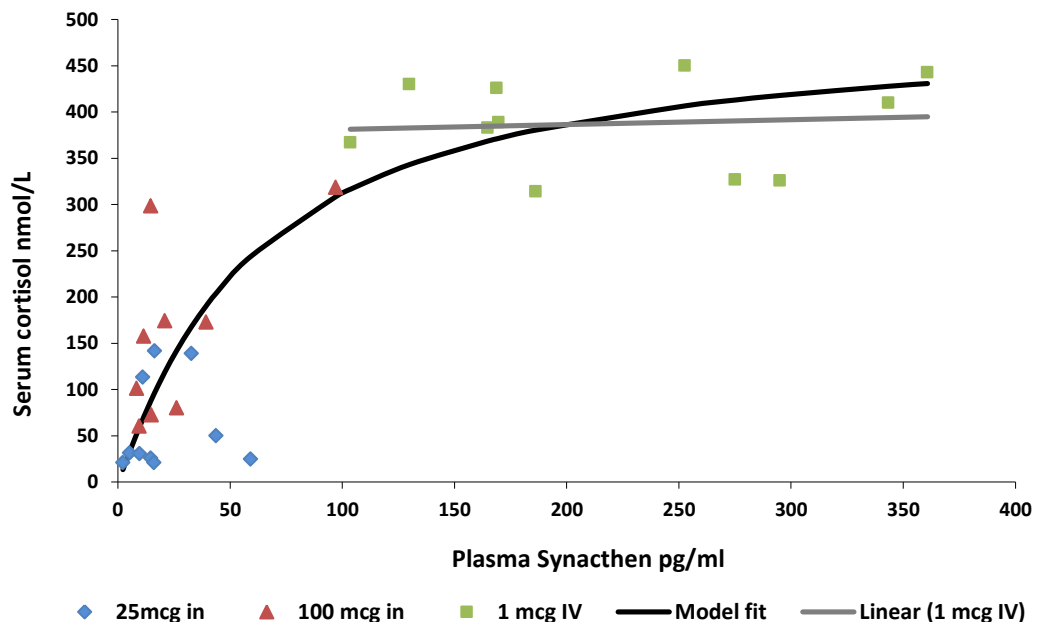


Figure 4.8: NesST Study individual peak plasma Synacthen plotted against peak serum cortisol following administration of three Synacthen doses

4.3.4 Characteristics of the i.v low-dose Short Synacthen Test

Performing a LDSST in 12 volunteers with 14 sampling times enabled an in-depth study of the test and its performance characteristics (*table 4.8*). On further analysis of the 1 mcg i.v data the peak plasma Synacthen was seen at five

minutes, the first sampling time after i.v injection, and returned to baseline by 20 minutes in all volunteers (*figure 4.6a*). The mean peak plasma Synacthen level was 222.7 pg/ml (SD 87 pg/ml) (range 103.6 to 360.8 pg/ml).

The Vd for Synacthen was very small and similar to typical blood volume. The clearance (CL) was intermediate, consequently the elimination half-life of the drug, calculated from $0.693 \cdot Vd/CL$, was very short with a median value of only 6.6 minutes. The related parameter 'Mean Residence time' (average time a molecule of Synacthen stays in the body) was also short at approximately 10 minutes.

Table 4.8: Summary of the PK (pharmacokinetic) parameters for 1 mcg i.v Synacthen

PK Parameter	Mean	SD	Median	Range
Clearance (ml/min)	675	313	680	324 - 1446
Volume of distribution (ml)	5275	2941	4680	2399 - 12050
Cmax (pg/ml)	222.7	101	178	103.6 – 360.8
Tmax (min)	< 5	0	<5	-
AUC _{0-t} (min*pg/ml)	1640	811	1443	691 - 3082
Mean Residence Time (min)	10.98	4.3	9.81	6.7 – 20.8
Elimination half-life (min)	8.24	8.28	6.6	2.14 – 33.39

The cortisol peak was seen to occur at different times: 18% (2/11) at 15 minutes, 18% (2/11) at 20 minutes, 45% (5/11) at 30 minutes and 18% (2/11) at 40 minutes. The mean peak cortisol was 383.8 (SD 48.7 nmol/l, range 314 to 450 nmol/l), lower than would be expected. All the volunteers achieved an

incremental rise from a suppressed baseline cortisol of more than 200 nmol/l (314-450 nmol/l) (*table 4.9*).

Table 4.9: NeSST Study individual data displaying operating characteristics of i.v LDSST (N=11). Subject 11 excluded for inadequate dexamethasone suppression

Subject number	Peak plasma Synacthen (pg/ml) (minus baseline)	Peak serum cortisol (nmol/L)	Timing of peak cortisol (minutes)	Cortisol increment (nmol/L)
1	343.4	410	30	384
2	186.2	314	15	314
3	295.1	326	30	326
4	169.6	389	30	389
5	129.9	430	20	430
6	164.8	383	20	358
7	275.0	327	40	327
8	252.6	450	15	450
9	360.8	443	30	443
10	168.7	426	40	393
12	103.6	367	30	339

4.3.5 Pharmacokinetic data comparing i.v and i.n routes

The data were analysed by the standard PK parameters for bioavailability and bioequivalence: T_{max}, C_{max} and AUC_{0-t}. The descriptive statistics for the i.v and i.n formulations and doses are shown in *table 4.10*.

Table 4.10: Summary statistics for bioavailability (F), C_{max} and AUC_{0-t} for the 25 mcg and 100 mcg nasal tests.

100 mcg dose (n = 11)								
	AUC_{0-t}		C_{max}		T_{max}		F	F (Uncorrected for dose)
Formulation	nasal	i.v	nasal	i.v	nasal	i.v		
Median	341	1443	14.9	178	17.5	5	0.00198	0.197
Min	152	691	5.96	103	15	5	0.00067	0.0669
Max	1237	3082	97	360	20	5	0.0179	1.78
25 mcg dose (n = 11)								
	AUC_{0-t}		C_{max}		T_{max}		F	F (Uncorrected for dose)
Formulation	nasal	i.v	nasal	i.v	nasal	i.v		
Median	148	1443	10.17	178	10	5	0.0023	0.0575
Min	14	691	2.91	103.6	10	5	0.00043	0.0108
Max	459	3082	18.6	360.8	10	5	0.0265	0.664

The median absolute bioavailability of the 100 mcg intranasal dose was approximately 0.2% of the 1 mcg i.v dose but there was a great deal of variability (range 0.0007 to 0.179%), with one subject having a higher bioavailability with the nasal dose. The time to maximum concentration (T_{max}) was longer for intranasal at 17.5 minutes compared to 5 minutes following i.v administration. The maximum concentration (C_{max}) was approximately 8.3% of i.v (14.9 pg/ml compared with 178 pg/ml). The median C_{max} ratio (nasal/i.v) was 0.088.

The data following administration with 25 mcg intranasal Synacthen must be viewed with caution as so little of the drug was absorbed. The median absolute bioavailability was 0.24% compared with i.v (similar to that seen with 100 mcg).

The Tmax was 10 minutes compared to 5 minutes i.v and the Cmax 5.7% of i.v (10.2 pg/ml compared with 178 pg/ml). The median Cmax ratio (nasal/i.v) was 0.044. Results of the pair-wise comparisons of the nasal formulation at the two dose levels are shown in *table 4.11*.

Table 4.11: Bioavailability assessment: point estimate and 90% confidence interval for ratio of geometric means for C_{max} and AUC_{0-t}

	C_{max}	AUC_{0-t}
100 mcg nasal versus i.v	0.087 (0.04-0.176)	0.226 (0.120 – 0.423)
25 mcg nasal versus i.v	0.044 (0.026 – 0.072)	0.065 (0.03 – 0.147)

The bioavailability of intranasal Synacthen was very low and if the same criteria were used as for bioequivalence, with acceptance criteria for the confidence intervals set at 0.8 to 1.25 (standard for PK analysis), then the nasal formulation would fail with respect to both Cmax and AUC.

4.4 Relationship between serum cortisol, salivary cortisol and salivary cortisone

Part of the process of validation of a non-invasive LDSST entails the demonstration of a close and reliable relationship between serum and salivary cortisol. At each time point paired serum and saliva samples were taken to enable relational analysis. In total 459 paired samples were obtained. The scatter plot of their correlation is shown below (*figure 4.9a*).

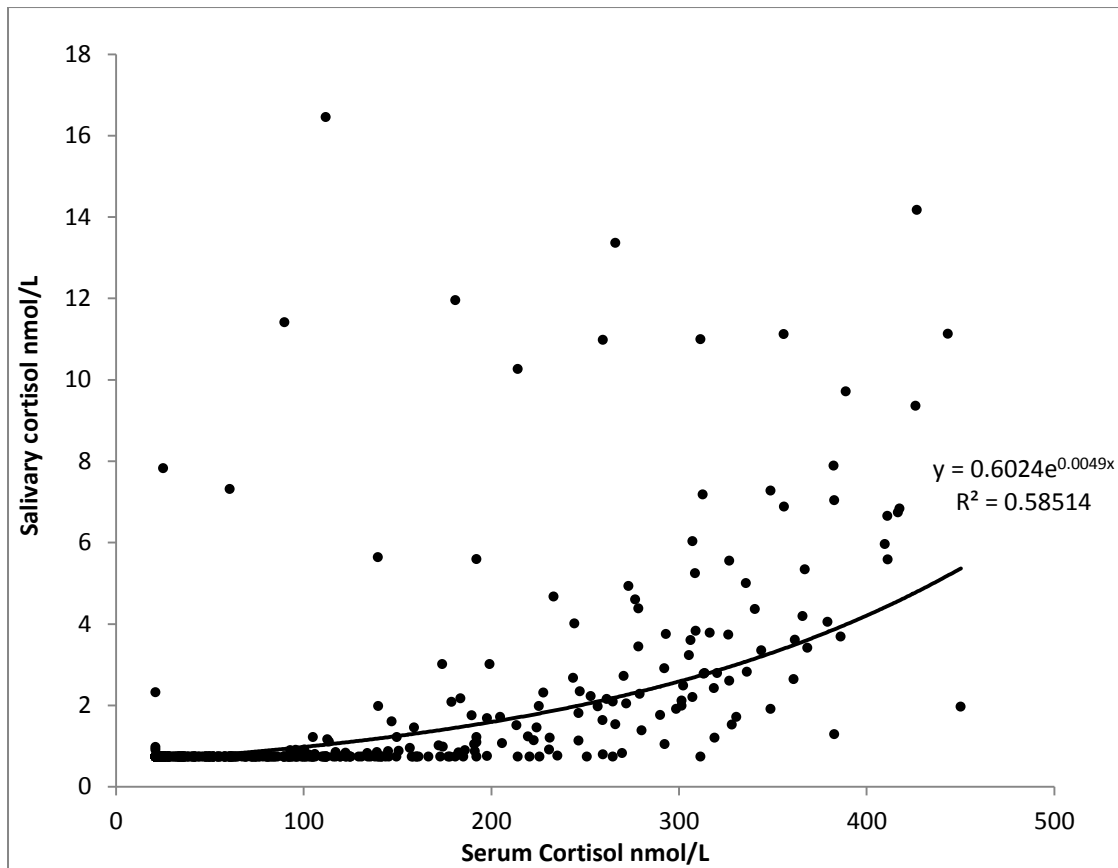


Figure 4.9a: Scatter plot of paired serum and salivary cortisol samples following Synacthen administration in the NeSST Study (salivary cortisol below the limit of assay detection displayed as 0.74 nmol/L and serum cortisol as 21 nmol/L).

There was a disappointing lack of a convincing correlation between serum and salivary cortisol, some of which may have been attributable to the time delay in cortisol reaching the saliva and some due to the skewing effect of so many samples with undetectable salivary cortisol. Reanalysis was performed with any value below the limit of detection excluded and correlating the peak serum and peak salivary cortisol, to take into account their different timings (N=19) (figure 4.9b). It shows a closer relationship, although that is based on many fewer data points.

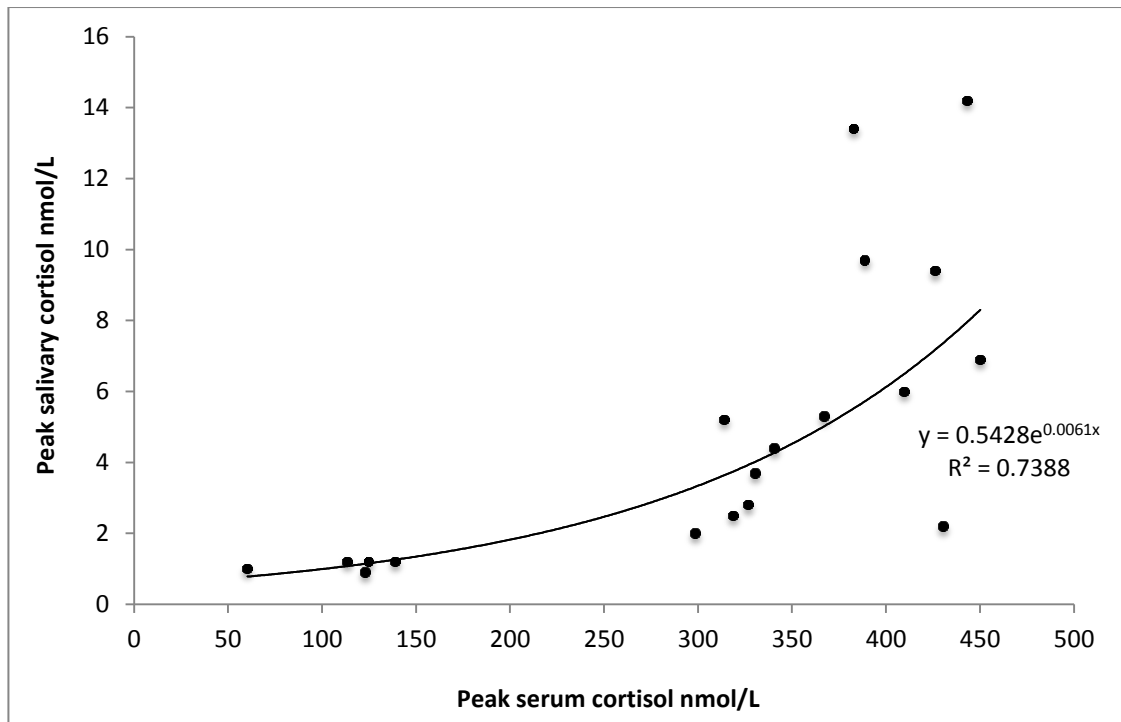


Figure 4.9b: Scatter plot of peak serum and peak salivary cortisol samples following Synacthen administration in the NeSST Study (salivary and serum cortisol below the limit of assay detection removed).

A closer relationship has been described between serum cortisol and salivary cortisone (Perogamvros, Keevil et al. 2010). *Figure 4.10a* displays the correlation of paired samples and *figure 4.10b* shows the correlation enhanced, albeit with many fewer data points, by removal of values below the lower limit of detection and the peak serum cortisol value correlated against the peak salivary cortisone.

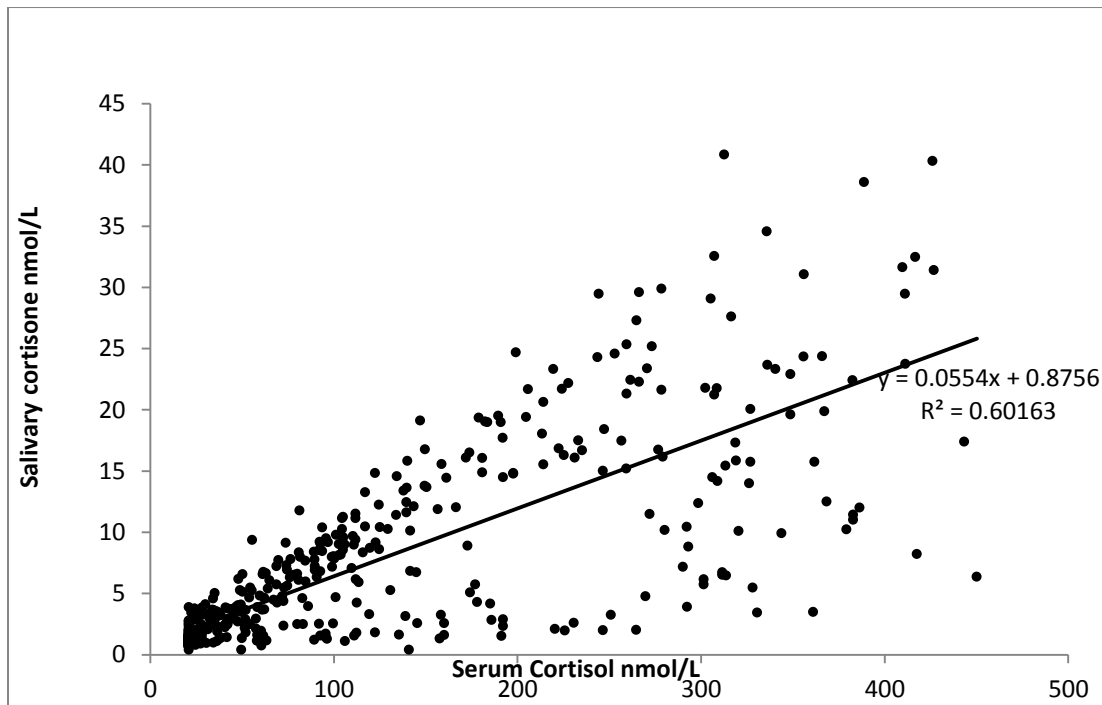


Figure 4.10a: Scatter plot of paired serum cortisol and salivary cortisone samples following Synacthen administration in the NeSST Study (salivary cortisone below the limit of assay detection displayed as 0.4 nmol/L and serum cortisol as 21 nmol/L).

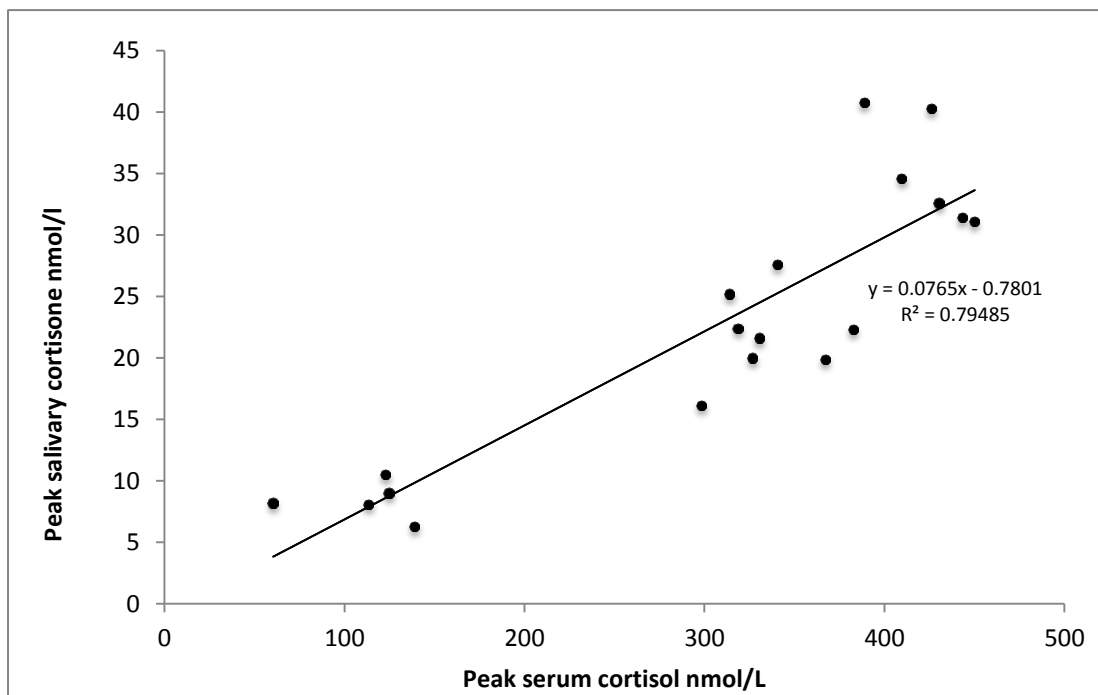


Figure 4.10b: Scatter plot of peak serum cortisol and peak salivary cortisone samples following Synacthen administration in the NeSST Study (salivary cortisone and serum cortisol values below the limit of assay detection have been removed).

The correlation between salivary cortisol and salivary cortisone was also sought and 475 paired samples analysed (*figure 4.11a*). Once again analysis was performed with any value below the limit of detection excluded and the peak salivary cortisol and peak salivary cortisone correlated (N=19) (*figure 4.11b*).

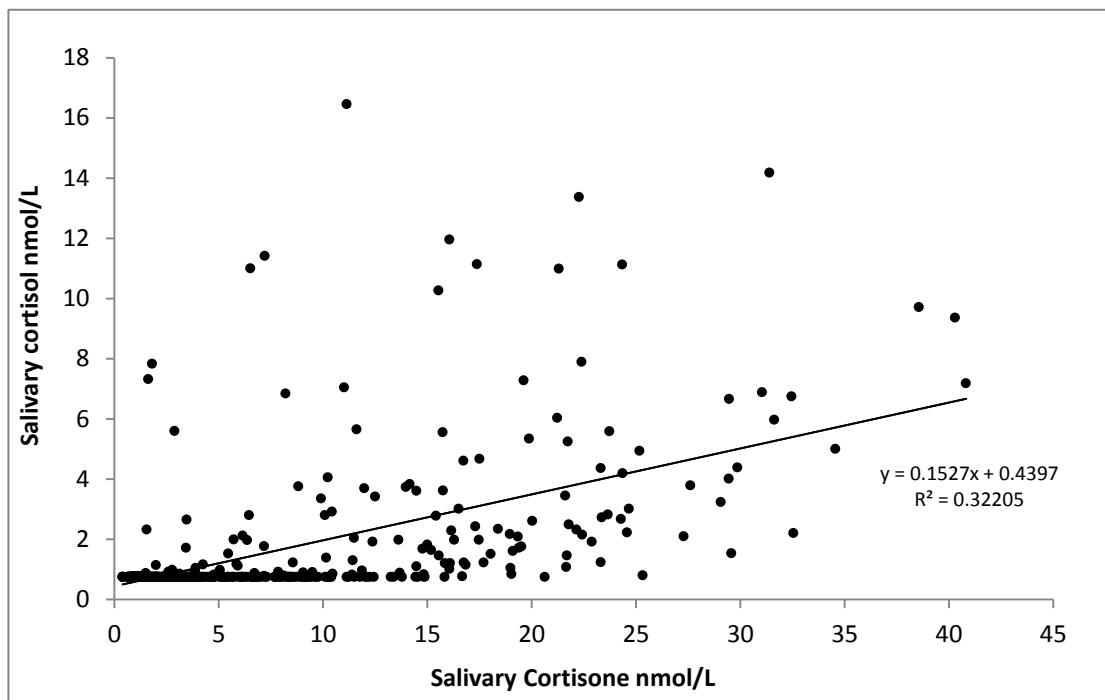


Figure 4.11a: Scatter plot of paired salivary cortisol and cortisone samples following Synacthen administration in the NeSST Study (salivary cortisone below the limit of assay detection displayed as 0.4 nmol/L and cortisol as 0.74 nmol/L).

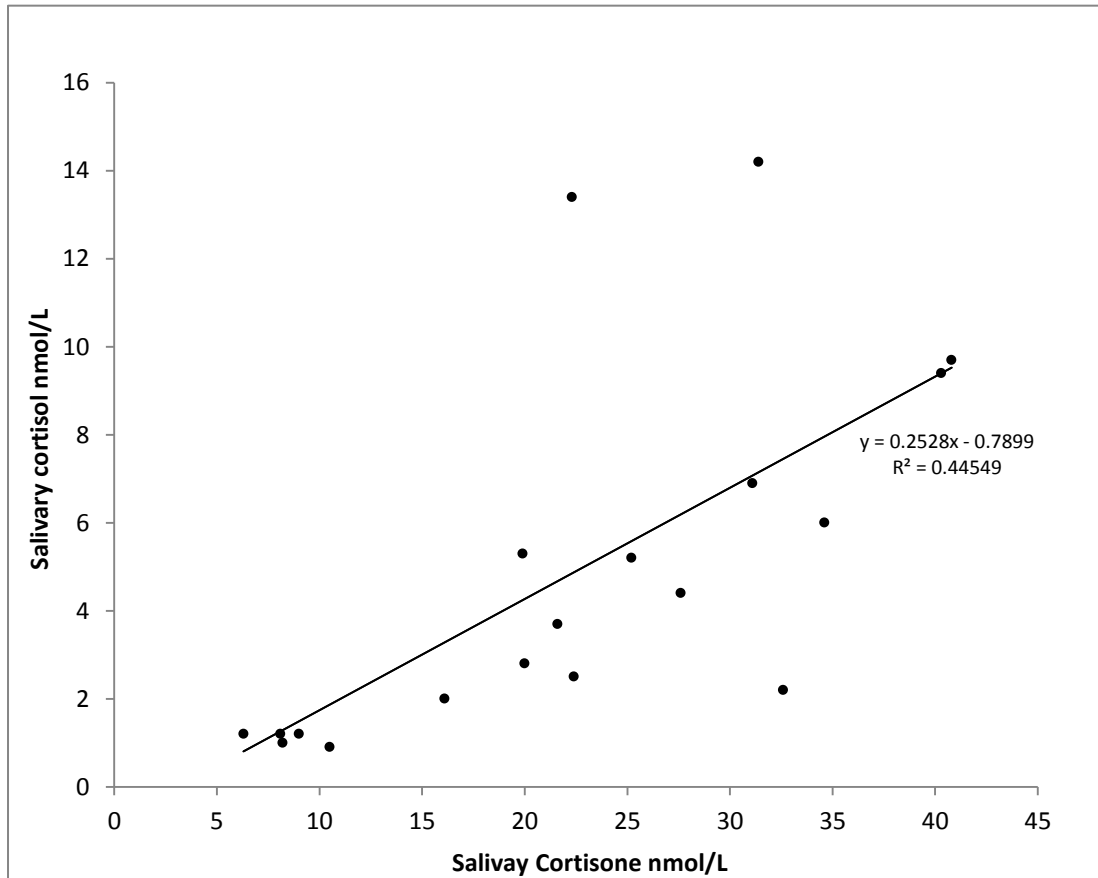


Figure 4.11b: Scatter plot of peak salivary cortisol and peak salivary cortisone samples following Synacthen administration in the NeSST Study.

The correlation between salivary cortisol and salivary cortisone was not as tight as predicted, although tightened by looking at the peaks, rather than paired samples. The relative timing of the peaks was then analysed.

4.4.1 Salivary cortisol and salivary cortisone peak timings

The timing of the peak serum cortisol following administration of Synacthen is likely to differ to that of peak salivary cortisol or cortisone. The timings of the salivary cortisol and cortisone peaks are displayed in *table 4.12*.

Table 4.12: Timings of the salivary cortisol and salivary cortisone peaks following administration with three different doses of Synacthen in the NeSST Study.

Minutes	Salivary Cortisol			Salivary Cortisone		
	1 mcg	100 mcg	25 mcg	1 mcg	100 mcg	25 mcg
20	0	1	0	0	0	0
30	7	0	0	1	0	0
40	4	1	0	10	3	2
50	1	0	0	1	3	2
75	0	0	0	0	1	0
No peak	0	9	11	0	4	7
Total	12	11	11	12	11	11

Following administration with 1 mcg Synacthen i.v the peak in salivary cortisol was seen in the majority at 30 minutes but with a third peaking at 40 minutes. Salivary cortisone peaked later and more reliably at 40 minutes.

When examining the salivary responses to all three doses of Synacthen it was observed that cortisone is the more sensitive marker of adrenocortical response at lower concentrations compared with cortisol. Many of the nasal tests had salivary cortisol responses below the limit of assay detection but a measurable cortisone response was seen. A flat response was observed in salivary cortisol for all 25 mcg tests and 82% of 100 mcg tests. There was a lack of discernable salivary cortisone peak seen in a third of study subjects following 100 mcg and two thirds following 25 mcg nasal Synacthen.

4.5 Study volunteer feedback

The nasal administration of Synacthen via a mucosal atomizer device was straightforward and required no formal training. All volunteers, except one who did not attend after the first visit, completed an anonymous, five-question, questionnaire. All responses affirmed that volunteers had found the administration of nasal Synacthen easy and the salivary samples straightforward to produce. The results of the questionnaire are tabulated and the free text answers summarised in *tables 4.13a-d* below.

Table 4.13a: Responses to question 1: How did you find having the nasal spray administered?

Very easy	Easy	OK	A little tricky	Difficult	Very difficult	TOTAL
8	2	1	0	0	0	11

Only four comments were made “Far easier than any spray I have had before”, “No problem, “Very quick to administer” and “It’s a bit painful if the hard tip is pushed against the septum so maybe make sure it goes in straight and isn’t pushed at an angle”.

Table 4.13b: Responses to question 2: How did you find receiving the nasal Synacthen?

No problem	Slightly unpleasant	Unpleasant	Very unpleasant	TOTAL
9	2	0	0	11

Four respondents commented on a vinegary smell/taste, due to the acetic acid, which forms a major constituent of the Synacthen preparation used. Those who commented said it was short-lived and not unduly unpleasant.

Table 4.13c: Responses to question 3: Did you experience any unpleasant side effects from the nasal Synacthen?

Stinging	Irritation	Sneezing	Soreness	Itching	Nasty taste	None	TOTAL
1	0	0	0	0	1	10	11

Subjects were asked to “tick all that apply” and a single respondent entered the stinging and nasty taste. All others indicated that they had experienced no unpleasant side effects, although three commented on the vinegary taste.

Table 4.13d: Responses to question 4: Overall how did you find the nasal Synacthen compared with the intravenous Synacthen?

Much better	Better	Much the same	Worse	Much worse	TOTAL
1	5	4	1	0	11

The respondent who answered “worse” qualified this by commenting “only slightly worse due to unpleasant vinegary smell”. Summarising a number of the respondents’ comments they felt that, although they did not find being cannulated unpleasant, they could see the nasal route being preferable for children or needle phobic adults.

4.5.1 General comments

Those who chose to comment complimented the running of the study.

4.6 Safety

There were no adverse events of any severity or type during the 34 visits.

4.7 Summary of results

The bioavailability achieved with 100 mcg and 25 mcg doses of nasal Synacthen were 0.2 and 0.24% respectively, compared to a 1 mcg i.v dose, this is more than 16 times less than suggested by the study conducted in rats (Wuthrich, Martenet et al. 1994). The C_{max} was 8.3% of i.v with 100 mcg and 5.7% with 25 mcg. The T_{max} was 17.5 minutes with 100 mcg and 10 minutes with 25 mcg compared with 5 minutes (first time point measured after iv administration) after i.v. Equivalence, in terms of a reliable SST dosing regimen, was not achieved. There was considerable variation in the height and timing of the individual's cortisol and Synacthen peaks.

5 Discussion

5.1 Proof of dexamethasone suppression

The hACTH RIA employed uses a single epitope near the N terminus of the molecule (amino acid sequence 5-18) for detection and was quoted as cross-

reacting 100% with Synacthen. Single-site RIAs are sensitive but may lack specificity. It was this lack of specificity, which was exploited to allow detection of Synacthen but required endogenous ACTH to be suppressed. Dexamethasone, at a standard suppression dose of 1mg on two occasions prior to the test, was used for this purpose.

Baseline (-1minute) ACTH and cortisol values were used to demonstrate adequate suppression. All but one of the volunteers (excluded from most analyses) had cortisol values of <50 nmol/L, the majority undetectable. In contrast to the cortisol, the ACTH values at -1 minute appeared unsuppressed for all volunteers. A number of factors have led to the supposition that there was assay interference by an unrelated yet structurally similar molecule. Both the ACTH (on the Immulite 2000 (Siemens) sandwich assay) and cortisol were shown to be adequately suppressed and thus the interfering molecule is likely to be unrelated to any HPA-axis molecule, due to its lack of suppression in the presence of dexamethasone. The values show remarkable intra-individual consistency but considerable inter-individual difference, implying interference with a molecule whose levels are person-specific, relatively constant within an individual but vary between individuals. Following administration of Synacthen the plasma values were seen to rise and then return to baseline by 20 minutes in all volunteers, allowing the inference that the interfering molecule was stable and unaffected by either dexamethasone or Synacthen.

The presence of assay interference compromises the scientific integrity of the method employed to measure Synacthen. MP Biomedicals, the hACTH RIA

manufacturer, was contacted and the data shared for their insights and advice. The information quoting 100% cross-reactivity with Synacthen, contained within the Directional Insert, was a supposition as the assay has never been tested against Synacthen. They had more than twenty-five years of experience with the same antibody but no records of similar problems and thus struggled to reproduce or explain the findings. They questioned whether the high baseline values might represent cross-reactivity with the dexamethasone and tested this reporting it not to be the case.

The presence of trasylol was tested and demonstrated higher results in the non-trasylol samples, although the values were not vastly different. Trasylol works to inhibit enzymatic degradation of Synacthen and as such one would expect that the samples with no added trasylol would contain less Synacthen. The difference in results is likely to represent assay variation rather than an inhibitory effect of trasylol. Only two samples were dual tested. Analysing more samples and those at -1 minute would have helped to more conclusively answer the question concerning assay interference.

A meeting to review all the data was convened and the expert opinion sought was that, due to the relative stability of the molecule, if baseline values are subtracted from all subsequent time points, then the inference that any rise is due to Synacthen remains valid.

One volunteer (subject 3) had much higher levels of ACTH detected compared with the other participants. There are two possible reasons for this; whatever is causing the assay interference is present in higher concentrations, or that he additionally has a heterophilic antibody (an endogenous antibody that binds assay antibodies) causing additional assay interference. As the ACTH antibody used in the assay is generated in rabbits it is possible that he has a heterophile antibody from previous rabbit exposure. His high ACTH levels skewed the data, causing non-parametric distribution and large standard deviations, and his data were excluded from analyses where relevant.

5.2 Comparison of intravenous and nasal administration routes

The plasma Synacthen levels attained following nasal administration were significantly reduced compared to the i.v route at both 5 and 10 minutes. The clearance of plasma Synacthen is very rapid and thus the large differences in plasma levels following the different routes of administration were not maintained over time. Despite differences in C_{max} and AUC there was no statistical difference shown between the two nasal doses when Synacthen level was compared at each time point. This is likely to be due to poor nasal absorption of both doses.

Some nasal absorption of Synacthen was achieved with both doses as evidenced by the resultant cortisol response. This was dose dependent with significantly higher serum cortisol levels attained with the 100 mcg nasal compared to 25

mcg. Serum cortisol values were higher following i.v administration compared to both nasal formulations at all time points after 5 and 10 minutes for 25 mcg and 100 mcg respectively.

5.2.1 Bioavailability of nasal Synacthen

The bioavailability of the nasal formulation was very low, less than 0.25% compared with the i.v formulation, as assessed by AUC corrected for dose differences. Despite minimal absorption of the 25 mcg dose the median relative bioavailabilities were very similar for the two nasal doses indicating that the very low absolute bioavailability is due to the drug not getting across the nasal mucosa rather than it being lost from the nose due to problems in administering the larger dose (e.g. dripping out).

Synacthen is a relatively large, polar molecule - not ideal characteristics for effective nasal absorption. The doses selected for the study were based on a single murine study (Wuthrich, Martenet et al. 1994). Nasal anatomy is subject to considerable interspecies variability. The three turbinates found in the human nose are relatively simplistic compared to the murine nose. The more sophisticated cell types lining the more complex maxilloturbinates of the rodent nose allow more efficient and effective disposal of airborne particles and thus likely more effective absorption of Synacthen (Harkema, Carey et al. 2006).

From the geometric means¹ for the AUC_{0-t} values at the two doses 0.226 (100 mcg) and 0.065 (25 mcg) there appears to be a reasonably linear effect between dose and exposure. The inference from this is that nasal absorption of Synacthen is, at least in part, a dose dependent phenomenon and by increasing the dose the bioavailability of Synacthen may increase in a predictable manner. The current study indicates that with a 5-fold increase in dose (500 mcg) a similar absolute Synacthen exposure compared to the 1 mcg i.v dose may be achieved. The constraints of a tolerable nasal volume (0.2ml per nostril) did not allow for higher doses as there is only one suitable commercially available Synacthen product.

Bioequivalence was not reached with either nasal dose. C_{max} of 0.087 and AUC_{0-t} of 0.226 are far below the confidence intervals set at 0.8 to 1.25. In the context of this study the comparative bioavailability is the important comparative parameter. The results for bioequivalence were at this stage academic as it is not necessary to demonstrate bioequivalence between an i.n and i.v formulation but to show the i.n formulation is safe and as efficacious as the LDSST.

5.3 Dose response relationship of Synacthen and cortisol

Although the methodologies used in the study were to determine pharmacokinetic parameters the data have provided the opportunity to examine some features of the LDSST. The LDSST has emerged as a popular test of

¹ Geometric mean – an average calculated using the product of the values of interest (as opposed to the arithmetic mean which uses the sum)

adrenocortical function, particularly within paediatric practice (Davies and Howlett 1996, Elder, Sachdev et al. 2012), yet there is paucity of data on the relationship between Synacthen and cortisol. There have been numerous studies describing the cortisol response to different doses of Synacthen yet, despite almost 50 years since the first description of the SST and its increasing popularity, few studies have focused on the relationship between ACTH/Synacthen and cortisol. Graphs depicting a straightforward causal relationship with ACTH rises followed by cortisol peaks appear in most endocrinology textbooks (Miller 2009) however it has been observed that cortisol can peak without a precedent ACTH rise and that even when the temporal relationship is clear, apparent proportionality may be lacking (Krieger, Allen et al. 1971). Whether these aberrations are attributable to inadequate assay sensitivity is unknown.

A logarithmic dose-response relationship between Synacthen and cortisol following the administration of different doses of Synacthen has been described previously, yet neither study correlated peak plasma Synacthen with peak cortisol (Leclercq, Bruno et al. 1972, Krishnan, Miller et al. 1993). The most in-depth relational study to date is a pharmacokinetic comparison of 250 mcg and 1 mcg Synacthen in ten healthy adults. The authors reported that neither the peak plasma level nor the AUC for Synacthen significantly correlated with any of the characteristics of the cortisol response, including peak cortisol level, AUC for cortisol, timing of the peak or incremental rise from baseline (Alia, Villabona et al. 2006). The NeSST Study results indicate that the dose-response relationship

was being masked in Alia *et al's* study by the use of Synacthen doses that resulted in maximal cortisol responses and therefore lie on the flat part of the dose-response curve. This is seen by the lack of relationship with 1 mcg but with a dose-response relationship at the lower doses.

Analysis of the NeSST Study data show that a plasma level of Synacthen of greater than 88 pg/ml, achieved with 1 mcg i.v LDSST, will result in near-maximal cortisol response. This is in keeping with two other studies in which the ACTH level required to near-maximally stimulate the adrenal gland has been quantified (Oelkers, Boelke et al. 1988, Darmon, Dadoun et al. 1999).

5.4 Characteristics of the 1 mcg i.v LDSST

5.4.1 Plasma Synacthen variability

Further analysis of the 1 mcg i.v data demonstrated that, despite all volunteers having similar demographics and receiving the same dose of i.v Synacthen, made up by the same method and by the same individuals, there was considerable variability in plasma Synacthen values, a phenomenon noted previously with administration of low doses (Krishnan, Miller et al. 1993). The single epitope RIA used in this study, like others used previously, lacks specificity and may account for some of the variability (Graybeal and Fang 1985, Krishnan, Miller et al. 1993, Darmon, Dadoun et al. 1999, Alia, Villabona et al. 2006). Other potential confounders include dilution or mixing inconsistencies whilst making up of the 1 mcg dose (Dickstein, Shechner et al. 1991), adsorptive losses of Synacthen on the

plastic equipment (Murphy, Livesey et al. 1998, Wade, Baid et al. 2010) or the unidentified interfering molecule. As it is cortisol that is used as the primary endpoint in clinical practice the significance of the variation in Synacthen peaks is uncertain but may add weight to the call for commercial production of a 1 mcg Synacthen dose.

It may be that the plasma Synacthen peak was missed following i.v administration by sampling at 5 minutes. With such rapid clearance it is likely that the real peak may lie between 1 and 3 minutes.

5.4.2 Timing of serum cortisol peak

The timing of the serum cortisol peak following 1 mcg i.v Synacthen varied and occurred at 30 minutes in 45% of study subjects. All experienced their peak between 15 and 40 minutes. Studies show the cortisol peak to occur between 15 and 30 minutes (Crowley, Hindmarsh et al. 1991, Daidoh, Morita et al. 1995, Nye, Grice et al. 1999, Park, Park et al. 1999). The BSPED survey (*vide supra chapter 2, section 1*) revealed that all units sample at 0 and 30 minutes, 52% at 20 minutes and 69% at 60 minutes, with a number of additional sampling times (Elder, Sachdev et al. 2012). The NeSST Study data show that sampling at only 0, 20 and 30 minutes would have missed a third of the peaks and a 60-minute sample would have been of no additional benefit being too late to detect any cortisol peaks. From our small adult cohort sampling times of 0, 15, 20, 30 and 40 minutes would have identified all the cortisol peaks.

5.4.3 Low peak serum cortisol

The serum cortisol results were universally low in terms of diagnostic cut-off. Although controversial and subject to considerable variation in practice, most endocrinologists practicing in the UK look both at the incremental rise in serum cortisol from baseline, usually taking greater than 200 nmol/L as normal, and a peak cortisol of more than 500 nmol/L or 550 nmol/L (Lashansky, Saenger et al. 1991, Miller 2009, Elder, Sachdev et al. 2012). All study subjects had a serum cortisol incremental rise of more than 200 nmol/L but none managed a peak of 500 nmol/L. As all subjects were healthy it is thought that this may be due to a blunting effect of the dexamethasone. It is important to consider that the diagnostic cut-offs quoted in the literature are not from a suppressed baseline and that the peak cortisol is a combination of baseline plus incremental rise and will therefore be lower when the baseline is artificially lowered. The effect of dexamethasone on the peak serum cortisol has been previously reported by some as having a potentiating effect (Landon, James et al. 1967, Leclercq, Bruno et al. 1972, Graybeal, Fang et al. 1985, Rosenfield, Helke et al. 1985, Crowley, Hindmarsh et al. 1991) and by others a blunting effect (Saito, Ichikawa et al. 1979, Copinschi, Beyloos et al. 1983, Crowley, Hindmarsh et al. 1991, Dickstein, Shechner et al. 1991). It is unclear how dexamethasone interacts with the cells of the adrenal cortex and whether there is a direct inhibitory effect (Saito, Ichikawa et al. 1979) or not (Dardis and Miller 2003) as has been shown in rat models (Loose, Do et al. 1980). In order to investigate this further a study in which volunteers undergo the LDSST with dexamethasone suppression and without would need to be conducted.

5.5 Relationship between serum cortisol, salivary cortisol and salivary cortisone

Whilst acknowledging that it is unlikely a blood value will equate exactly with a tissue concentration and the serum and salivary assays will have different precision profiles, relational analysis (correlation) has been used, as by others previously, to assess whether the different measures agree and at different cortisol concentrations.

The relationship observed between salivary cortisol and serum cortisol was exponential, as seen in previous studies, and is due to the Cortisol Binding Globulin (CBG) effect (Vining, McGinley et al. 1983, Perogamvros, Aarons et al. 2011). At lower concentrations cortisol is bound by serum CBG, however at higher concentrations, when CBG is saturated, a more rapid increase in salivary concentration is seen. The change in curve slope “tipping point” appears to be at approximately 360nmol/L of serum cortisol, which is lower than the previously reported 500nmol/L (Perogamvros, Owen et al. 2009, Perogamvros, Owen et al. 2010). A tighter and linear correlation was seen between salivary cortisone and serum cortisol, as would be expected because cortisone more closely reflects serum free cortisol and is unaffected by changes in CBG. The closest correlation has been shown to be between serum free cortisol and salivary cortisone but serum free cortisol is not routinely quantified because to do so is time-consuming, cumbersome and expensive (Perogamvros, Keevil et al. 2010). The correlations achieved in this study are weaker than those quoted in the

literature, despite being published by the same group who performed the salivary cortisol and cortisone analysis for NeSST. The reason for this discrepancy is unclear, however the relationship was tightened by correlating the serum and salivary peaks rather than the paired samples indicating that enzymatic conversion and absorption effects may have played a role.

5.5.1 Salivary cortisol and salivary cortisone peaks

Salivary cortisone was seen to be the more sensitive marker of adrenocortical response at lower values as peaks were detected when no salivary cortisol response was seen. Salivary cortisone has an approximately two-fold greater sensitivity, when measured by mass spectrometry, compared with salivary cortisol and the 11β -HSD-2 activity in saliva causes a 3-4-fold increase in cortisone over cortisol (personal communication, Professor Brian Keevil). Additionally the rate of enzymatic activity varies between individuals (personal communication, Professor Brian Keevil). In contrast serum cortisone has been found to decrease following ACTH administration suggesting that ACTH stimulates restoration of inactive cortisone to biologically active cortisol (Vogeser, Zachoval et al. 2001). Following the initial serum rise in cortisol, parotid gland 11β -HSD-2 activity may convert cortisol to cortisone and be partly responsible for the greater sensitivity. The timing of the peak salivary cortisol was seen between 30 and 40 minutes and between 40 and 50 minutes with salivary cortisone. The slightly later peak with cortisone is likely to be due to its enzymatic conversion from cortisol.

5.6 Limitations and further work

The PK data show that nasal Synacthen is absorbed in a dose-dependent manner. To improve the bioavailability an increase in the dose would likely increase the absolute amount of Synacthen available systemically. The relative bioavailability indicates that a 5-fold increase in dose (500 mcg) would be required to achieve the same exposure as a 1 mcg i.v dose. The C_{max} value, when the dose was increased four-fold (25 mcg to 100 mcg), did not increase four-fold. This would, in normal circumstances, allay fears that increasing the dose to achieve bioequivalence would result in very high initial concentrations. Potential problems with toxicity when increasing the dose are not a concern in this study as Synacthen is routinely given in supraphysiological amounts (250 mcg i.v and a 1 mg depot preparation) and is known to be safe. The scope for an increase in the dose of nasal Synacthen is limited by the currently available formulation (250 mcg/ml vials only). Due to the restriction on volume for nasally administered drugs (0.2ml per nostril) equivalence in the adrenal stimulation with the LDSST could not be achieved just by increasing the volume five-fold, manufacturing a more concentrated formulation would be required. The addition of a nasal drug absorption enhancer may additionally increase the bioavailability of Synacthen.

5.7 Conclusion

This study in 12 healthy, male adult volunteers has shown nasal Synacthen to be safe and easy to administer. Some systemic absorption was achieved as a

resultant and dose-dependent cortisol response was seen however the Synacthen bioavailability was only 0.2% of that seen with i.v administration. When corrected for the increased dose given, it is postulated that a five-fold increase in the 100mcg dose is required to reach an acceptable bioavailability, however the considerable individual variability seen with nasal absorption is of concern.

By studying the dose-response relationship of plasma Synacthen and serum cortisol at doses below 1 mcg a dose-response relationship between Synacthen and cortisol was demonstrated which supports evidence that 1 mcg Synacthen maximally stimulates the adrenal gland and give further credence to the use of the LDSST in clinical practice.

Chapter 5: NeSST2 study

*The second clinical
study in the
development of a non-
invasive Short
Synacthen Test*

1 Introduction

The first clinical study (NeSST, *chapter 4*) showed nasal Synacthen to be easily administered and well tolerated but poorly absorbed and therefore exhibited low bioavailability. The estimated appropriate nasal dose to yield a similar cortisol response to the 1 mcg i.v dose was 500 mcg. The scope for a dose increase was limited by the available formulation of the Synacthen and tolerable nasal volume, 500 mcg would require 1 ml per nostril of 250 mcg/ml commercial preparation, far exceeding the recommended 0.2 ml per nostril (Personal communication, Archimedes Pharmaceuticals Ltd)(Wolfe and Braude 2010). Manufacture of a more concentrated formulation of Synacthen appropriate for nasal administration was therefore investigated. This offered the opportunity to further improve the absorption/bioavailability by the addition of a nasal drug enhancer, with the possibility of reducing the inter-individual variability in plasma Synacthen seen in the first NeSST Study (*vide supra chapter 4, section 5.4.1*).

Formulating a novel compound, performing appropriate stability testing, obtaining regulatory approvals and manufacturing clinical study grade product was a considerable undertaking. It required additional grant income and input from a number of different colleagues and commercial companies. This chapter details the process of manufacture of the new compound and the results of administering the novel IMP (Investigational Medicinal Product) to human subjects in a clinical study – the NeSST2 Study.

The generic name tetracosactide, rather than Synacthen (proprietary name), is used to describe the active pharmaceutical ingredient in the newly formulated IMP.

2 Aims and Objectives

2.1 Aim:

To investigate the use of a higher dose of nasal Synacthen (500 mcg) and the addition of a nasal drug enhancer in the development of a non-invasive alternative to the 1-microgram (low-dose) intravenous Short Synacthen Test (LDSST).

2.2 Objectives:

- To compare the bioavailabilities of three different nasal formulations of tetracosactide with the 1 mcg i.v SST.
- To assess the effect of dose escalation on the absorption of nasal tetracosactide.
- To assess the effect of a nasal drug enhancer, chitosan, on the absorption of nasal tetracosactide.
- To establish the first bioavailability and pharmacokinetic data of nasal tetracosactide with chitosan in humans.
- To establish the inter-individual variability of cortisol response to nasal tetracosactide with chitosan in humans.

3 Materials and Methods

The methodology employed in the NeSST2 Study was very similar to NeSST Study (*vide supra chapter 4, section 3*). The pre-study set-up and essential methodological differences are described below.

3.1 Pre-study work on Investigational Medicinal Product

Before recruitment or subject visits took place tetracosactide was sourced and imported; a nasal drug enhancer was selected and sourced; manufacturing was tendered and a manufacturer chosen; legal contracts were written; the novel combinations stability were tested; appropriate documentation was written; regulatory approvals (REC, MHRA and R+D) were gained and manufacture of the IMPs undertaken.

Archimedes Development Ltd (linked with the Nottingham University School of Pharmacy) has expertise in nasal drug delivery. Meetings were held with their Director of Pharmaceutical Development and Vice President to share the NeSST Study results and garner advice about improving the absorption of nasal Synacthen, the viability of a nasal enhancer and what the process would involve. A number of enhancers were researched and then discussed. The majority of their experience is with chitosan and they were confident that its addition would significantly increase the bioavailability of nasal Synacthen. They estimated a bioavailability of 5-10% (an increase of 25-50 fold) based on experience with peptide molecules of comparable solubility, molecular weight, and polarity. It

was also thought that chitosan would reduce the variability of nasal absorption seen in the first NeSST study.

After having chosen to combine tetracosactide with chitosan quotes were requested from three NHS manufacturing pharmacies and Archimedes Development for the development and manufacturing work. Despite a number of enquires only Archimedes tendered and their quote was felt to be reasonable and thus they formulated the tetracosactide/chitosan nasal solutions (TNS), conducted stability testing and manufactured the clinical supplies. Archimedes Development Ltd performed all the necessary pre-clinical work, the information from which formed the basis of the Investigator's Brochure, a detailed document submitted to the MHRA with comprehensive information about the IMP.

The tetracosactide was sourced from Bachem (Bubendorf, Switzerland) and imported (after frantic negotiations during a medical student lecture on a Friday afternoon, when it was seized by HM Customs and Excise on entry to UK!). Archimedes Development Ltd supplied the Chitosan glutamate, manufactured under the trade name PROTASAN™ by FMC BioPolymer (NovaMatrix) Norway. Its use in the IMP required legal permissions from FMC Biopolymer (Philadelphia, USA) and involved lengthy negotiations and a tripartite agreement. The manufacture, packaging, quality control testing and batch release of the IMP formulations were conducted in accordance with current Good Manufacturing Practice at Archimedes Development Limited, Nottingham, UK. After more than 18 months the new CTIMP (Clinical Trials of a Investigative Medicinal Product)

study was successfully awarded REC, MHRA and R+D approval and the manufactured clinical supplies delivered to SCH.

3.2 Study design

The NeSST2 Study had a similar design to NeSST (*vide supra chapter 4, section 3.1*); a pharmacological bioavailability study using an open label, quadruple arm, crossover design (*figure 5.1*), as recommended by EMA (European Medicines Agency 2010). In keeping with pharmacokinetic trials of this kind the subjects were not blinded and did not receive a placebo. In contrast to the NeSST Study the subjects received the first three formulations (1 mcg i.v, 100 mcg tetracosactide and chitosan and 500 mcg tetracosactide) in a randomised order and the fourth and final formulation (500 mcg tetracosactide and chitosan) only after interim analysis showed this to be required. Randomisation was felt to improve the scientific integrity of this bioavailability study and was performed by the Pharmacy department at SCH using an on-line programme (www.randomization.com). As before it was a single-centre study conducted from SCH, Sheffield, UK.

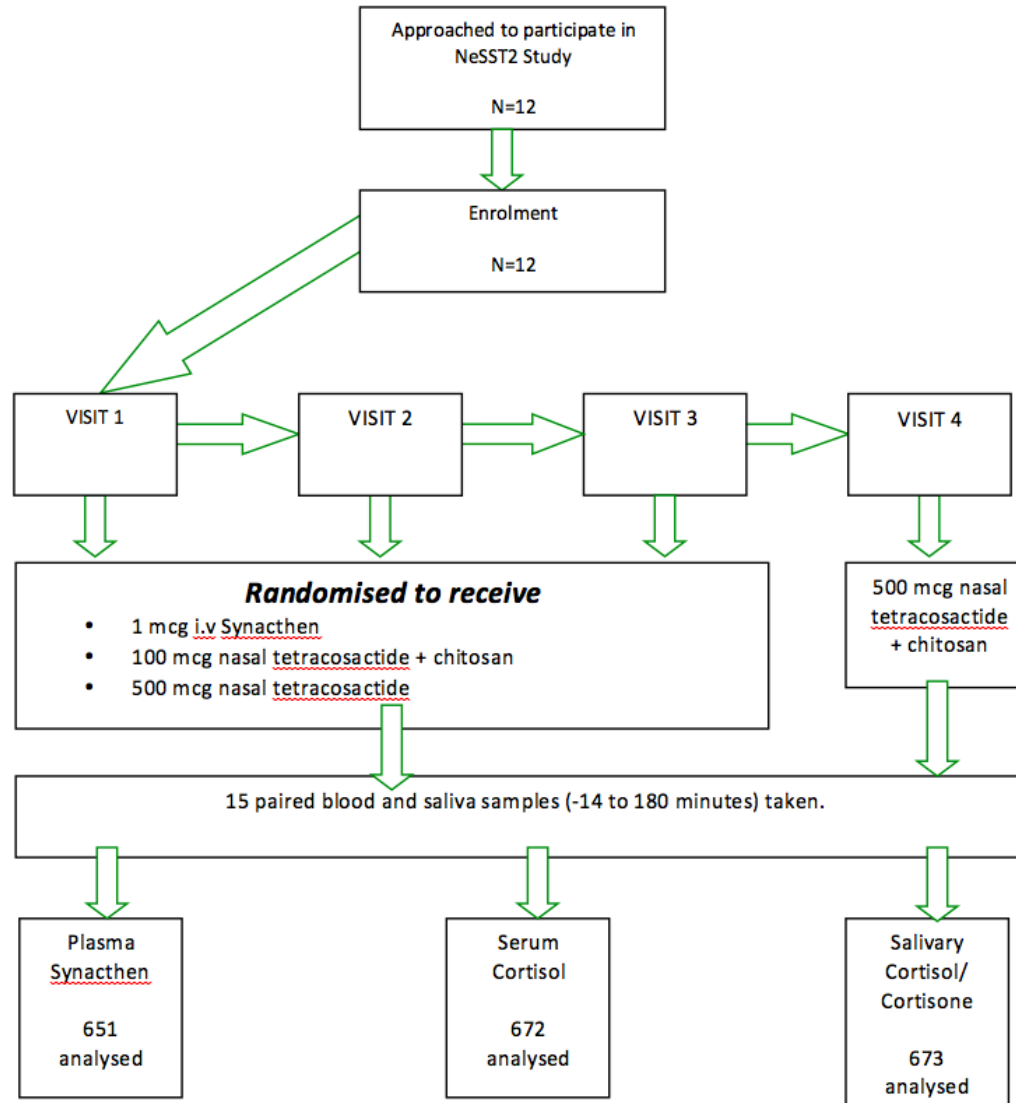
3.3 Subjects and the visits

In most respects the subjects and their visits were as they had been for the NeSST Study (*chapter 4, section 3.2 and 3.3*). The differences are detailed below and the recruitment and volunteer pathway shown in *figure 5.1*.

- A routine MHRA inspection of the Research Department at SCH was undertaken in November 2010, during which the NeSST study was heavily scrutinised, as the only drug trial being conducted in and sponsored by the Trust. A concern was raised about informed consent being obtained after a drug (dexamethasone) had been administered. This was revised for the NeSST2 Study subjects, whereby written consent was obtained following enrolment but prior to visit 1 and before the dexamethasone was given.
- After re-consulting the literature and the clearance data of both dexamethasone and tetracosactide the fortnight between visits was reduced to a single week.
- Following peer-review of the NeSST Study results a suggestion was made to include a -14 minute sample in addition to the -1 minute sample before the tetracosactide is given. This was to confirm both the ACTH and cortisol were suppressed following dexamethasone administration.
- The IMPs (TNS - Tetracosactide Nasal Solutions) and i.v comparator given at the visits are described below:
 - A low-dose SST, with 1 mcg i.v Synacthen (i.v comparator visit)
 - A nasal SST with 100 mcg of tetracosactide with chitosan to examine the effect of the addition of chitosan on nasal tetracosactide absorption and cortisol response (100 mcg had been given in the NeSST Study).
 - A 500 mcg nasal SST to examine the effect of the increased dose alone on nasal tetracosactide absorption and cortisol response.

- If interim analysis indicated, a 500mcg tetracosactide with chitosan nasal SST to examine the cumulative effect of increased tetracosactide dose and the addition of a nasal drug enhancer on nasal tetracosactide absorption and cortisol response.
- The plasma Synacthen samples were analysed in December 2011 and July 2012 (final visit); the serum cortisol samples were processed between January and February 2012 and July 2012 and the salivary cortisol/cortisone samples were batch analysed in April and July 2012.

Figure 5.1: Recruitment flow chart and volunteer pathway for NeSST2 Study



3.4 Tetracosactide Nasal Solution (TNS)

The pharmacokinetic, metabolic, toxicological and clinical properties of tetracosactide following i.v injection are well known and are described in the Summary of Product Characteristics (Alliance Pharmaceuticals 2011). Tetracosactide nasal solution (TNS) has the same active pharmaceutical ingredient but with the addition of an excipient, chitosan, to aid absorption when administered nasally.

Pharmacokinetic extrapolation using NeSST Study AUC data estimated that 500 mcg of intranasal Synacthen would be required to give an equivalent response to the 1 mcg i.v LDSST. TNS is an aqueous solution containing either 0.5 mg/ml (providing 50 mcg tetracosactide) or 2.5 mg/ml (containing 250 mcg of tetracosactide) in the form of the acetate salt. In order to deliver 100 mcg and 500 mcg one spray (of 0.1ml) per nostril (0.2 ml in total) was required. Two TNS solutions also contained chitosan glutamate (a cationic biopolymer which acts as a mucoadhesive/bioadhesive agent, (*vide supra chapter 1, section 5.4.3*). In addition, the three TNS solutions contained sodium chloride for tonicity adjustment, benzalkonium chloride as a preservative, and acetic acid and sodium acetate as a buffer to adjust/maintain pH.

The TNS 0.5 mg/ml with chitosan, TNS 2.5 mg/ml, and TNS 2.5 mg/ml with chitosan solution were packaged as 0.5 ml aliquots in 2 ml glass vials and at the

point of use, 0.1ml of the solution was withdrawn from the vial into a 1 ml syringe attached to a Mucosal Atomizer Device (Wolfe-Tory Medical, USA) (*chapter 4, figure 4.2*) to facilitate administration of 0.1 ml to each nostril. Each sealed vial was individually packaged in a labelled carton and stored refrigerated in the clinical trials refrigerator in the SCH pharmacy department.

The 1 mcg i.v and 100 mcg nasal dose chosen for the study were below that of the i.v licensed dose, 250 mcg, and therefore toxic effects of Synacthen “overdose” were not anticipated. The 500 mcg nasal dose, although higher, when administered nasally was thought likely to yield much lower systemic levels of tetracosactide compared with the 250 mcg i.v dose. The 500 mcg dose with chitosan was only given because interim analysis revealed that systemic absorption with chitosan alone or with 500 mcg alone had produced an insufficient cortisol response in three of the volunteers.

Tetracosactide is used as the acetate salt form in the existing injection product (Synacthen, Alliance Pharmaceuticals, UK). This salt form is reported to be sparingly soluble in water (approximately 10-30 mg/ml), however this solubility is sufficient to provide a solution suitable for intranasal administration. Two of the three TNS formulations contained chitosan (in the glutamate salt form). A chitosan glutamate concentration of 5 mg/ml was selected for the TNS formulations based on previous investigations and experience at Archimedes Development Limited (Nottingham, UK).

Table 5.1: Composition of each 0.5ml vial of Tetracosactide Nasal Solution.

Component	Reference to Standard	Function	Quantity per 0.5 ml		
			TNS 0.5 mg/ml + chitosan	TNS 2.5 mg/ml	TNS 2.5 mg/ml + chitosan
Tetracosactide acetate	Ph.Eur.*	Active	0.25 mg	1.25 mg	1.25 mg
Chitosan glutamate	HSE (SPEC/0003) [§]	Bioadhesive	2.5 mg	--	2.5 mg
Sodium chloride	Ph.Eur.*	Osmolality adjustment	2.7 mg	2.7 mg	2.7 mg
Benzalkonium chloride	Ph.Eur.	Preservative	0.075 mg	0.075 mg	0.075 mg
Acetic acid	Ph.Eur.	pH adjustment	2.55 mg	2.55 mg	2.55 mg
Sodium acetate trihydrate	Ph.Eur.	pH adjustment	1.05 mg	1.05 mg	1.05 mg
Water for injections	Ph.Eur.	Vehicle	To 0.5 ml	To 0.5 ml	To 0.5 ml

* Ph. Eur – European pharmacopoeia (monograph 0644, pages 3059-3060 of European Pharmacopoeia 2010, 7th Edition, Council of Europe, Strasbourg.)

[§] in-house reference standard at Archimedes Drug Development, Nottingham, UK

3.4.1 Physicochemical characteristics for Tetracosactide drug compound

<i>Molecular formula</i>	C ₁₃₆ H ₂₁₀ N ₄₀ O ₃₁ S (net)
<i>Molecular mass</i>	2931.6 g/mol (monoisotopic mass, net)
<i>Salt form</i>	Acetate salt (between 4 and 8 moles of acetic acid may be present per mole of peptide)
<i>Physical form</i>	White to yellow powder
<i>Polymorphism</i>	Amorphous powder, as tetracosactide is isolated by lyophilisation. No crystalline or polymorphic forms are known.
<i>Appearance of solution</i>	Clear and colourless (1 mg/mL in water)

3.4.2 Physiochemical characteristics of the chitosan used in the NeSST2 Study

<i>Generic name:</i>	Chitosan glutamate
<i>Chemical name:</i>	β -(1 \rightarrow 4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose glutamate; β -(1 \rightarrow 4)-linked <i>N</i> -acetyl-D-glucosamine and D-glucosamine glutamate
<i>Manufacturers name:</i>	PROTOSAN™ UP G 213
<i>CAS registry:</i>	84563-76-8
<i>Molecular weight:</i>	The molecular weight (g/mol) of the monomer unit is 161 for D-glucosamine and 203 for <i>N</i> -acetyl-D-glucosamine. Glutamate has a molecular weight of 146. Typically, the molecular weight for PROTASAN UP G 213 is in the range 200000-600000 g/mol.
<i>Description:</i>	PROTASAN UP G 213 is a white or off-white powder that forms a clear, colourless to slightly yellowish solution when dissolved in water. The solution may become thick, i.e. viscous depending upon the concentration of PROTASAN UP G 213 used.
<i>pH of aqueous solution:</i>	The pH of an aqueous solution (1%) of PROTASAN UP G 213 is between 4 and 6.
<i>Solubility:</i>	Solubility of PROTASAN UP G 213 is a function of the concentration (g/l or mg/ml), ionic strength of the solution and the presence of buffering ions.

3.4.3 Chitosan safety

Chitosan glutamate has been tested for safety and toxicity in a number of animal species using different routes of administration. The preclinical data augment the

considerable clinical data on intranasal chitosan in both human volunteers and patients. Nasal formulations containing chitosan, both in the form of solutions containing 5 mg/ml chitosan as well as powders, have been administered to over 1000 people in clinical trials and in excess of 2900 doses have been administered. Collectively no safety or tolerability issues were identified in any study and these data do not identify any specific hazard that would preclude the use of chitosan in further clinical trials (personal communication, Peter Watts, Archimedes Development Ltd).

3.5 Plasma Synacthen dilutions

Dose escalation and the addition of chitosan resulted in a number of plasma Synacthen results above the limit of detection of the assay. When spun and separated the plasma had been split and frozen into two aliquots and thus the stored second aliquot samples were run following a 1:10 dilution in the MP Biomedicals zero standard. Twenty-five microlitres of plasma Synacthen sample were diluted in 225 microlitres of zero standard diluent and the results adjusted by multiplying by ten.

3.6 Statistical analysis

The PK and non-PK analyses were the same as those used in the NeSST Study (*chapter 4, section 3.5*): C_{max}, T_{max}, AUC, terminal half-life, mean residence time, bioavailability and clearance (for definitions see *chapter 4, section 3.5.1*).

The addition of the -14 minute sample meant there were two possible baseline values to subtract from subsequent values when cleaning the data. Generally the -14 and -1 minute values were very similar. For consistency with the PK data analysis the -1 minute result was chosen as baseline unless it was at odds with the rest of the data in which case the -14 minute value was used.

4 Results

4.1 Recruitment data

Ten healthy, adult males who contacted the author, were eligible for inclusion and were duly enrolled and two were re-recruited from the first NeSST Study (*figure 5.1*). On discussion at later study visits, it became evident that two volunteers were experiencing mild dyspeptic symptoms soon after taking the dexamethasone, presumed to be a side effect. Both were excluded from any further visits (1 mcg and final visit for subject 11 and the final visit only for subject 1). No replacement subjects were recruited due to financial constraints (the i.v comparator visit would have had to be repeated). All other subjects participated in all four visits (*table 5.2*). Interim analysis was performed after subjects had completed three visits and supported continuation with administration of 500 mcg tetracosactide and chitosan to the remaining subjects.

Table 5.2: NeSST2 Study recruitment data

	Enrolled	Attended 1 mcg i.v visit	Attended 100 mcg + chitosan visit	Attended 500 mcg nasal visit	Attended 500 mcg + chitosan visit
Number	12	11	12	12	10

4.2 Demographic and anthropometric data

Basic data were collected from all volunteers at the first visit: age, ethnic origin, height and weight, from which body mass index (BMI) and body surface area (BSA) were calculated. *Table 5.3* summarises the 12 volunteers' demographic and auxological data with ranges, means and standard deviations given.

Table 5.3: NeSST2 Study subjects' demographic and anthropometric data

	Number of volunteers	Range	Mean	Standard deviation +/-
Age (years)	12	22-46	25	7
Height (m)	12	1.760-2.060	1.816	0.085
Weight (kg)	12	64.1-86.0	76.5	7.1
BMI (kg/m ²)	12	20.2-25.7	23.2	1.9
BSA (m ²)*	12	2.27-1.79	1.9	0.3
Ethnic origin	12	10 = white British, 1 = black African, 1 = white other (American)		

* Dubois Formula: Body Surface area (m²) = 0.007184 × (height in cm)^{0.725} × (weight in kg)^{0.425}

4.3 Data derived from patient specimen sampling

Paired blood and saliva sampling was performed at 15 time points from -14 to 180 minutes. If all samples on all volunteers had been successfully obtained

there would have been 2160 data points, however 209 were missing, 135 because the subjects were excluded from attending any further visits and 74 due to human error (*table 5.4*). The total number of plasma Synacthen samples analysed was 651, serum cortisol was 672 and salivary samples was 673.

Table 5.4: Samples collected for each subject at each NesST2 Study visit with missing data points and reasons given. "Complete" indicates all 15 samples of plasma Synacthen, serum cortisol and salivary cortisol/cortisone (N=45) were successfully collected. (pl S = plasma Synacthen, SerC=serum cortisol, Sal=salivary sample)

Subject No	1 mcg i.v visit	100 mcg + chitosan visit	500 mcg nasal visit	500 mcg + chitosan visit
1	No -14 min pl S ^{&}	Complete	Complete	-
2	No -14 min pl S ^{&}	Complete	Complete	Complete
3	Complete	Complete	Complete	No -1 min pl S ^{&}
4	Complete	Complete	Complete	Complete
5	Complete	Complete	Complete	Complete
6	Complete	No 40, 150, 180 min pl S + SerC* No 50 min sal [@]	Complete	No -14 min pl S ^{&}
7	Complete	Complete	No 10 min salivary [@]	No -14-75 min pl S [%]
8	Complete	Complete	Complete	Complete
9	Complete	Complete	Complete	Complete
10	Complete	Complete	Complete	Complete
11	-	Complete	Complete	-
12	Complete	Complete	Complete	No 5,10,15,20,30,40 min pl S [£]

* Problem with cannula bleeding back

£ Nurse forgot to take samples

& Problem during plasma centrifugation and plasma separation

% Lost samples

@ Saliva sampling missed due to problem with blood sampling

4.3.1 Proof of dexamethasone suppression

The baseline plasma Synacthen samples were high in the NeSST Study. This was thought to be due to interference with an unrelated molecule (*chapter 4, section 5.1*). *Table 5.5* displays the baseline plasma ACTH and cortisol for all volunteers at -14 and -1 minute (prior to Synacthen administration).

Table 5.5: Plasma ACTH and serum cortisol values at -14 and -1 minute for 12 NeSST2 Study volunteers. Results for the 4 visits given for each volunteer (top left-hand box visit 1, top right-hand visit 2, bottom left-hand box visit 3 and bottom right-hand visit 4). ACTH normal range <10pg/ml. Cortisol normal range <50 nmol/L. High values shown in red. “-“ indicates no sample.

Volunteer Number	Plasma ACTH (pg/ml)				Serum Cortisol (nmol/L)			
	-14 minute samples		-1 minute samples		-14 minute samples		-1 minute samples	
1	-	87.9	86.7	87.4	48	<22	41.9	<22
	79.1	-	91.6	-	26.2	<22	22.5	-
2	-	73.5	59	59	<22	23.2	<22	22.7
	54	174	68.3	298.9	22.4	78.4	<22	73.1
3	91.7	>max	77.4	96.1	122.6	26.1	84.3	<22
	93	244.8	91.1	-	<22	29.3	<22	23.3
4	234.6	188	209.2	152.5	66.1	<22	39.9	<22
	162.5	367.8	171.1	468.4	<22	<22	<22	<22
5	99.2	79.2	116.9	89.7	<22	<22	<22	<22
	131.6	166.3	131	171.1	<22	<22	<22	<22
6	126.2	110.7	131.8	124.7	<22	<22	<22	<22
	128.5	-	117.5	281.3	<22	<22	<22	<22
7	108.7	94.6	127.4	90.4	<22	<22	<22	<22
	97.3	-	120.2	-	<22	28.8	<22	26.3
8	324.3	123.9	160.7	96.5	33.4	382.8	33.1	374.9
	71.5	89.8	87.4	186.1	35.4	237.5	36.2	202.3
9	65.1	57.6	58.6	58.2	<22	<22	<22	<22
	65.7	81.3	148.4	191.7	<22	<22	<22	<22
10	124.2	145.6	122.9	143.4	32.4	31.7	29.8	23.7
	157.8	112.1	160.5	119.1	27.8	22.6	24.9	23.6
11	274.7	257.8	212.5	239.5	<22	<22	<22	<22
	-	-	-	-	-	-	-	-
12	124.4	104.2	88.5	99.3	22.6	<22	<22	23.5
	115.7	113.4	114.4	107.5	25	<22	<22	23.8

For all volunteers the baseline ACTH values were higher than would be expected but in the majority the low cortisol values in *table 5.5* indicate that they had taken their dexamethasone and consequently suppressed their cortisol. In five visits of the 45 the cortisol prior to Synacthen administration (-14 and -1 minute samples) were either only partially suppressed or unsuppressed. None of the four volunteers had a Cushingoid habitus, they were all reportedly healthy and it is likely that they were either non-adherent with the request to take dexamethasone or had another, undisclosed, exogenous source of corticosteroid (although none had unsuppressed baselines at all visits).

- Subject 2 had a slightly raised baseline cortisol (78.4, 73.1 nmol/L) at his final visit (500 mcg plus chitosan) but showed a good response to Synacthen and his data have been retained for analysis.
- Subject 3, appears to have taken his dexamethasone just prior to his 100 mcg plus chitosan visit – with baseline cortisols of 122.6 then 84.3 nmol/L and then displaying a fairly flat response to Synacthen; his data from this visit have been excluded from further analysis.
- Subject 4, had a slightly elevated baseline cortisol (66.1 nmol/L) but by -1 minute it is suppressed (39.9 nmol/L) at his 100 mcg plus chitosan visit, his data have been included for analysis.
- Subject 8 appears not to have taken his dexamethasone before either his 1 mcg i.v or his 500 mcg plus chitosan visits. His data from these visits has been excluded from relevant analyses.

The plasma Synacthen values showed more intra-individual variability than in the NeSST Study and were universally high.

4.3.2 Plasma Synacthen assay

The Synacthen assay methodology had changed between the NeSST and NeSST2 studies with the adoption of Synacthen standards made up using the zero standard from the RIA kit as a diluent. The results of the only repeated formulation, 1 mcg i.v, gave the opportunity to compare the workings of the assay. The mean plasma Synacthen values following 1 mcg i.v. Synacthen in both studies is shown in *figure 5.2 (vide supra chapter 3, table 3.2.3)*. The high baseline, presumed due to an interfering molecule, is seen in both studies. The shape and height of the curves are similar with divergence after 90 minutes. There is overlap of error bars throughout.

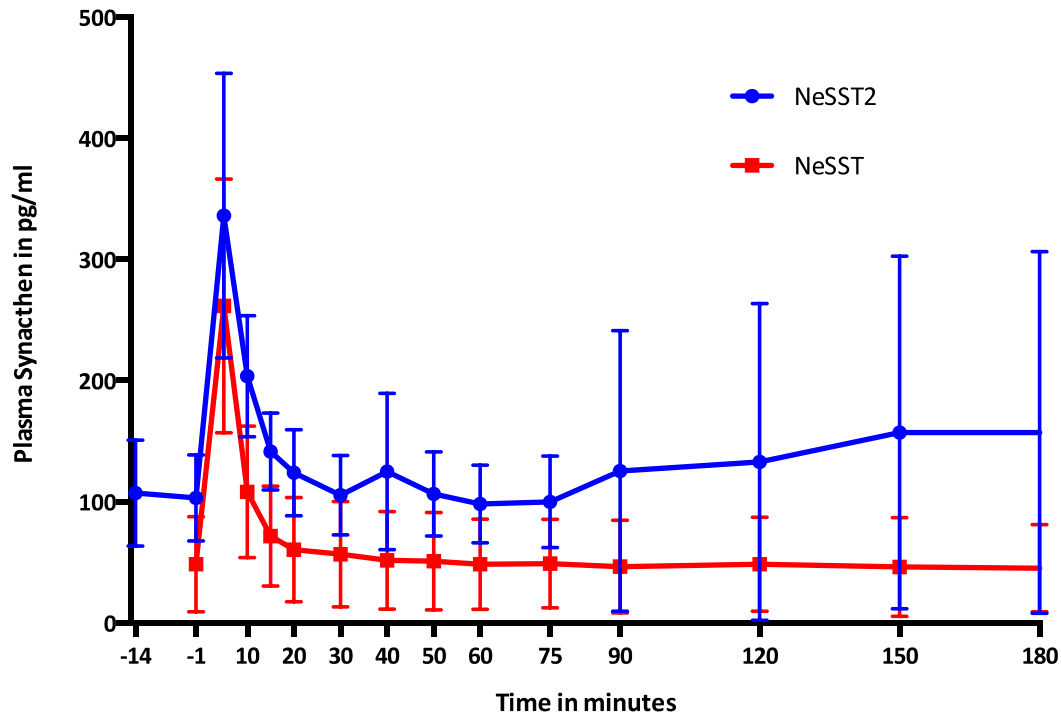


Figure 5.2: Mean Plasma Synacthen values following 1 mcg i.v. Synacthen in both NeSST and NeSST2 studies. Error bars are shown.

4.3.3 Comparison of intravenous and nasal administration routes

Following the nasal administration of 1 mcg i.v. Synacthen, 100 mcg tetracosactide with chitosan and the 500 mcg tetracosactide nasal solutions interim analysis was performed. It was evident that there had been a poor response in some individuals and thus the decision was taken to proceed with the final visit and administer the 500 mcg tetracosactide and chitosan formulation. Individual plasma Synacthen levels and cortisol responses to the four different doses are displayed in *figure 5.3*.

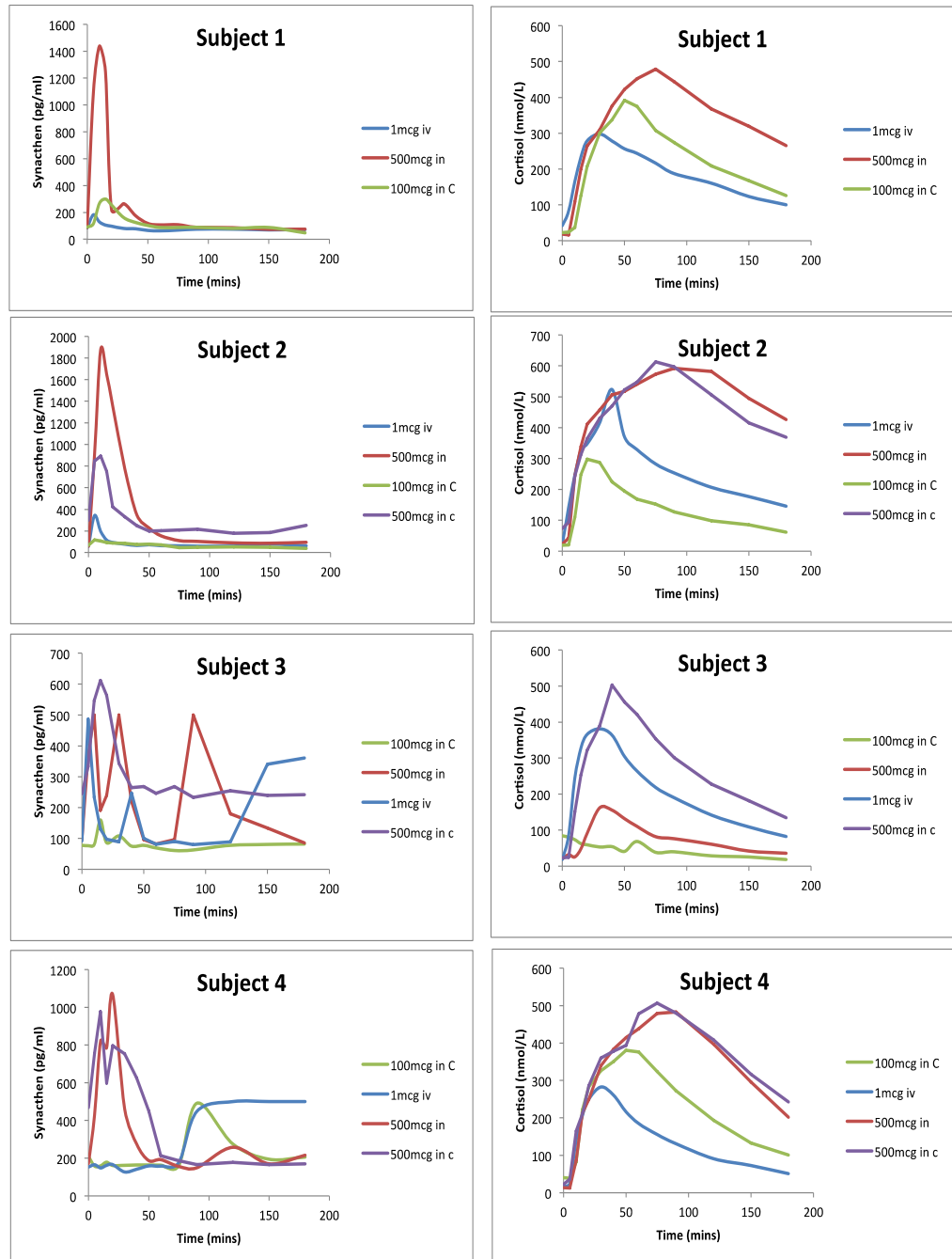


Figure 5.3: Individual study volunteers' plasma Synacthen levels and serum cortisol responses to 1 mcg i.v Synacthen and three doses of nasal Synacthen (100 mcg with chitosan, 500 mcg and 500 mcg with chitosan).

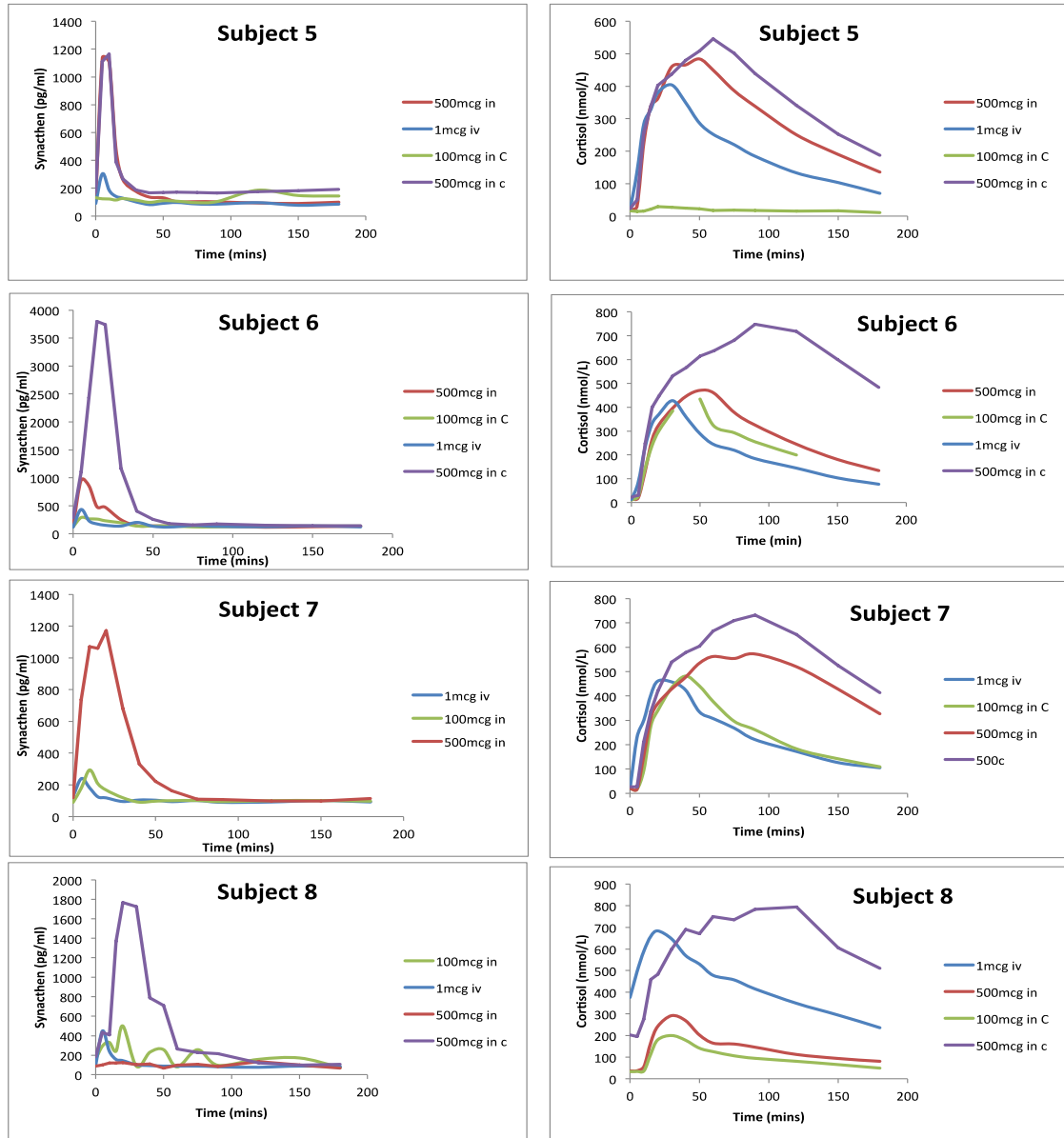


Figure 5.3 (continued): Individual study volunteers' plasma Synacthen levels and serum cortisol responses to 1 mcg i.v Synacthen and three doses of nasal Synacthen (100 mcg with chitosan, 500 mcg and 500 mcg with chitosan).

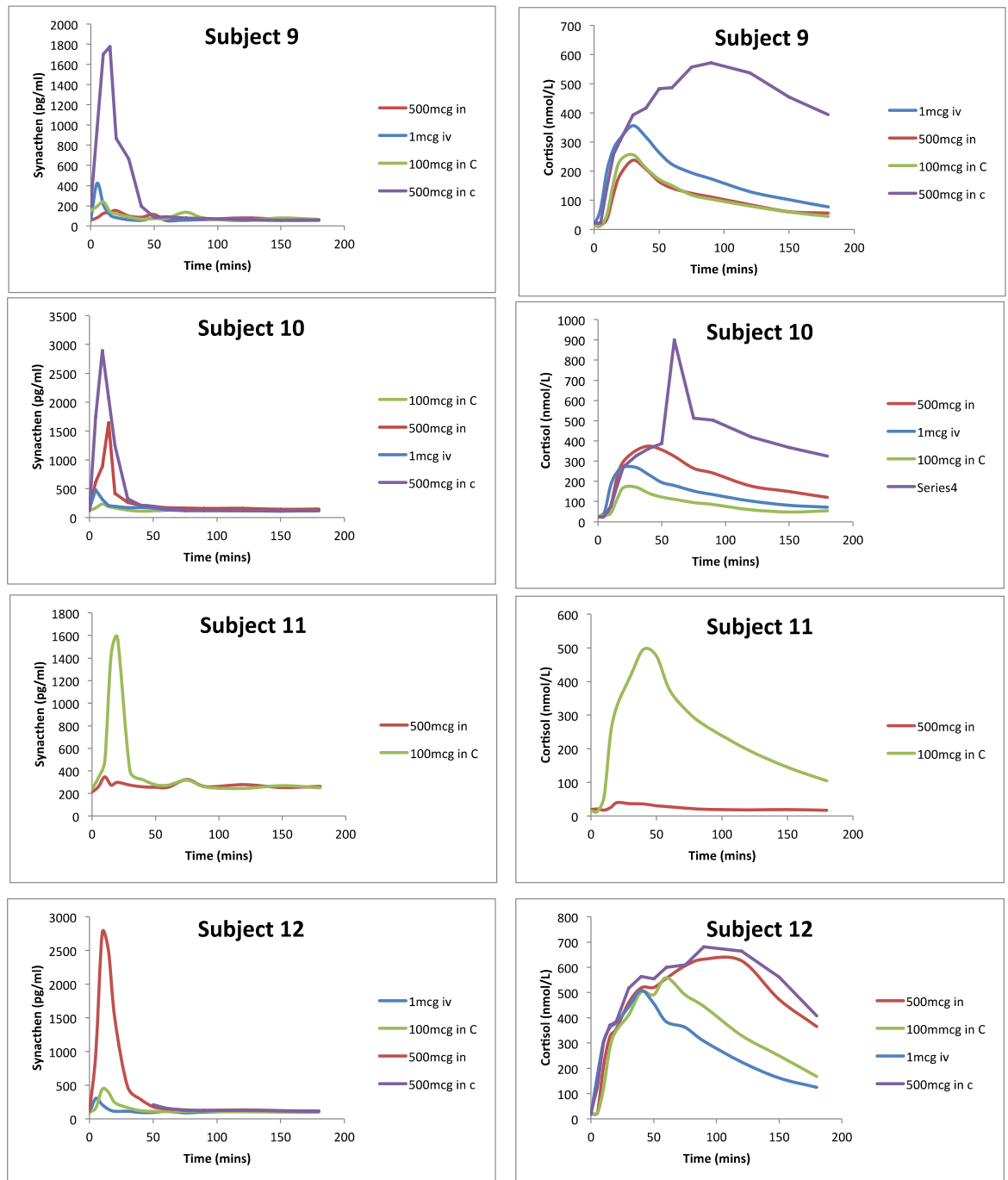


Figure 5.3 (continued): Individual study volunteers' plasma Synacthen levels and serum cortisol responses to 1 mcg i.v Synacthen and three doses of nasal Synacthen (100 mcg with chitosan, 500 mcg and 500 mcg with chitosan).

The interim PK analysis demonstrated that, although the PK parameters of AUC and Cmax were similar between the 100 mcg plus chitosan nasal dose and the 1 mcg i.v dose, there were a number of non-responders (subjects 3, 5 and 8), or those with a sub-optimal response (subject 2). In the majority of research volunteers the plasma Synacthen levels seemed higher following nasal administration with 500 mcg tetracosactide than after i.v and the cortisol response was higher, however once again there were non-responders (subjects 3 and 11), or those with a sub-optimal response (subject 8). It was felt that administration of the 500 mcg tetracosactide with chitosan might overcome this individual variability.

The individual plasma Synacthen responses to each of the four doses of Synacthen are shown in *figures 5.4a-d*. The y-axis scales are different on each graph due to the considerable variability of plasma levels following administration with different doses. High baselines and large individual variation can be seen. The individual cortisol responses to each of the four doses of Synacthen are shown in *figures 5.5a-d* and show greater variability with nasal administration, especially for the 100 mcg with chitosan and 500 mcg tetracosactide doses. The mean plasma Synacthen (*figure 5.6a*) and mean serum cortisol (*figure 5.6b*) over time following administration with each of the three doses of Synacthen are shown with standard deviations.

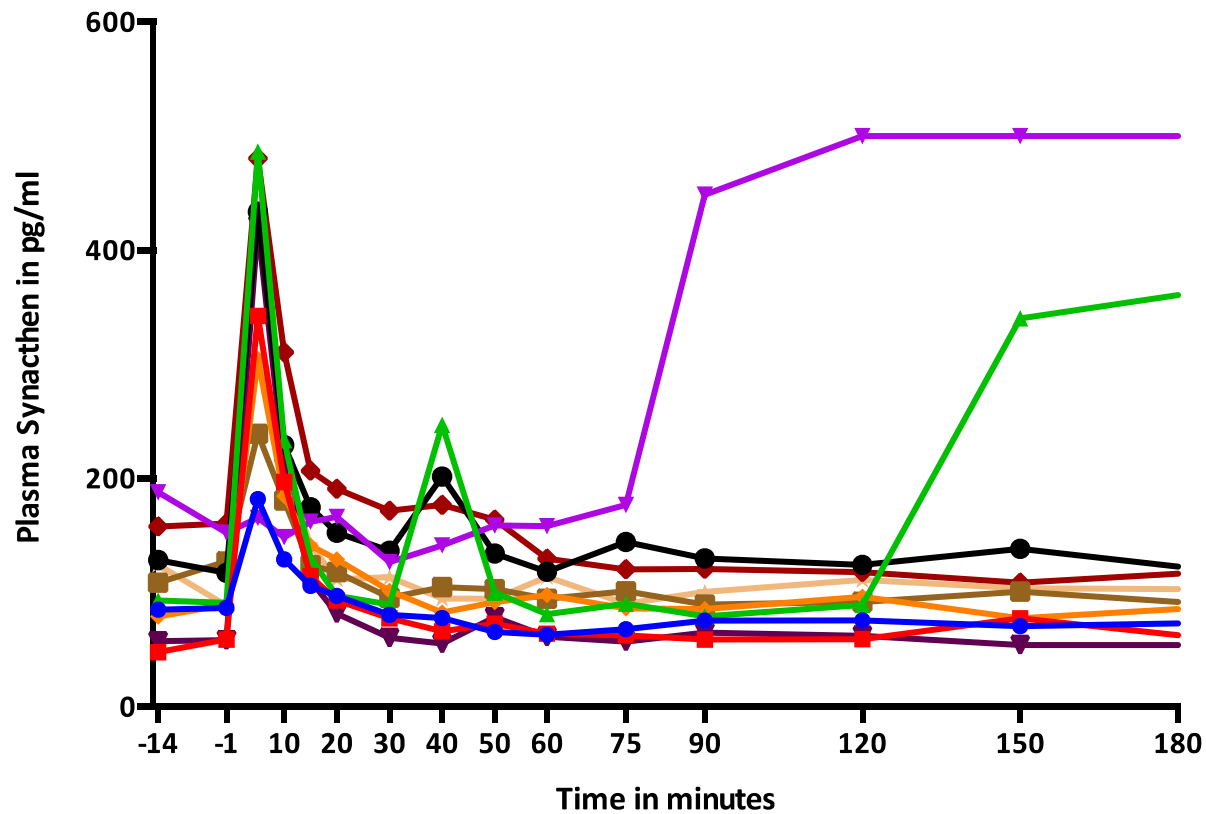


Figure 5.4a: Graph of the individual plasma Synacthen responses to i.v administration with 1 mcg Synacthen in the NeSST2 Study (baseline not subtracted). Each coloured line depicts a study subject.

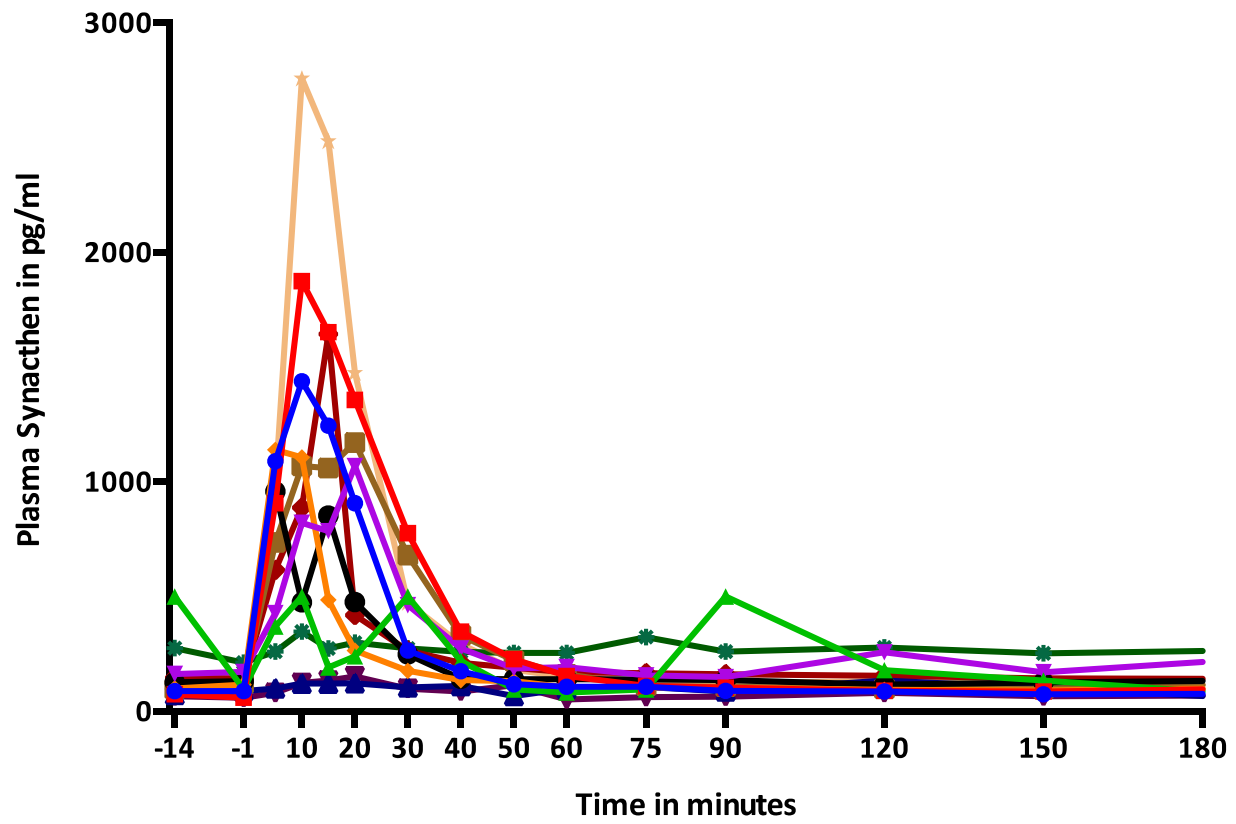


Figure 5.4b: Graph of the individual plasma Synacthen responses to i.n administration with 500 mcg tetracosactide (baseline not subtracted). Each coloured line depicts a study subject.

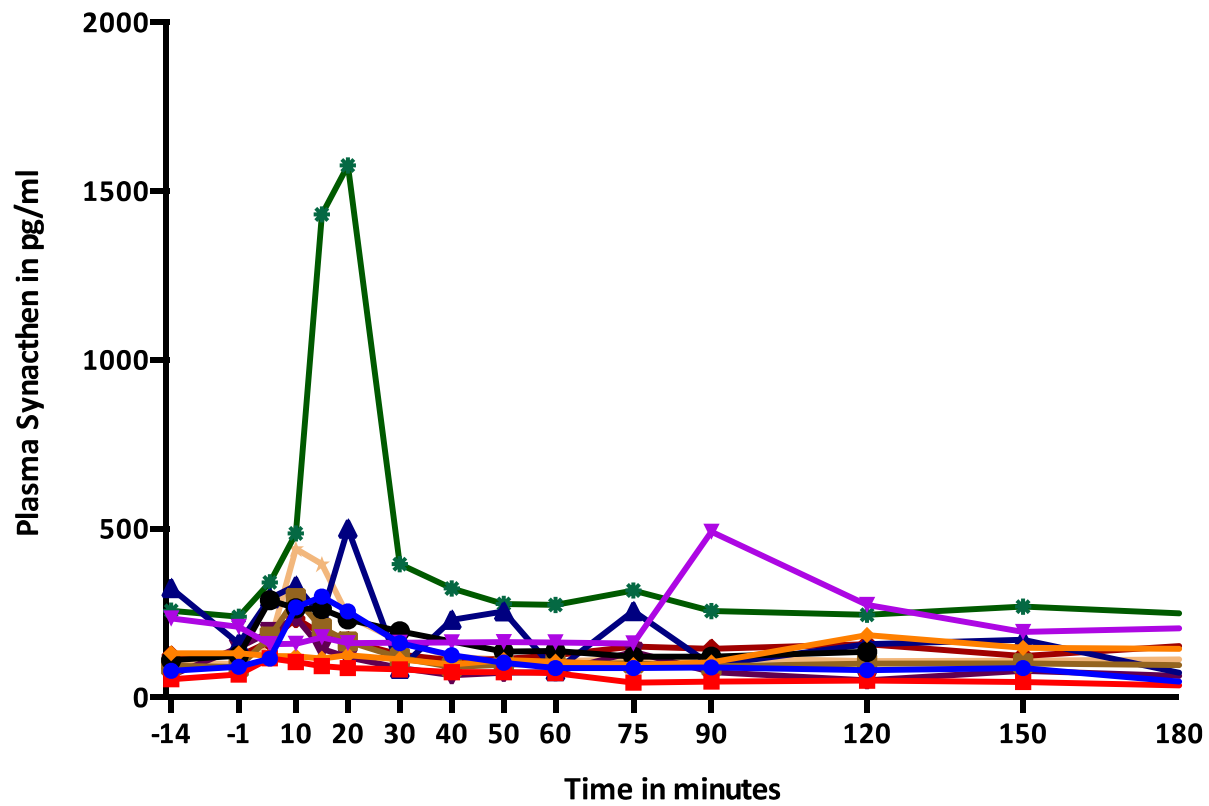


Figure 5.4c: Graph of the individual plasma Synacthen responses to i.n administration with 100 mcg tetracosactide + chitosan (baseline not subtracted). Each coloured line depicts a study subject.

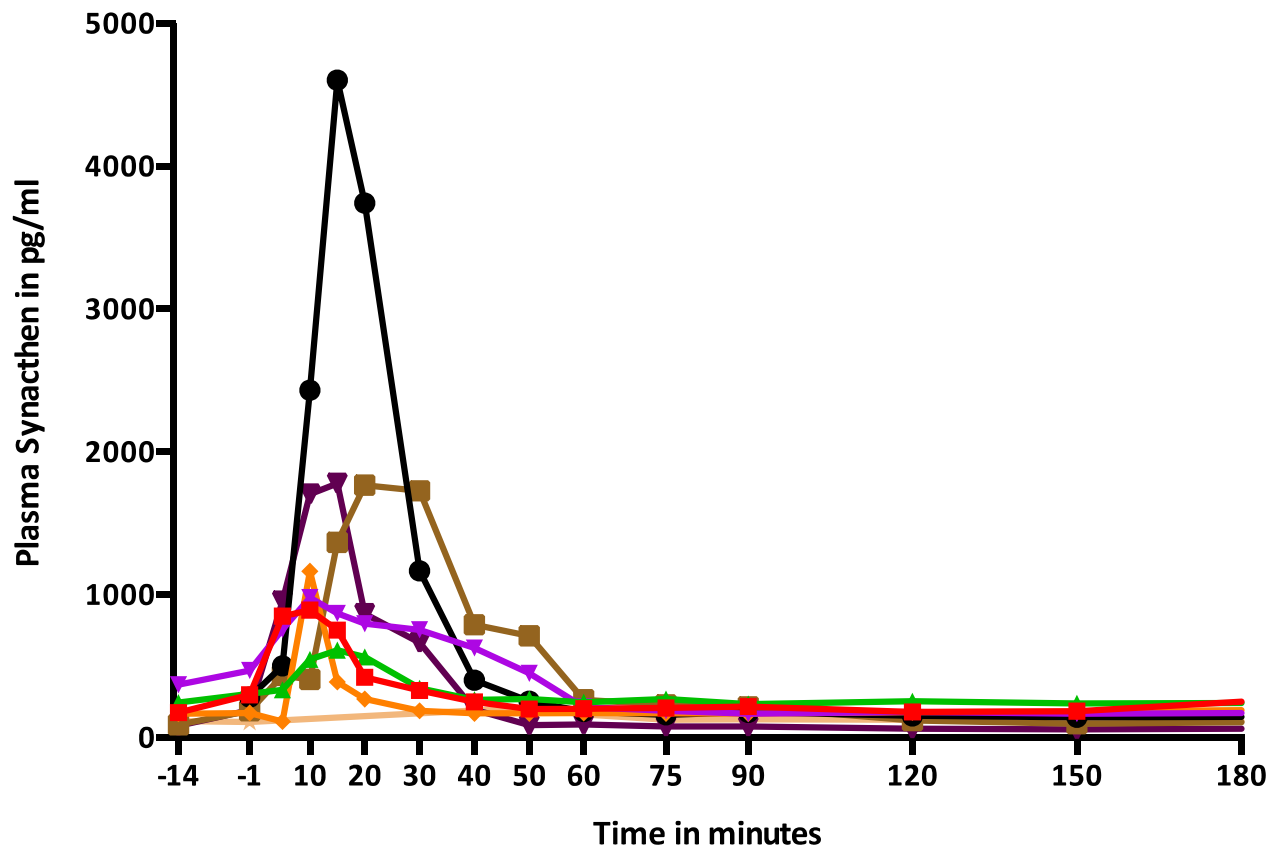


Figure 5.4d: Graph of the individual plasma Synacthen responses to i.n administration with 500 mcg tetracosactide + chitosan (baseline not subtracted). Each coloured line depicts a study subject.

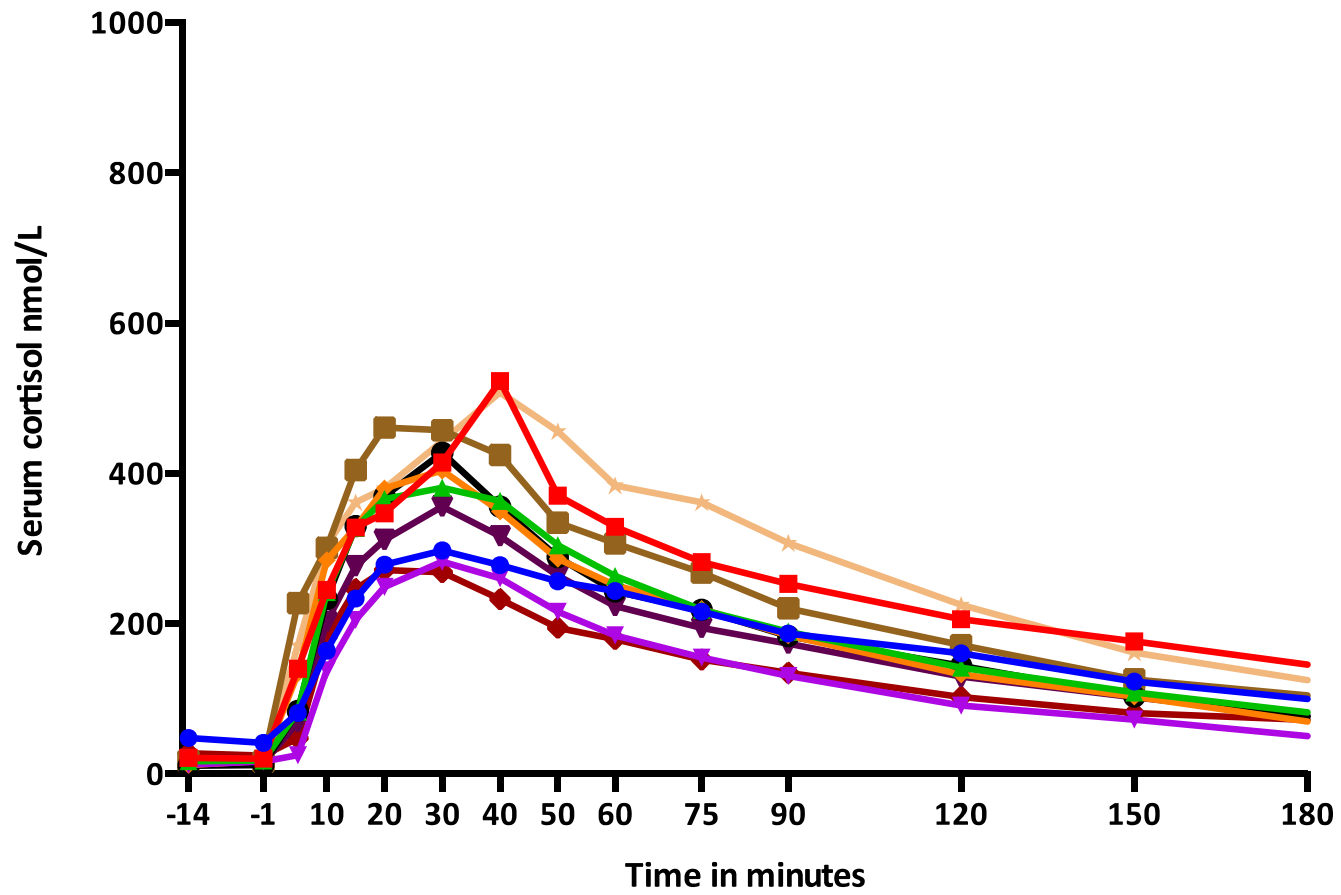


Figure 5.5a: Individual serum cortisol responses following i.v administration with 1 mcg Synacthen in the NeSST2 Study (N=10).

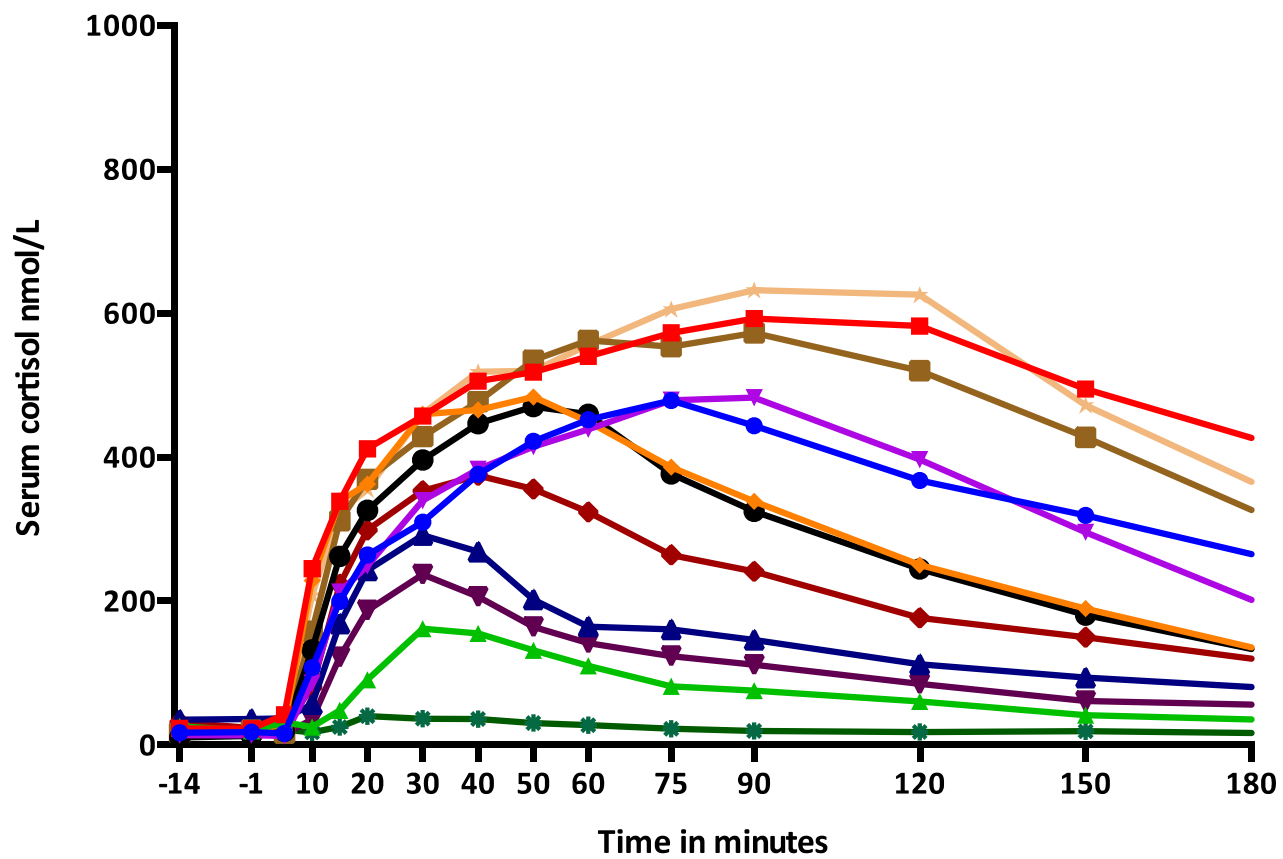


Figure 5.5b: Individual serum cortisol responses following nasal administration with 500 mcg tetracosactide (N=12).

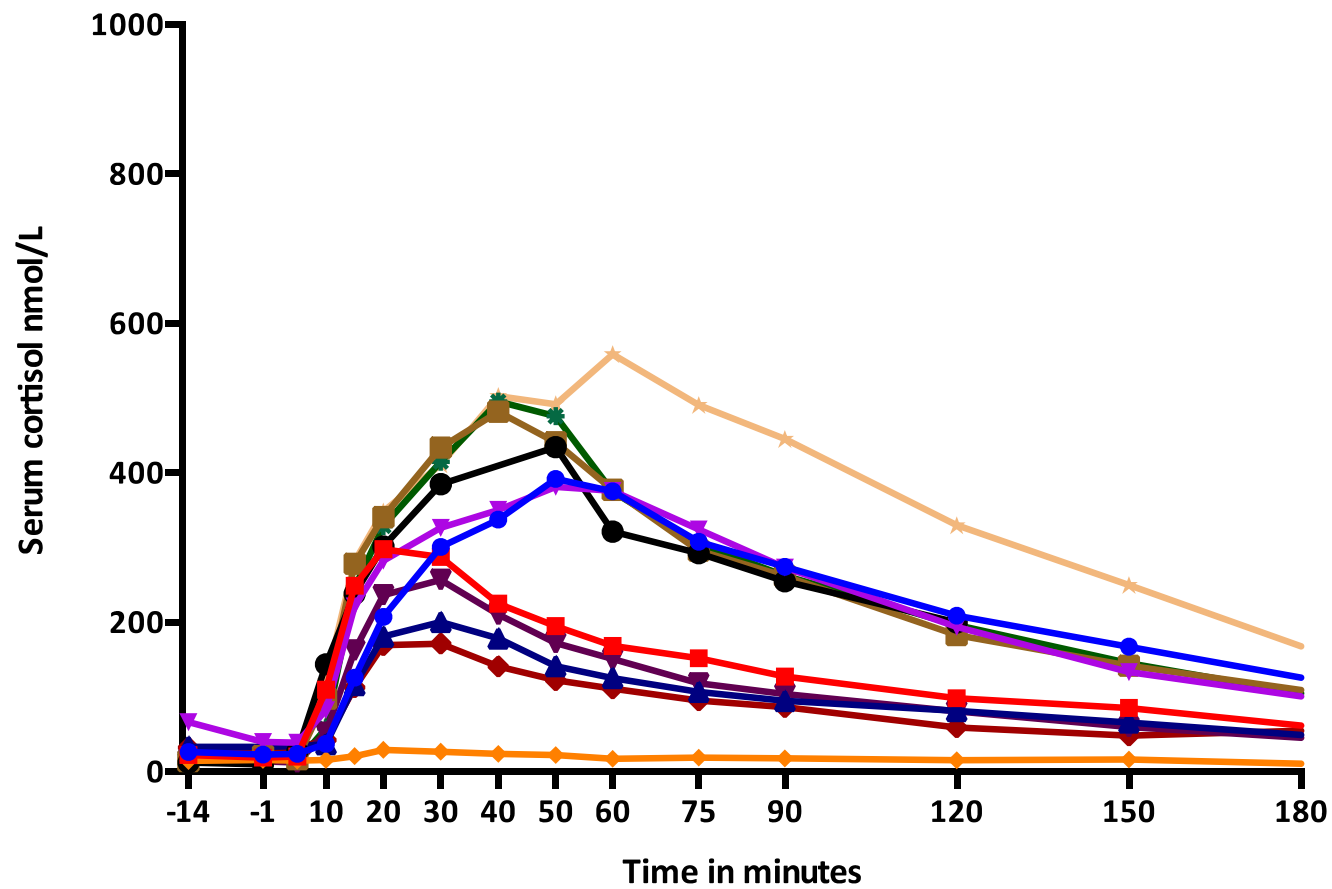


Figure 5.5c: Individual serum cortisol responses following nasal administration with 100 mcg tetracosactide and chitosan (N=11).

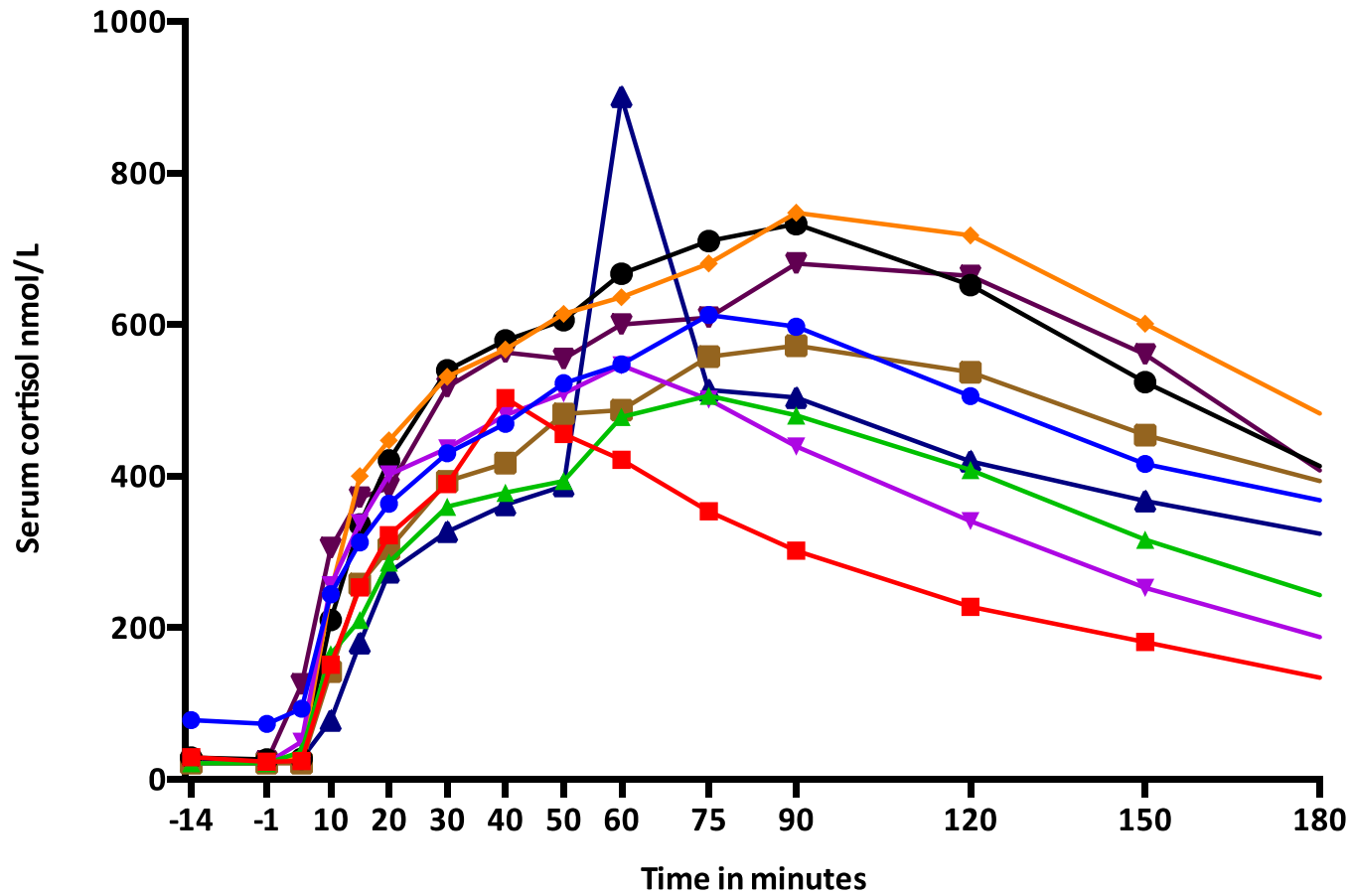


Figure 5.5d: Individual serum cortisol responses following nasal administration with 500 mcg tetracosactide and chitosan (N=9).

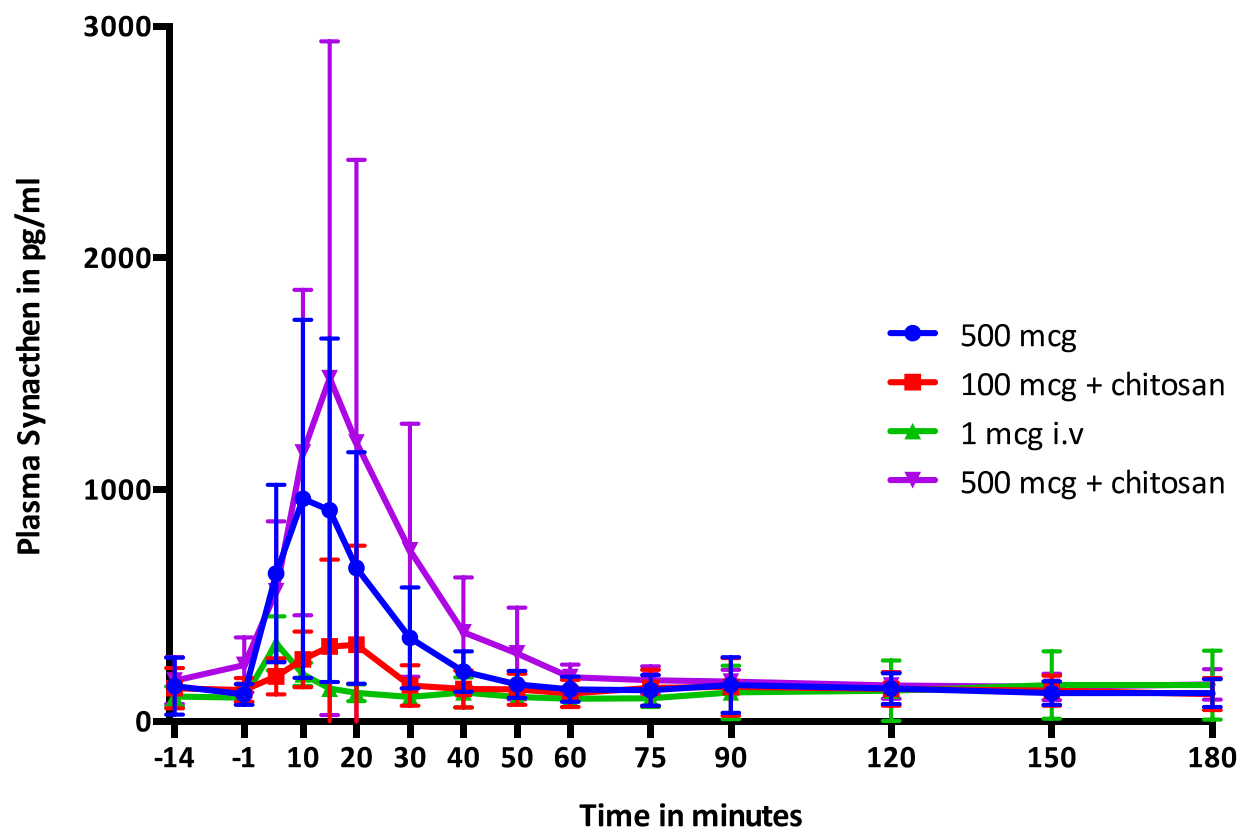


Figure 5.6a: Graph of the mean rise of plasma Synacthen from a suppressed baseline in subjects following administration with 1 mcg i.v, 500 mcg tetracosactide i.n, 100 mcg tetracosactide + chitosan i.n and 500 mcg tetracosactide + chitosan i.n. Standard deviations shown as error bars.

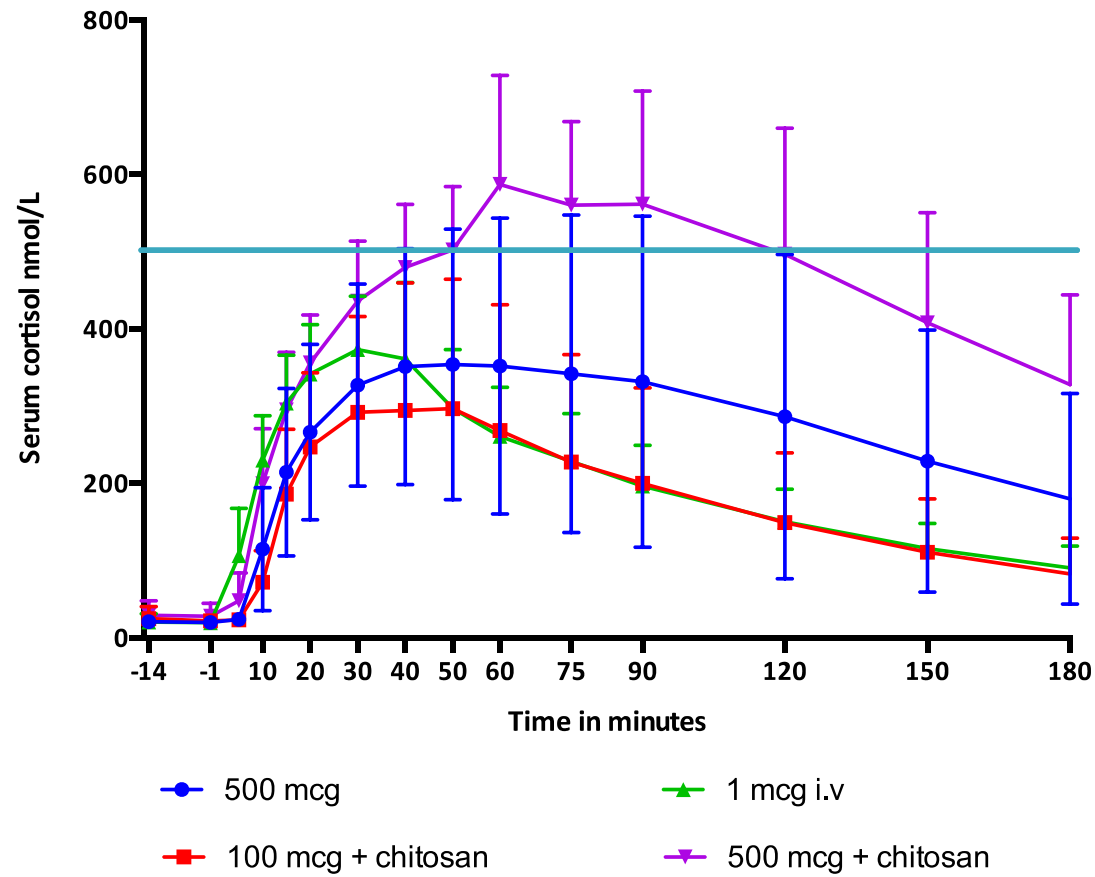


Figure 5.6b: Graph of the mean rise of serum cortisol from a suppressed baseline in subjects following administration with 1 mcg i.v, 500 mcg tetracosactide i.n, 100 mcg tetracosactide + chitosan i.n and 500 mcg tetracosactide + chitosan i.n. Standard deviations shown as error bars. Light blue line depicts a "normal" response on LDSST - 500 nmol/L

The mean plasma Synacthen responses to the four different doses of Synacthen are heightened both by the increase in dose and the addition of chitosan. The resultant cortisol response was similarly affected by both dose and the addition of chitosan. The peak in plasma Synacthen following administration with 1 mcg i.v Synacthen is much lower compared with the nasal formulations however the cortisol peak is similar to those seen following 100 mcg with chitosan and 500 mcg nasal Synacthen.

To assess whether chitosan improved the variability of Synacthen absorption the coefficient of variation has been calculated from the mean and standard deviations of the peak plasma Synacthen for the different doses, including those administered in the first NeSST Study. The Synacthen and cortisol peaks are compared in *table 5.6*. Baseline Synacthen (-1 minute) values have been subtracted from all subsequent data points.

Table 5.6: Comparison of means, standard deviations (SD), ranges, coefficient of variation (CV) and timing of the peak for peak plasma Synacthen and serum cortisol values in six different Synacthen tests performed in the NeSST and NeSST2 studies. CV expressed in %. Ranges and modes displayed in parentheses.

Visit	Number of subjects	Mean, SD, range & CV for plasma Synacthen peak in pg/ml	Median with IQR and mode timing of the Synacthen peak in mins	Number of subjects	Mean, SD, range & CV for serum cortisol peak in nmol/L	Median with IQR and mode timing of the cortisol peak in mins
1 mcg i.v NeSST	12	222.7 ±87 (103-360.8) 39.1%	5 ±0 (5)	11	389.8 ±51.2 (314-450) 13.1%	30 ±10 (30)
1 mcg i.v NeSST2	10	258.0 ±102.6 (95.2-393.4) 39.8%	5 ±0 (5)	10	392.3 ±92 (271.8-532.4) 23.5%	30 ±0 (30)
100 mcg i.n NeSST	10	55.6 ±104.4 (6.0-374.0) 187.8%	20 ±5 (20)	10	159.6 ±95 (60.5-318.7) 59.2%	30 ±7.5 (30)
100 mcg + chitosan NeSST2	11	171.7 ±90.4 (48.4-336.3) 52.6%	10 ±7.5 (10)	11	336.1 ±160.8 (29.1-558.7) 47.8%	40 ±20 (50)
500 mcg i.n NeSST2	12	974.7 ±780.5 (46.1-1800.2) 80.1%	10 ±10 (10)	12	401.6 ±183 (40-632.1) 45.6%	50 ±60 (90)
500 mcg i.n + chitosan NeSST2	6	1393.6 ±1498.6 (366.7-4318.7) 107.5%	12.5 ±5 (10)	9	645.4 ±132.7 (503.1-900.6) 20.6%	75 ±30 (90)

The two 1 mcg i.v tests produced broadly similar plasma Synacthen responses (*figure 5.2*), both with a suppressed cortisol response. Considerable variability in plasma Synacthen levels with all formulations and serum cortisol response following nasal administration are demonstrated by the large ranges and SDs and can be seen in *figures 5.4a-d, 5.5a-d* and by the large error bars in *figure 5.6a* and *b*. The calculation of CVs gives a measure of variability and is considerably smaller for the i.v dose than the nasal formulations. When chitosan is added to 100 mcg tetracosactide it reduces the variability of peak plasma Synacthen values (CV falls from 187.8% to 52.6%) however with the 500 mcg tetracosactide dose the addition of chitosan appears to worsen variability (80.1% to 107.5%). The addition of chitosan reduced the variability of peak serum cortisol at both doses (100 mcg 59.2% to 47.8% and 500 mcg from 45.6% to 20.6%). The timing of the cortisol peak is seen to vary more after nasal Synacthen than following i.v administration.

The cortisol response to the higher dose of nasal Synacthen given in the first NeSST Study (100 mcg), the combined mean of the two 1 mcg i.v tests from both studies and the three nasal doses from the NeSST2 Study are displayed in *figure 5.7* for a visual comparison of the effect of increase dose and addition of a nasal drug enhancer. The cortisol responses are further compared for the four NeSST2 Study doses in *table 5.7* by paired t-test with Bonferroni correction.

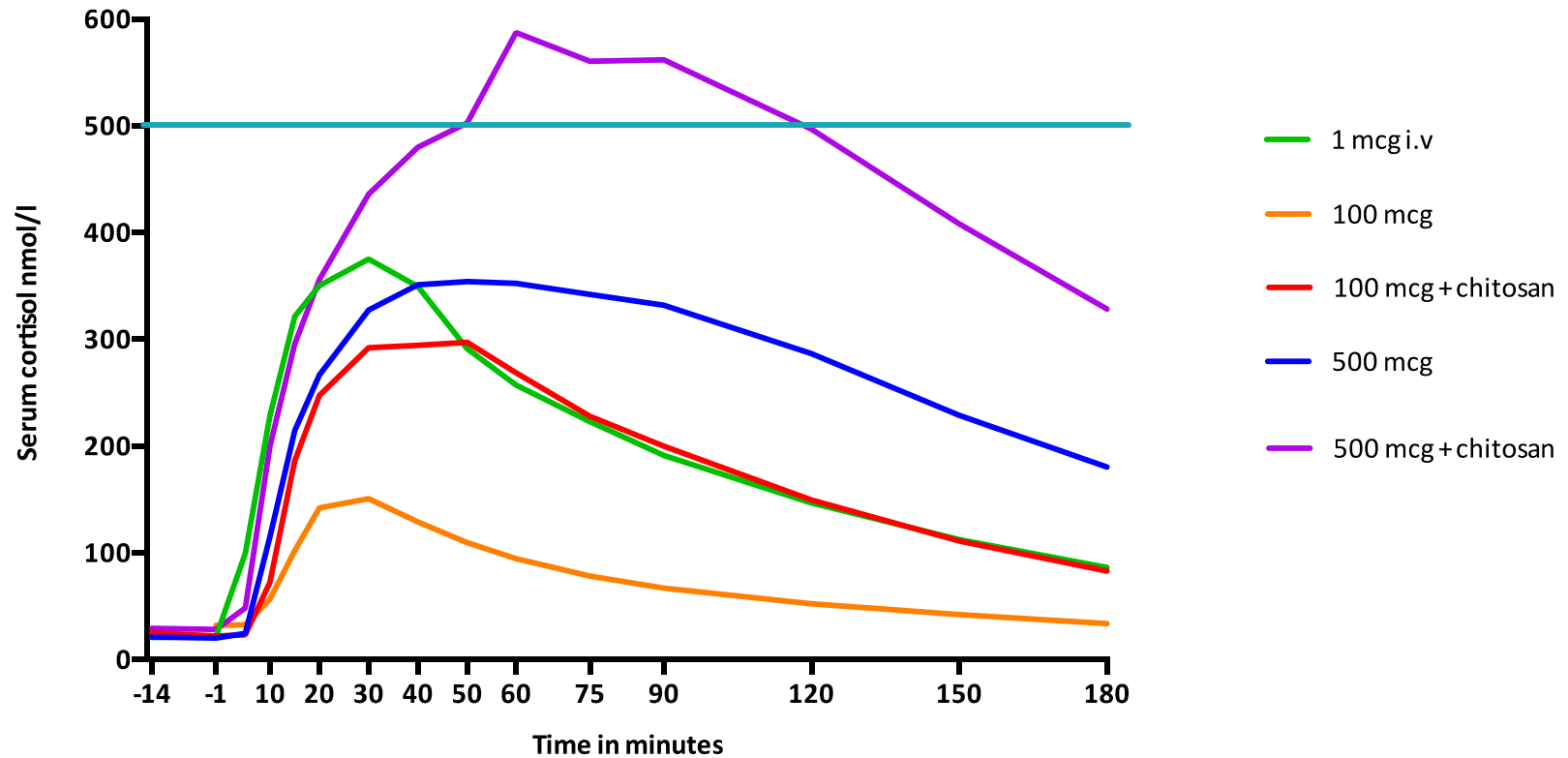


Figure 5.7: Mean rise of serum cortisol from a suppressed baseline in subjects following Synacthen administration with 1 mcg i.v (data from NeSST and NeSST2), 100 mcg i.n (NeSST), 100 mcg tetracosactide + chitosan i.n, 500 mcg tetracosactide i.n and 500 mcg tetracosactide + chitosan i.n. Standard deviations not shown for clarity. Light blue line depicts a "normal" response on LDSST - 500 nmol/L.

Table 5.7: Paired t-test with Bonferroni correction results comparing the mean cortisol responses at 13 different time points of the four tests. Statistical significance defined as p value <0.004 and denoted in red.

Tests compared	Sampling time (minutes)												
	5	10	15	20	30	40	50	60	75	90	120	150	180
1) 1 mcg i.v 2) 500 mcg tetracosactide + chitosan	0.057	0.057	0.323	0.574	0.001	0.002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
1) 1 mcg i.v 2) 100 mcg tetracosactide + chitosan	0.006	<0.0001	0.04	<0.0001	0.74	0.284	0.971	0.820	0.949	0.849	0.990	0.817	0.617
1) 1 mcg i.v 2) 500 mcg tetracosactide	0.002	0.001	0.048	0.132	0.666	0.432	0.036	0.011	0.009	0.008	0.010	0.028	0.010
1) 500 mcg i.n tetracosactide 2) 500 mcg tetracosactide + chitosan	0.061	0.004	0.103	0.061	0.052	0.07	0.046	0.016	0.007	0.011	0.027	0.026	0.025
1) 100 mcg tetracosactide + chitosan 2) 500 mcg tetracosactide + chitosan	0.081	0.001	0.019	0.030	0.005	0.014	0.004	0.004	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

The 500 mcg tetracosactide and chitosan nasal formulation resulted in significantly higher cortisol response at all time points after 30 minutes compared with the 1 mcg i.v preparation. The 100 mcg tetracosactide and chitosan formulation resulted in statistically lower cortisol values at 10 and 20 minutes when compared to 1 mcg i.v but otherwise there was no statistically significant difference between the two. The comparison between 500 mcg tetracosactide and the i.v preparation showed statistically significantly higher serum cortisol at 5 and 10 minutes only. The addition of chitosan to 500 mcg tetracosactide appeared, when examining the increase in serum cortisol seen in the graph (*figure 5.7*), to enhance the cortisol response considerably, however the difference is not statistically significant at any of the time points. This is likely due to large error bars and small subject numbers. An increase in dose from 100 mcg to 500 mcg increased the cortisol response significantly at 10 minutes and then all time points after 75 minutes. As some of the differences were thought to be due to the different patterns of absorption between the formulations the mean peak cortisol for the tests were compared (*table 5.8*).

Table 5.8: Paired t-test results comparing the mean peak cortisol responses of the four formulations. Statistical significance defined as p value <0.05 and denoted in red.

	Tests compared					
	1) 1 mcg i.v 2) 100 mcg tetracosactide + chitosan	1) 1 mcg i.v 2) 500 mcg tetracosactide	1) 1 mcg i.v 2) 500 mcg tetracosactide + chitosan	1) 100 mcg i.n tetracosactide + chitosan 2) 500 mcg tetracosactide + chitosan	1) 500 mcg i.n tetracosactide 2) 500 mcg tetracosactide + chitosan	1) 500 mcg i.n tetracosactide 2) 100 mcg tetracosactide + chitosan
P value	0.333	0.904	<0.0001	0.001	0.007	0.604

The difference between mean peak cortisol response when administering 1 mcg i.v. Synacthen and nasal 500 mcg tetracosactide or 100 mcg tetracosactide plus chitosan or those two nasal formulations were not statistically different, however 500 mcg tetracosactide and chitosan leads to statistically significantly higher peak cortisol than both other nasal formulations and 1 mcg i.v.

4.3.4 Pharmacokinetic data comparing i.v and i.n routes

A summary of the PK analysis is displayed in *table 5.9*, which includes data from the first NeSST study for comparison. The AUC data for the different formulations have been compared using Box and Whisker plots (*figure 5.8*). The clearance (CL) and volume of distribution (Vd) data are included for completeness sake but are difficult to interpret due to probably assay interference, variability of Synacthen results and small numbers.

Table 5.9: Plasma Synacthen PK data for NeSST and NeSST2 studies. (All data are median values plus range)

	NeSST & NeSST2	NeSST Study		NeSST2 Study		
Dose	1 mcg iv	25 mcg in	100 mcg in	100 mcg i.n with chitosan	500 mcg i.n	500 mcg i.n with chitosan
Numbers of subjects analysed	11* [§]	10	10	8	10	7
AUC _{0-∞} (min/pg/ml)	1442 (691-3082)*	148.4 (19.6 – 459)	341.2 (152.2– 1237)	2916 (1041 – 20937)	16191 (1285 – 47411)	20371 (7628 – 68479)
	2901 (864 -3198) [§]					
Cmax (pg/ml)	178 (104 – 361)*	10.2 (2.9 – 18.6)	14.92 (5.96 – 97.02)	170.3 (48.4 – 1336.8)	960 (34 – 2643)	1287 (366 – 3519)
	316 (95 – 395) [§]					
Tmax (min)	5	10 (5 – 15)	10 (5 – 20)	10 (5 – 20)	10 (5 – 20)	12.5 (10 – 20)
	5					
Bioavailability (F)		0.0024 (0.0006–0.027)	0.00198 (0.0007-0.0179)	0.007231 (0.0036–0.051)	0.0125 (0.0008–0.051)	0.019 (0.0052-0.0475)
CL/F (ml/min)		139273 (53285 – 1150431)	289013 (62774 – 541017)	34288 (4765 – 90657)	26415 (10543 – 331929)	339 (312 – 1132)
Vd/F (ml)		1703027 (946475 – 5385143)	5584221 (971911 – 16325569)	293259 (42167 – 2142164)	293198 (130072 – 12157505)	3253 (1247 – 7657)
Mean Residence Time (min)	7.2 (4.2-16.9)*	12.2 (8.1-25.7)	19.7 (13.7-30.2)	17.02 (9.8-20.0)	19.79 (11.1-26.1)	16.69 (9.2-29.4)
	9.5 (7.0-12.3) [§]					
Elimination half-life (min)	5.52 (3.0-41.6)*	10.68 (2.9-17.7)	16.39 (4.5-39.1)	7.53 (3.7-21.7)	8.66 (5.3-72.9)	5.32 (3.53-34.3)
	5.04 (2.5-8.1) [§]					

* NeSST Study [§] NeSST2 Study

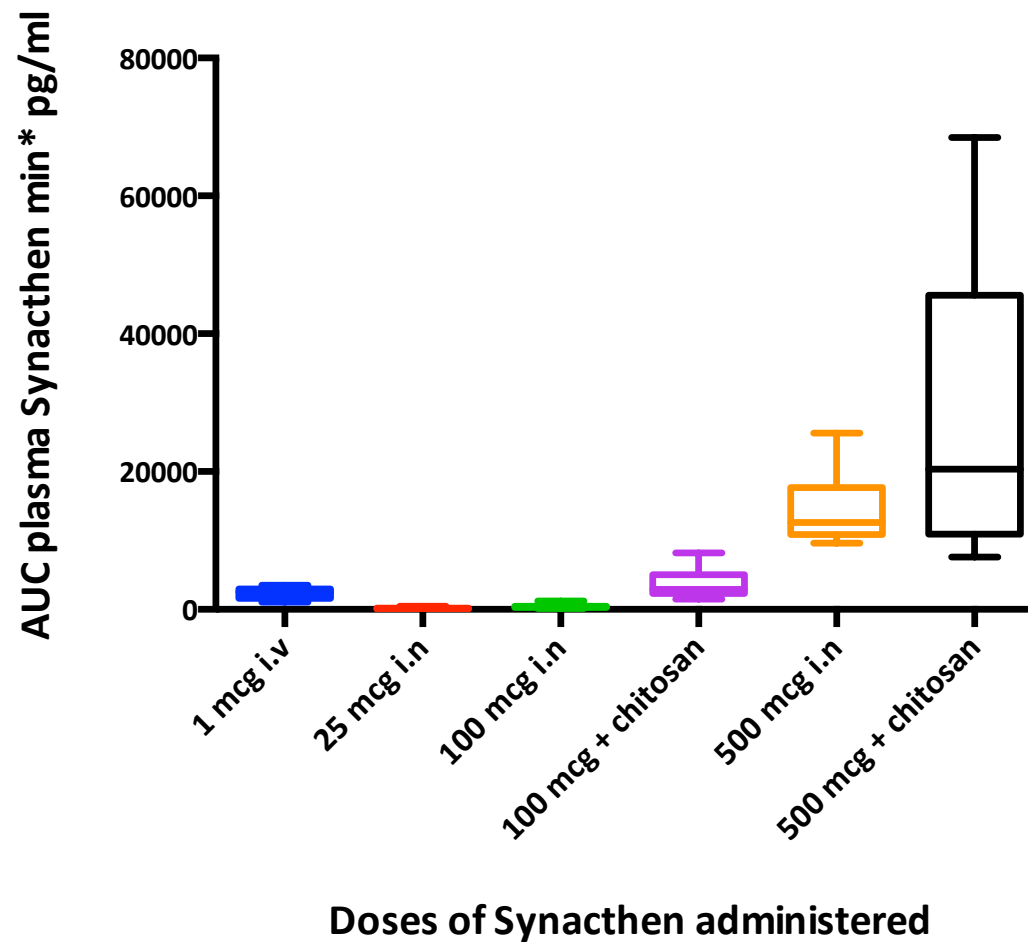


Figure 5.8: Box and Whisker plot of plasma Synacthen Area Under the Curve for different doses of Synacthen administered in NeSST and NeSST2 Studies

As observed during interim analysis, the 100 mcg tetracosactide with chitosan intranasal dose had the closest relevant PK parameters (AUC, C_{max}) to the 1 mg i.v dose, although not for all the study volunteers. The bioavailability was markedly increased following the NeSST2 study doses compared with those given in the NeSST Study.

The median absolute bioavailability of the 100 mcg with chitosan intranasal dose was approximately 0.72% (range 0.36 to 5.1%) of the 1 mcg i.v dose. The bioavailability with 500 mcg tetracosactide showed the greatest variability, 1.25% (0.08-5.1%) and the highest bioavailability was achieved with the 500 mcg tetracosactide and chitosan formulation, 1.9% (0.52-4.8%). The time to maximum concentration (T_{max}) was longer for intranasal formulations, 10-12.5 minutes, compared with five minutes following i.v administration and was longest with the 500 mcg tetracosactide and chitosan preparation (12.5 compared to 10 minutes). The maximum concentration (C_{max}) was approximately 53.9% following 100 mcg with chitosan, 303.8% after 500 mcg tetracosactide and 407.3% following 500 mcg tetracosactide with chitosan, much greater than those seen with the nasal doses given in the NeSST Study.

The bioavailability of the 100 mcg Synacthen intranasal dose increased by 3.65-fold following formulation with chitosan, although the populations were not the same as the results are combined from the NeSST and NeSST2 studies. The bioavailability of the 500mcg intranasal dose was higher than anticipated

without the addition of chitosan and the addition of the nasal enhancer only increased the bioavailability by 1.5-fold.

Mean residence time (MRT) measures the time that molecules of Synacthen remain in the body after injection/nasal ingestion to elimination and therefore, as one would expect, the MRT was longer for the nasal formulations than for the i.v preparation. At both 100 and 500 mcg the MRT was reduced following the addition of chitosan, but dose escalation appeared not to influence it. Similarly the elimination (or terminal) half-life of plasma Synacthen was shorter following the addition of chitosan to both the 100 and 500 mcg doses. It was almost the same for the i.v and 500 mcg tetracosactide with chitosan formulations. Despite a shorter half-life, the cortisol response appeared more prolonged with the higher nasal doses.

Following both the 100 mcg tetracosactide with chitosan and the 500 mcg tetracosactide doses individuals showed a similar cortisol response to the 1 mcg i.v test but consistency was lacking. As expected there was more variability in the data following intranasal administration compared with i.v.

4.4 Relationship between serum cortisol, salivary cortisol and salivary cortisone

The relational analysis of the NeSST Study salivary samples, both to each other and the paired serum sample revealed a correlation, but not as tight as had been anticipated. The data from the NeSST2 Study were analysed in the same way

(figures 5.9a-c). The NeSST Study and NeSST2 Study data were combined to give approximately 1150 paired samples. These data are not displayed here as the trends are very similar and the NeSST study values serve to add numerous samples below the lower limit of assay detection. Tighter relationships than previously were seen between serum and salivary cortisol (figure 5.9a), salivary cortisol and cortisone (figure 5.9b) and serum cortisol and salivary cortisone (figure 5.9c).

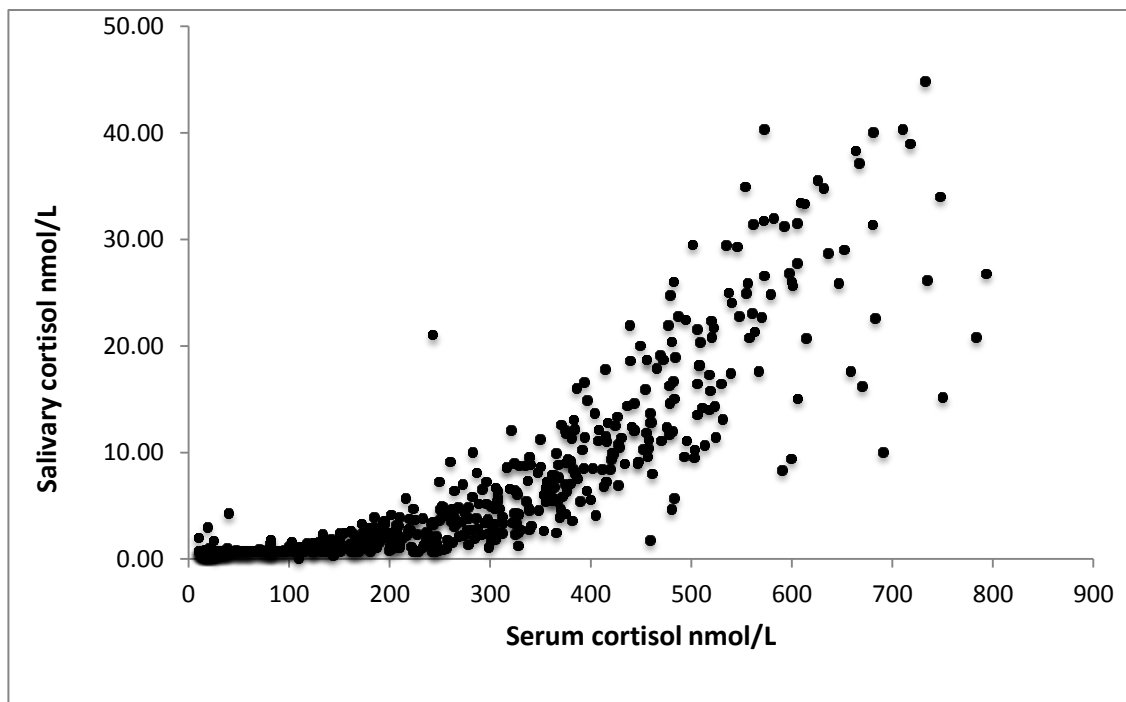


Figure 5.9a: Scatterplot of paired serum and salivary cortisol samples following administration with 4 formulations of Synacthen in the NeSST2 Study (salivary cortisol below the limit of assay detection displayed as 0.74 nmol/L and serum cortisol as 21 nmol/L) N=671.

A clear exponential, or biphasic response is seen between salivary and serum cortisol. A tight linear relationship between salivary cortisol and cortisone is observed and between serum cortisol and salivary cortisone.

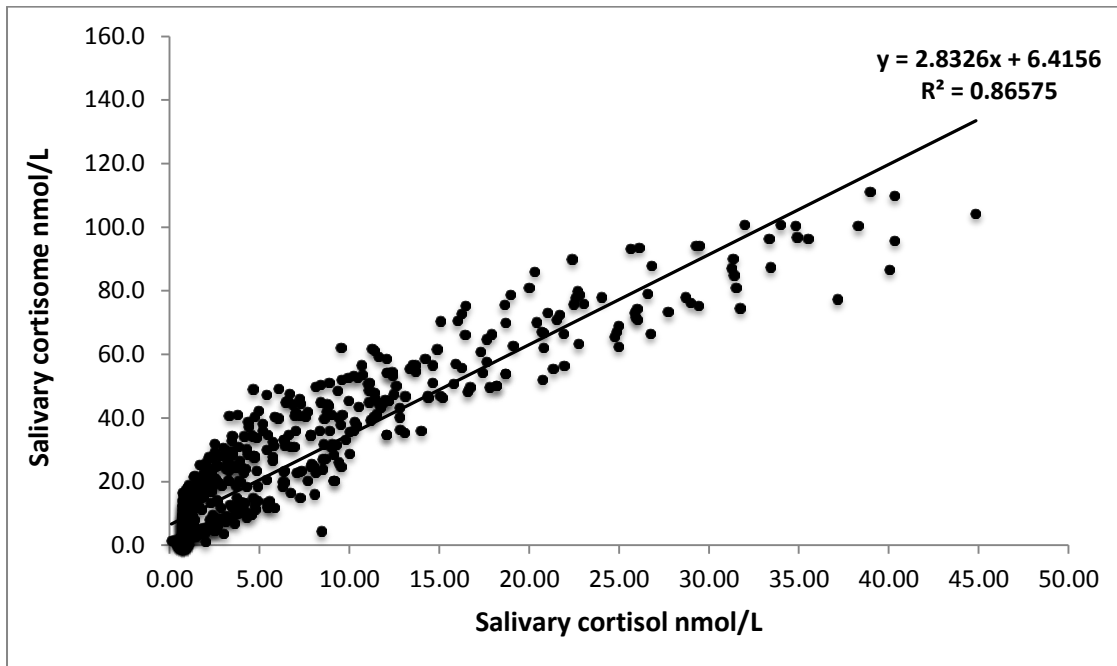


Figure 5.9b: Scatterplot of paired salivary cortisol and cortisone samples following administration with 4 formulations of Synacthen in the NeSST2 Study (salivary cortisol below the limit of assay detection displayed as 0.74 nmol/L and salivary cortisone as 0.4 nmol/L) N=674.

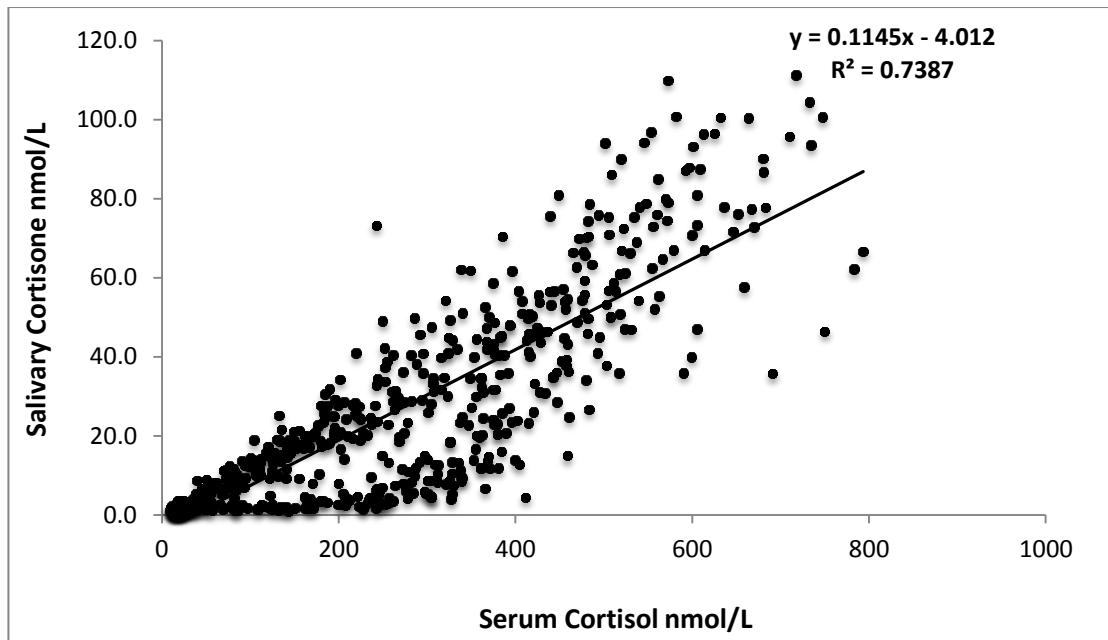


Figure 5.9c: Scatterplot of paired serum cortisol and salivary cortisone samples following administration with 4 doses of Synacthen in the NeSST2 Study (serum cortisol below the limit of assay detection displayed as 21 nmol/L and salivary cortisone as 0.4 nmol/L) N=670.

4.4.1 Salivary cortisol and salivary cortisone peaks and timings

The relationship between the timing of the serum cortisol peak and the peaks in salivary cortisol and cortisone following administration with 500 mcg tetracosactide and chitosan were examined in a number of ways to determine recommended sampling times (table 5.10, figure 5.10).

Table 5.10: Times of peak serum cortisol, salivary cortisol and salivary cortisone following nasal administration with 500 mcg tetracosactide and chitosan (N=9). Mode and median, with interquartile ranges (IQR) have been calculated and are displayed in red.

	Peak cortisol/cortisone times in minutes		
	Serum cortisol	Salivary cortisol	Salivary cortisone
Subject 1	75	75	75
Subject 2	40	50	60
Subject 3	75	75	75
Subject 4	60	75	60
Subject 5	90	120	120
Subject 6	90	90	90
Subject 7	90	90	90
Subject 8	60	75	75
Subject 9	90	90	120
Mode	90	75	75
Median (IQR)	75 (30)	75 (15)	75 (15)

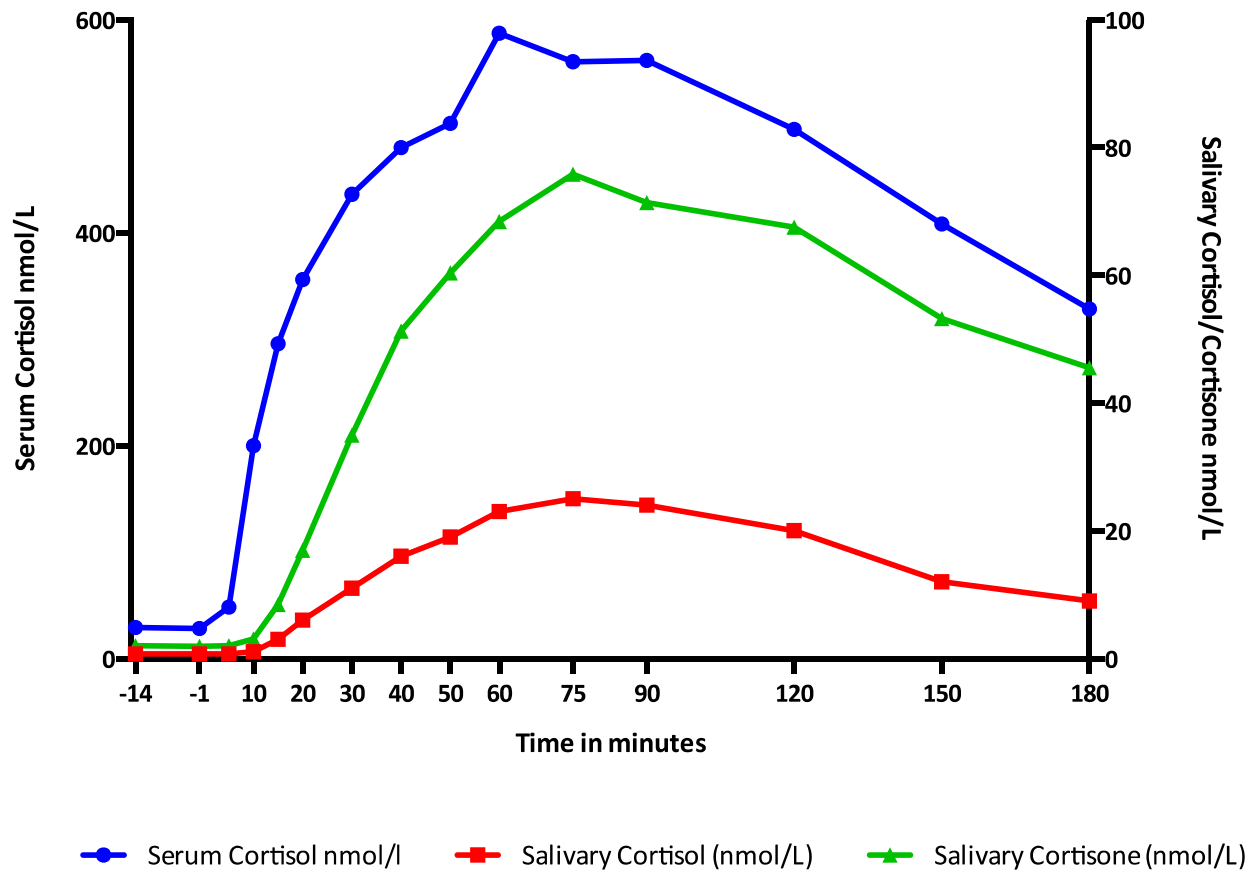


Figure 5.10: Mean serum cortisol, salivary cortisol and salivary cortisone responses over time following nasal administration with 500 mcg tetracosactide with chitosan (N=9).

When viewing the graph of mean cortisol response to 500 mcg tetracosactide and chitosan (*figure 5.10*) the peak times for peak salivary cortisol and cortisone appear to occur slightly later, at 75 minutes, compared to the 60 minute peak seen when measuring the response in serum. However the modal peak time occurs at 90 minutes when measuring serum cortisol and earlier at 75 minutes for the salivary markers, but at 75 minutes for all markers when the median is used.

4.5 Study volunteer feedback

The study volunteers were asked to complete a 5-question anonymous questionnaire after the third visit. A summary of the responses is given below (*tables 5.11a-d*) and comparisons to the responses given to the same questions in the NeSST Study (*figures 5.11a-d*).

Table 5.11a: Responses to qu 1: How did you find having the nasal spray administered?

Very easy	Easy	OK	A little tricky	Difficult	Very difficult	TOTAL
5	4	3	0	0	0	12

The following comments were made “very vinegary” “somewhat unpleasant”, “difficult not to sneeze afterwards but overall it was fine” “less uncomfortable when nurse administers compared to when held the nasal device myself”, “smelt a tad like vinegar but no irritation or reaction”, “a fair amount went down the back of my throat”.

The responses were compared to those given by the NeSST Study volunteers (*figure 5.11a*) and show, despite the drug being given using the same atomiser device NeSST2 study volunteers did not find it as easy. This may have been borne out of confusion in the question wording as many appeared to be answering about the nasal Synacthen rather than the method of administration.

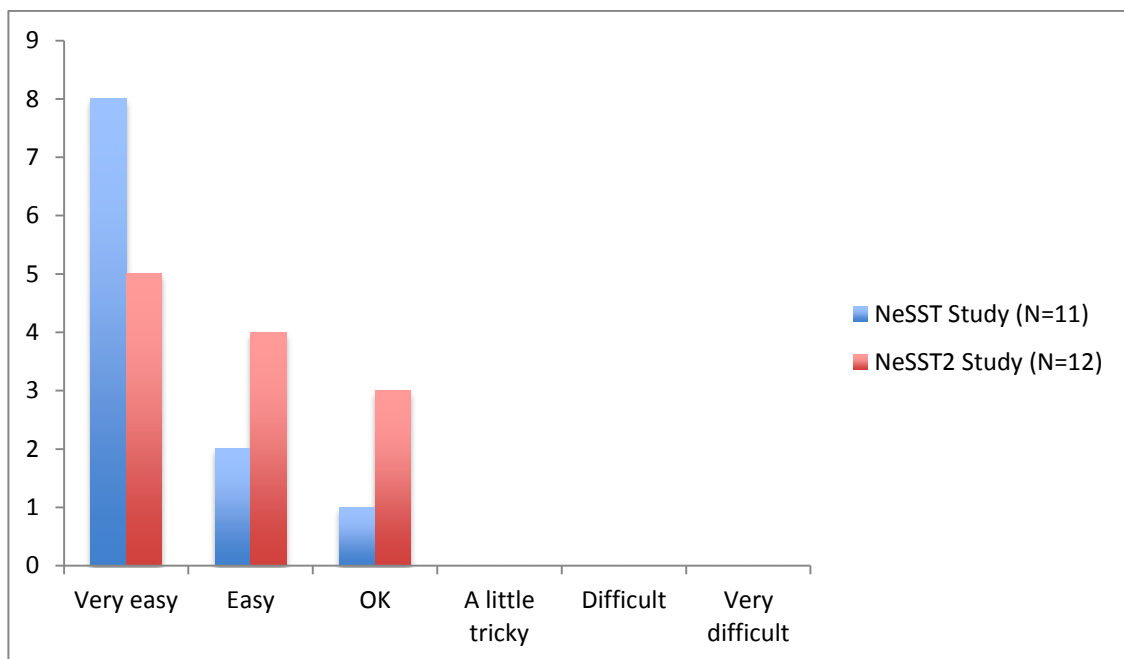


Figure 5.11a: Volunteer responses from the NeSST Study and NeSST2 Study when asked “How did you find having the nasal spray administered?”

Table 5.11b: Responses to question 2: How did you find receiving the nasal Synacthen?

No problem	Slightly unpleasant	Unpleasant	Very unpleasant	TOTAL
3	8	1	0	12

Comments: “Fine”, “strong vinegary smell, makes your eyes water and makes you want to sneeze”, “was a little uncomfortable but easily tolerable”, “made me

sneeze once”, “slight sting but only lasted a second or two”, “induced a sneezing fit the second time”.

Again the responses were compared to those given in the NeSST Study (*figure 5.11b*) and show that, whilst the majority in the NeSST Study found nasal Synacthen administration to be of no problem, most found it to be slightly unpleasant NeSST2 Study.

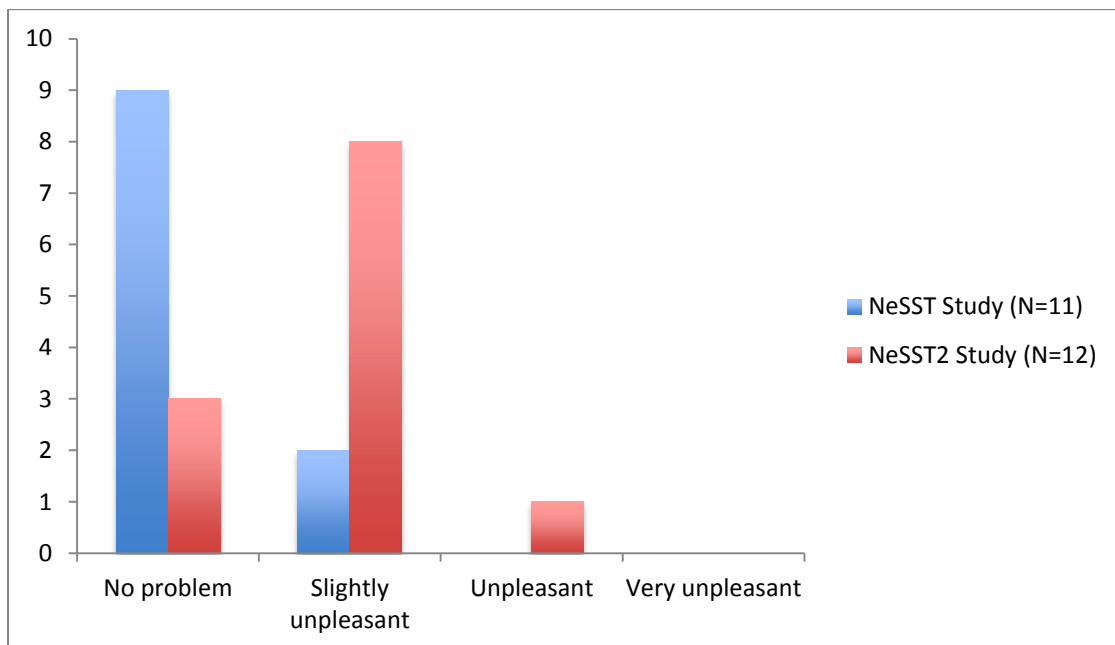


Figure 5.11b: Volunteer responses from the NeSST Study and NeSST2 Study when asked “How did you find receiving the nasal Synacthen?”

Table 5.11c: Responses to question 3: Did you experience any unpleasant side effects from the nasal Synacthen?

Stinging	Irritation	Sneezing	Soreness	Itching	Nasty taste	None	TOTAL
7	2	3	0	0	0	3	15

Subjects were asked to “tick all that apply” and a number ticked several responses, whilst three experienced no unpleasant side effects.

The comparison with the NeSST Study responses (*figure 5.11c*) show more volunteers complaining of sneezing, nasal irritation and stinging compared to the majority who had complained of no unpleasant side effects in the NeSST Study.

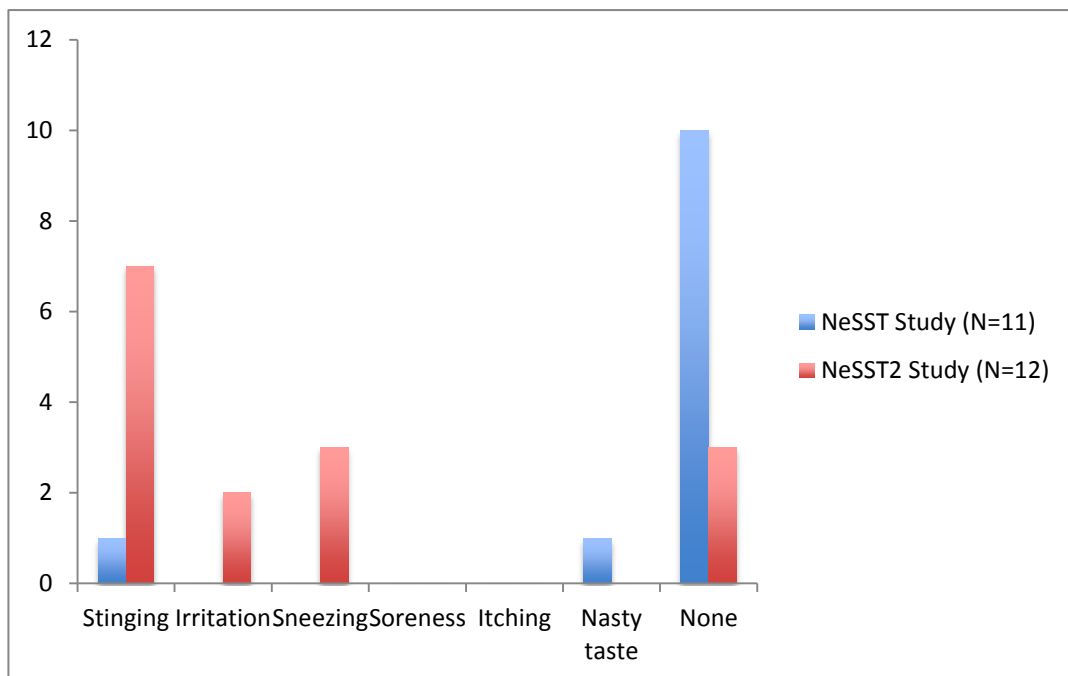


Figure 5.11c: Volunteer responses from the NeSST Study and NeSST2 Study when asked “Did you experience any unpleasant side effects from the nasal Synacthen?”

Table 5.11d: Responses to question 4: Overall how did you find the nasal Synacthen compared with the intravenous Synacthen?

Much better	Better	Much the same	Worse	Much worse	TOTAL
3	0	5	3	0	11

Free text comments: A number (N=5) of respondents felt that whilst neither method was unpleasant nasal administration is preferable to avoid cannulation “...if people struggle with cannulas/needles nasal would be a much more preferable option”. Others felt there was no preferable option.

The two study comparison (*figure 5.11d*) revealed more of a spread of responses for NeSST2 volunteers with more feeling nasal Synacthen was preferable to i.v but more also thinking it was inferior.

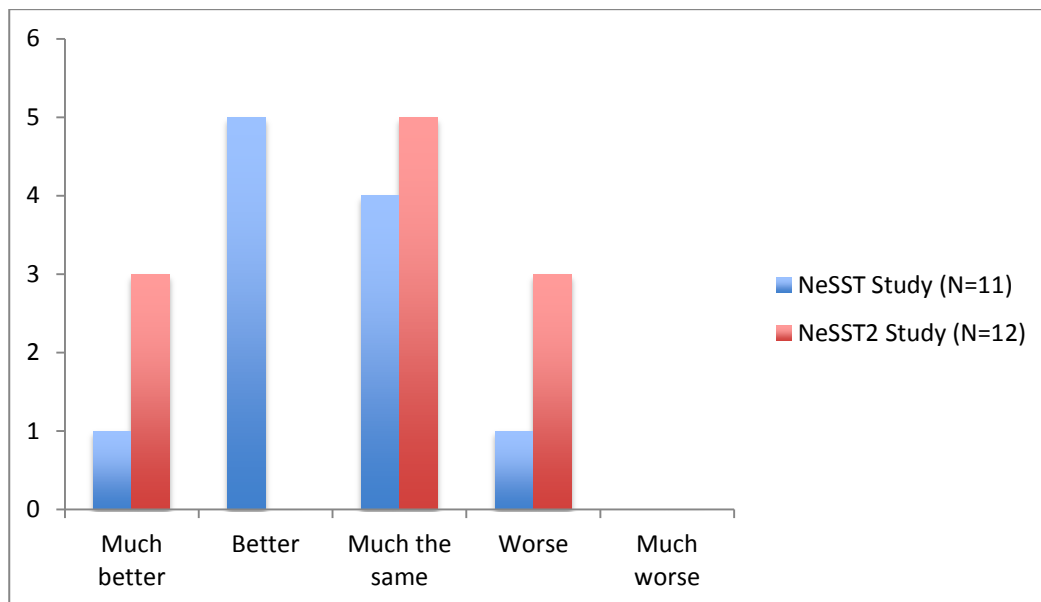


Figure 5.11d: Volunteer responses from the NeSST Study and NeSST2 Study when asked “Overall how did you find the nasal Synacthen compared with the i.v Synacthen?”

4.6 Safety

No adverse events attributable to the administration of Synacthen/tetracosactide were recorded for the NeSST2 Study. On two occasions volunteers admitted, on direct questioning, to dyspeptic symptoms following

dexamethasone self-administration. Both subjects were excluded from further study.

4.7 Summary of results

Although variable, both the 500 mcg tetracosactide and the 100 mcg tetracosactide with chitosan formulations showed a good and comparable cortisol response to the 1 mcg i.v dose in some individuals. A dose escalation to 500 mcg tetracosactide and chitosan, giving much higher plasma Synacthen C_{max} and AUC, was required to achieve a cortisol response of over 500 nmol/L in all volunteers. This was not achieved with the 1 mcg i.v dose. The bioavailabilities calculated for the 100 mcg tetracosactide with chitosan, 500 mcg tetracosactide and 500 mcg tetracosactide and chitosan respectively were 0.72%, 1.25% and 1.9%, considerably higher than seen with the doses given in the NeSST Study. As expected the T_{max} was longer with the nasal formulations and more variable than with the i.v dose. The nasal formulations were less well tolerated with more volunteers complained of stinging and a vinegary taste but there were no serious adverse events.

5 Discussion

There was disappointingly a smaller data set for the 500 mcg tetracosactide with chitosan dose compared to the other three formulations due lost samples,

nursing errors, volunteers excluded for presumed non-adherence to the pre-test dexamethasone and those unable to continue due to dyspeptic symptoms.

5.1 Synacthen assay and proof of dexamethasone suppression

As observed in the NeSST Study the baseline results (-14 and -1 minute samples) were universally high, although HPA-axis suppression was shown by the low baseline cortisol results in the majority of volunteers. For a more extensive discussion see *chapter 4, section 5.1*. In contrast to the NeSST Study, the intra-individual consistency of the high plasma Synacthen baseline results is not so striking. The trend of rise from and fall back to baseline observed in both studies following Synacthen administration, seen most clearly after i.v administration, gives credence to the theory of an unrelated and stable interfering molecule, unaffected by either dexamethasone or Synacthen, and remains the most plausible explanation.

In the 2 years between analysis of the NeSST and NeSST2 Study samples the hACTH RIA was validated for quantifying Synacthen, albeit with significant flaws (*vide supra chapter 3*). The Synacthen standards used in NeSST2 sample analysis, made from the RIA kit zero standard, and used to derive the standard curve from which the volunteer samples were calculated, improved the scientific integrity of the Synacthen sample analysis. However the different assay standards employed need to be taken into consideration when making direct comparisons between NeSST and NeSST2 Study data.

The reliability of the assay to give similar results when measuring the same sample on different occasions, inter-assay precision, has not been demonstrated. It was only in retrospect that the author recognised the missed opportunity to test inter-assay precision. All plasma Synacthen samples were split following separation and frozen and therefore a selection of the remaining frozen samples could have been rerun on the assay during the analysis of diluted high samples and the results compared to the first analysis. The Synacthen standards, which were from one batch, have acted as a surrogate for inter-assay precision and were very similar to one another (*figure 3.16*).

Overall the results following the administration of 1 mcg i.v Synacthen, the only dose to be performed in both NeSST studies, were similar, a surrogate marker of Synacthen test and assay reliability. However in both studies considerable individual plasma Synacthen variability was seen possibly reflecting inherent biological variability. Mean plasma Synacthen was seen to rise towards the end of the testing time in the NeSST2 Study only and the error bars were much larger, this was due to two individuals with aberrant high results at the end of testing, possibly due to human error and a mix up of samples (*figure 5.2a*). The acknowledged flaws in the assay: variability in Synacthen responses to the same dose of tetracosactide, even the i.v formulation, and unresolved issue of the high baseline values, have resulted in plasma Synacthen results that must be viewed with some caution. Trends and general comparisons are valid however over interpretation and direct comparisons, are not.

5.2 Comparison of intravenous and nasal administration routes

Despite the individual variability observed in both plasma Synacthen (all formulations) and serum cortisol results, particularly seen with nasal formulations, it is clear that both dose escalation and the addition of the nasal drug enhancer, chitosan, increased plasma Synacthen levels, and therefore the resultant cortisol response, following nasal administration.

Statistical analysis comparing the difference in mean serum cortisol of the formulations at each time point revealed that cortisol rises earlier and therefore was significantly different, when Synacthen was given i.v. However the differences were not significant when comparing i.v with the 500 mcg tetracosactide plus chitosan dose, presumably because the additive effect of the high dose and the nasal drug enhancer caused an early high rise in plasma Synacthen levels, not dissimilar to that seen with i.v Synacthen. Both formulations of the 500 mcg tetracosactide elicited a good cortisol response but exhibited a slower clearance time and prolonged raised serum cortisol. This may be due to slower with nasal formulations and more prolonged absorption (due to reduced mucociliary clearance) when chitosan is added. This may lead to prolonged adrenocortical stimulation or reflect supraphysiological stimulation with upregulation of glucocorticoid synthesis (Alia, Villabona et al. 2006).

There was no significant difference in the peak cortisol when comparing the 1 mcg i.v test to both the 100 mcg tetracosactide with chitosan and the 500 mcg tetracosactide doses, indicating clinical parity between the formulations.

However when individual's data are studied both tests, in particular the 100 mcg tetracosactide with chitosan, included non-responders and thus the reliability of the formulation was questioned. The 500 mcg tetracosactide with chitosan produced a significantly higher cortisol peak than all three of the other formulations, with all subjects reaching above 500 nmol/L peak cortisol, despite their suppressed baseline, and thus "passing" their LDSST.

5.2.1 Pharmacokinetic data: the effects of dose escalation and the addition of chitosan

The bioavailability of the 100 mcg (NeSST Study data) and 500 mcg doses were improved by the addition of chitosan but the effect was less pronounced for the 500 mcg dose. The bioavailability of the 100 mcg intranasal dose increased by 3.65-fold following formulation with chitosan, however it must be noted that, as this required combining NeSST and NeSST2 data, neither the populations nor the assay methodology are the same. The five-fold dose increase between the two studies improved the bioavailability considerably and, perhaps surprisingly, the addition of chitosan to the 500 mcg dose only increased the bioavailability by 1.5-fold. The dose escalation had a greater effect on absorption of nasal Synacthen shown by the better bioavailability of the 500 mcg dose without chitosan compared to the 100 mcg dose plus chitosan.

The bioavailability of both doses of nasal Synacthen in the NeSST Study was approximately 0.2%. The expectation therefore was that the bioavailability seen

with the 500 mcg tetracosactide dose in the NeSST2 study would be similar, however it was 1.3%, 6.5-fold higher and was 3.4-fold lower than in the murine study (Wuthrich, Martenet et al. 1994). If the result is a true reflection of the relative bioavailabilities it may be that the high concentration alone results in improved absorption lessening the effect of the enhancer i.e. a dose effect causing a larger concentration gradient and consequent saturation of an efflux mechanism. Whether Synacthen is a substrate for the known drug efflux transporter (P-gp) present in the nasal mucosa is unknown (Kandimalla and Donovan 2005). Additional contributing factors may be the difference in the assay methodology, the inherent assay variability or the difference in the populations being compared. Alternatively the tetracosactide may be subject to pre-absorption losses e.g. peptidase activity in the nasal cavity, which would have a relatively greater effect on the lower dose and thus explain the higher bioavailability seen with the 500 mcg tetracosactide dose.

It was hoped that the addition of chitosan to tetracosactide would not only enhance absorption but would improve variability. When comparing the CVs for the different nasal formulations chitosan reduced the variability of mean peak serum cortisol in both the 100 and 500 mcg doses. When considering clinical practice and the applicability of a non-invasive SST it is peak serum cortisol variability that can yield borderline cases and false positives and negatives. The addition of chitosan and the reduction of variability are important, however neither formulation had a CV as low as seen with i.v administration.

The reduction in mean residence time observed with the addition of chitosan is likely to be the result of more rapid absorption, as would be expected with an enhancer which aids paracellular transport across the nasal mucosa. There is no clear relationship between terminal half-life and dose escalation. When the dose is increased from 100 mcg to 500 mcg the half-life reduces, possibly a reflection of saturation of the elimination pathway, yet the results for equivalent doses with the addition of chitosan confound this theory. One would expect a longer half-life with chitosan, as more is absorbed and faster, resulting in higher plasma concentrations to be eliminated. The prolongation of the cortisol response at higher doses may be explained by the greater Synacthen concentrations achieved with the higher doses remaining above the minimum concentration that evokes a cortisol response.

5.3 Relationship between serum cortisol, salivary cortisol and salivary cortisone

An exponential or biphasic relationship was observed between serum and salivary cortisol when paired samples were compared, as previously reported (Vining, McGinley et al. 1983; Gozansky, Lynn et al. 2005; Perogamvros, Owen et al. 2010). The non-linearity is due to the presence of Cortisol Binding Globulin (CBG), binding serum free cortisol at low concentrations but becoming saturated at higher concentrations allowing free cortisol to become available for absorption into the saliva. When paired samples of salivary cortisol and salivary cortisone were plotted a linear relationship was seen, as in the NeSST Study, but

with a considerably tighter correlation. This is also true for the relationship seen when serum cortisol and salivary cortisone were correlated. The serum cortisol and salivary cortisol/cortisone analyses were carried out in NHS clinical chemistry laboratories, on assays which remained the same between the two studies, and thus the tighter relationships observed in the NeSST2 paired samples are likely to be due to a greater spread of values, with many more samples of higher values, and fewer outliers.

Although individual variance in CBG is unlikely to have affected the results in this healthy, adult, male population it has been reported previously (Dhillon, Kong et al. 2002). It is possible that the tighter correlation observed in NeSST2 is due to the higher values, where CBG is less of a concern in contrast to low cortisol levels where it is not always straightforward to delineate what is free and what is bound (personal communication, ML).

Cortisone is emerging as the preferred salivary marker of adrenocortical function and the data from the NeSST and NeSST2 studies support this. Cortisone is more sensitive at the lower end of both the physiological and the assay range, due to the effect of 11β -HSD-2 causing it to have a 3-4 fold higher concentration than cortisol. This sensitivity makes it better suited to the detection of adrenal insufficiency (Arafah, Nishiyama et al. 2007, Perogamvros, Owen et al. 2010, Blair, Lancaster et al. 2013) and it has a close and linear relationship with serum cortisol removing interference from CBG variance (Perogamvros, Owen et al. 2009). If the slope of the correlation curve, when plotting paired serum cortisol and salivary cortisone data from the NeSST2 Study, is used a correction factor of

0.115 would need to be employed e.g. 57.5 nmol/L salivary cortisone equates to 500 nmol/L serum cortisol.

5.3.1 Sampling times

The mean, mode and median time for the salivary cortisol and cortisone peak to occur following administration with 500 mcg tetracosactide with chitosan was 75 minutes. Serum cortisol was more variable with the mean peak occurring at 60, the median at 75 and the mode at 90 minutes. Sampling times of 60, 75 and 90 minutes for serum or salivary cortisol would have caught 8/9 of the peaks in this cohort of healthy male adults. Additional sampling at 120 minutes would have increased the capture for salivary cortisone peaks from 7/9 to 9/9.

5.4 Side effects

Although the distribution of responses was different, there was no discernable difference in volunteers' comparison of nasal and intravenous administration. The vinegary smell and taste was noted in the feedback from both studies and was due to the acetic acid present in all formulations. Volunteers in the NeSST2 Study were more likely to find nasal Synacthen administration "slightly unpleasant", complain of nasal irritation, sneezing and stinging, compared with the NeSST participants. This may have been due to the increased concentration of tetracosactide or have been side effects attributable to the chitosan. The questionnaire was given out after the completion of three visits, as it was not known whether interim analysis would support the testing of a further dose.

Unfortunately the questions did not differentiate between visits and therefore it is not clear whether the nasal symptoms are related to the chitosan, the increased concentration or another, unknown factor. In one questionnaire the volunteer detailed the test in which they had experienced the nasal symptoms (100mcg with chitosan). Chitosan has been found in extensive studies to be non-toxic and is very well tolerated (Davis and Illum 2003).

5.5 Limitations and further work

The number of research participants in the final analysis, especially in the 500 mcg tetracosactide with chitosan group, was disappointing and below the 12 anticipated. Had the non-adherence to the dexamethasone been anticipated it may have been possible to organise for a baseline cortisol to be taken for immediate analysis and the visit abandoned if it been high. The laboratory where the serum cortisol samples were analysed (SCH) was only set up to batch analyse research samples a maximum of once per day. Additionally the timing this extra step would have required was not feasible.

In order to determine the differential effect of chitosan the desired methodology is to give the different formulations to the same individuals, who act as their own internal control. When comparing the 100 mcg doses both NeSST and NeSST2 data were used, and the studies involved different individuals. When comparing the two 500 mcg doses numbers were limited due the

exclusions made. The fact that the comparative analysis has been performed on different individuals or on small numbers limit the conclusions that can be drawn.

Despite our healthy male cohort of study volunteers, variations in CBG levels may have been present. It has previously been shown, in a mixed cohort of healthy adults, that CBG levels vary significantly between and within individuals and enough to affect the results of a SST (Dhillon, Kong et al. 2002). A more in depth study of the relationship between serum and salivary cortisol may have been possible with correction for the CBG variable. However it is not usually necessary to correct for CBG, particularly in a healthy, male population and congenital CBG deficiency is very rare.

The manufacture of a novel drug also offers the opportunity for a formulation with a more pleasant flavouring and odour to negate the vinegary taste and smell produced from the acetic acid which recipients would find more palatable.

5.6 Conclusion

The results of the NeSST2 Study show that increasing the dose and adding the nasal drug enhancer, chitosan, both improve nasal Synacthen absorption and therefore the height of the cortisol response, making it similar to that seen with 1 mcg i.v, albeit individually variable. The cumulative effect of the five-fold dose escalation and the addition of chitosan resulted in plasma Synacthen levels and cortisol responses higher than seen with 1 mcg i.v, all volunteers “passing” the LDSST. The chitosan additionally reduced the variability of peak serum cortisol. A

bioavailability of 1.9% was achieved with 500 mcg tetracosactide with chitosan, almost ten-fold that seen with 100 mcg nasal Synacthen in the NeSST Study, but below the 5-10% predicted by Archimedes Drug Development Ltd and equivalence was not achieved. Despite the lower than expected bioavailability and difficulty interpreting the PK data due to low numbers and “noise” created by the assay, the cortisol responses (in particular the 500 mcg tetracosactide with chitosan formulation) were good and, as cortisol is the primary endpoint in clinical practice, reflect success in the first step in the development of a non-invasive SST.

Chapter 6

Summary

and further

work

The first five chapters of this thesis present the background to the development of a non-invasive Short Synacthen Test, some small studies looking at local and national practice, the validation of a Synacthen assay and the two clinical studies undertaken. The material presented is discussed in detail within each chapter. This final chapter serves as a summary of the work achieved so far and a discussion of further work required in the development of a non-invasive SST and its utility in research to help delineate the children prescribed ICS who are most at risk of adrenal suppression.

1 What has been learnt from the NeSST and NeSST2 Studies?

1.1 Validation of a Synacthen assay

In order to generate comparative pharmacokinetic data it was necessary to measure plasma Synacthen levels following the administration of the different formulations and doses of Synacthen. There were considerable and unanticipated difficulties in the validation of the method and there continue to be concerns regarding flaws in the assay.

Serial dilution experiments failed to produce consistent and interpretable results until Synacthen standards were made up using a single vial of Synacthen and the zero standard from the hACTH RIA kit. Although this enabled validation of the assay it still contravenes the basic premise of competitive RIA, whereby the

antibody and sample compete for binding as identical molecules. Despite this, the Synacthen standards have yielded reliable and repeatable results with an interpretable curve from which to derive Synacthen values for the study. Plasma Synacthen levels showed considerable variability between individuals, which may in part be the assay, but is likely to reflect biological variance.

The other concern, which remains unresolved, is the lack of a suppressed baseline plasma Synacthen for all research subjects, despite other evidence of adequate dexamethasone suppression. The most likely explanation is assay interference by an unrelated yet structurally similar molecule, however the intra-individual consistency was not seen as clearly in the NeSST2 Study as in the NeSST Study. It was felt, after discussion with laboratory colleagues, that if baseline values were subtracted from all subsequent time points, then the rise seen after Synacthen administration was likely to be due to the administered Synacthen rather than changed levels of interference and this was the methodology adopted throughout the studies.

Quantifying plasma Synacthen levels is a research tool and does not have a role in clinical medicine. Cortisol is the clinical endpoint of interest in adrenal insufficiency and, as the measurement of plasma Synacthen is time-consuming, expensive and remains unreliable, future studies are likely to focus on cortisol measurement only. Future studies with glucocorticoids as the sole endpoint would enable volunteers not to undergo dexamethasone suppression. This is likely to reduce the number of volunteers excluded for both side effects and non-

adherence to medication. Additionally it would remove the blunting effect on the glucocorticoid response.

1.2 Bioavailability and pharmacokinetics of nasal Synacthen

Synacthen is a relatively large, polar molecule and not is ideally suited to nasal administration. Although poorly absorbed, some bioactive Synacthen was achieved with both the 25 mcg and 100 mcg doses in the NeSST Study, as evidenced by the resultant cortisol response. The responses of both plasma Synacthen and serum cortisol were dose dependent, with significantly higher serum cortisol levels attained with i.v compared to nasal and with the 100 mcg nasal compared to 25 mcg doses. The bioavailability of both doses of nasal Synacthen in the NeSST Study were very low, approximately 0.2%. The results were disappointing but showed a proof of concept – nasal Synacthen could be absorbed and was well tolerated.

Both dose escalation and the addition of a nasal drug enhancer were trialed in the NeSST2 Study and both enhanced the bioavailability and cortisol response. The bioavailabilities calculated for the 100 mcg tetracosactide with chitosan, 500 mcg tetracosactide and 500 mcg tetracosactide and chitosan respectively were 0.72%, 1.25% and 1.9%. Although variable, both the 500 mcg tetracosactide and the 100 mcg tetracosactide with chitosan formulations showed a good and comparable cortisol response to the 1 mcg i.v dose in most research subjects. The combination of 500 mcg tetracosactide with chitosan, gave a much higher

plasma Synacthen Cmax and AUC, and was the only formulation, including i.v, with which all volunteers achieved a cortisol response of over 500 nmol/L. Dose escalation improved the absorption of nasal Synacthen more than the addition of chitosan, and together there was a cumulative effect. The addition of chitosan improved the variability of the cortisol response.

1.3 Salivary cortisol and cortisone

The relationship observed between salivary and serum cortisol, in both studies, was exponential. This is expected and is due to the Cortisol Binding Globulin (CBG) effect. Salivary cortisone is less affected by CBG, reflecting serum free cortisol and therefore a tight and linear correlation was seen between salivary cortisone and serum cortisol. The correlations achieved in the NeSST Study were weaker than expected and improved in the NeSST2 Study, with no change in methodology and was attributed to having more data points in the upper ranges. Salivary cortisone was the more sensitive marker of adrenocortical response at lower values and may be the more appropriate marker of adrenocortical inadequacy.

2 Further work – NeSST3 and beyond

A validated non-invasive SST would enable large-scale research in healthy children of different ages, both sexes and different pubertal stages to establish normative data, currently unviable due to the invasive nature of the test. These

data could be used to compare the adrenocortical responses of children with asthma on different doses of ICS to ultimately enable stratification of patients into risk groups with their adrenal monitoring tailored to their risk; negating unnecessary investigation of low-risk patients and allowing close monitoring for high-risk individuals.

The following future research stream is proposed:

- *NeSST3 Study*: Perform three non-invasive LDSSTs (500 mcg tetracosactide plus chitosan) in the same individuals (N=6) to establish the intra-individual variability.
- *NeSST4 Study*: Perform non-invasive LDSST, with 1 mcg i.v comparator, in 20 children of both sexes and varying ages in order to establish that the chosen dose, peak cortisol response, bioavailability and pharmacokinetics are similar in the paediatric population compared with adult males. The PK data would require measurement of Synacthen, which needs further consideration before being undertaken.
- *NeSST5 Study*: In order to interpret the findings of the SST in children with possible adrenal insufficiency one must know the response in normal, healthy children. Approximately ten male and ten female subjects in each of the following age bands: 6 months-2 years, 3-5 years, 6-8 years, 9-11 and 12-15 years will undergo a non-invasive SST to establish the first normative data for the LDSST in the paediatric population. The effects of age, sex and pubertal status should be studied as previously pubertal

stage and age have been shown to impact on salivary cortisol concentration (Tornhage 2002) and cortisol response to Synacthen (Lashansky, Saenger et al. 1991, Blair, Lancaster et al. 2013).

- *NeSST6 Study*: Having established normative data, studies in children on ICS can be undertaken to investigate what dose may potentially cause adrenal suppression and the role of age, sex and pubertal stage. A recent study has shown sex but not age to impact on likelihood of an abnormal response to LDSST in children on ICS and age and sex to impact on peak cortisol levels (Blair, Lancaster et al. 2013). Children of both sexes, different ages (6 months – 15 years) and on varying doses of ICS will undergo a non-invasive SST, although the number required will be determined once normative data from NeSST5 Study have been analysed as means and standard deviations are required for power calculations.

Additional research within the above stated streams:

- The 500 mcg tetracosactide plus chitosan dose yields higher serum cortisol results compared to 1 mcg i.v and therefore may be higher than the dose required to maximally stimulate the adrenal gland. The exact dose of Synacthen required for maximal stimulation is a controversial area (*chapter 1, section 4.4*) and likely to shown some biological variability between individuals. Assessing patients with known adrenal insufficiency with the non-invasive test and calculating the sensitivity and specificity of the test would be important step in its validation.

- The plasma Synacthen levels in both the NeSST and NeSST2 studies show considerable individual variability. Some of this biological variability may be due to genetic variance of HPA-axis receptors, e.g. CRH and glucocorticoid. Variation in *CRHR1* (CRH receptor 1) has been shown to significantly contribute to the degree of improvement in lung function with ICS. (Tantisira, Lake et al. 2004). Future studies should consider the inclusion of genotyping as this may allow individual responses to Synacthen to be analysed and future prediction of dose responsiveness to be anticipated.

3 Recommended non-invasive LDSST.

This work supports further work validating a nasal alternative to the i.v LDSST. It demonstrates that Synacthen can be absorbed nasally in the amounts necessary to produce a reassuring cortisol response in healthy adult males. Further validation in non-dexamethasone suppressed adults, children and non-healthy populations is required. Although found to be palatable and safe in the adult volunteers investigated here, larger-scale safety studies would need to be conducted. From the results presented in this thesis further work to validate a non-invasive low-dose Short Synacthen Test, performed with nasally administered 500 mcg tetracosactide plus chitosan and salivary cortisone sampling at 60, 75 and 90 minutes would be recommended, with the diagnosis of

adrenal insufficiency taken as a peak cortisone of less than 57.5 nmol/L (or lower using a correction factor of 0.115).

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Acknowledgments

Taking the kernel of an idea and first transforming it into a scientifically valid research protocol; then negotiating the project through the necessary regulatory approvals; evolving it from paper to reality and having to right all the assumptions made was challenging. To then have that rewarded with disappointing results was a considerable set back and required reworking of the project, the timescales, additional funding and further regulatory approvals to be sought. However satisfying collaborative relationships were forged and a novel formulation developed which yielded much more satisfying results. The overall process was at times disheartening, frustrating and lonely, however there were moments of great joy and satisfaction. Without the support of many people the former emotions may have eclipsed the latter.

None of this would have been possible without the idea, support and supervision of Dr Neil Wright. He has always made time for me, given me the freedom and autonomy to direct the project and provided an excellent meta-perspective when required. Dr Jerry Wales has been a wise and experienced second supervisor answering queries and returning drafts with unnerving speed. Professor Nick Bishop has provided thoughtful general academic supervision over the five years and has been unfailingly supportive.

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This project and thesis would not have been possible without all these people and numerous others who have contributed in their way, so a big and very heartfelt thank you to them all.

Charlotte Elder, February 2014

Appendices

1. British Society of Paediatric Endocrinology and Diabetes SST survey
2. Survey of GP knowledge of adrenal suppression and inhaled corticosteroids
3. Emergency Standard Operating Procedure
4. Volunteer end of study questionnaire (NeSST and NeSST2 studies)

British Society of Paediatric Endocrinology and Diabetes SST survey

April 2009

Dear Paediatric Endocrinology Colleague,

We are currently surveying all British paediatric endocrinology departments about their use of the Short Synacthen Test (SST). We are interested in looking at how many of us now use the low dose SST, how the low dose is prepared and whether there is much variation in what we consider a normal result. The questionnaire should only take you a few minutes.

We have approached all BSPED members but only require one reply per department.

We would be grateful if you would email it or post it back to the address given below.

Thank you in advance for your help.

Charlotte Elder, Pooja Sachdev and Neil Wright – Sheffield Children's Hospital.

e-mail: C.J.Elder@sheffield.ac.uk

Postal address: Dr C Elder,
Clinical Research Fellow,
Academic Unit of Child Health,
Stephenson Wing,
Sheffield Children's Hospital,
Western Bank,
Sheffield S10 2TH.

Questionnaire

1. What is the name of your hospital?

.....

2. Does your centre carry out Short Synacthen Tests? Please circle as appropriate

Yes

No

If you have answered “no” please return the questionnaire at this point, you do not need to fill in any further answers. If “yes” please continue. Thank you.

3. What dose of Synacthen does your department use? Please circle answer.

low dose (1mcg)

standard dose (250mcg)

another dose

If you have answered “another dose” please state the dose

.....

4. If you use a dose less than 250mcg please describe how this is made up:

e.g. 250mcg (1ml) Synacthen mixed with 250mls 0.9% saline to make up

1mcg/ml

.....

.....

.....

.....

5. At what times do you take your cortisol samples? Please circle all appropriate

0 min 10 min 20 min 30 min 60 min

other (please specify)

.....

6. What cut off for normal do you use?

a. Peak cortisol (please circle as appropriate)

>500nmol/l >550nmol/l >580nmol/l other (please specify).....

b. Rise from baseline (please circle as appropriate)

>200nmol/l Other (please specify).....

c. For a normal result do you require (please circle tick appropriate box)

- i. Only peak cortisol
- ii. Only rise from baseline
- iii. Both peak and rise from baseline
- iv. Either peak or baseline but not both

5. Are you aware of the recommendations introduced in 2005 by the Committee on the Safety Medicines (CSM) that children on high dose inhaled corticosteroids (HDICS) should carry a steroid alert card and have Short Synacthen Tests performed?

Yes

No

6. Have you noted an increase in the number of SSTs requested in your hospital since these recommendations were introduced in 2005?

Yes

No

7. Have you detected adrenal suppression in children on HDICS (but with no other obvious cause)? If "yes" please give the number of cases.

Yes

No

Survey of GP knowledge of adrenal suppression and inhaled corticosteroids

Dear GP/asthma nurse,

Adrenal suppression in children on inhaled corticosteroids (ICS) is an area of emerging interest in paediatrics. However which children are at particular risk is unknown and how much of a part steroid dose, age and gender play is yet to be discovered. A large study looking to answer these questions is underway at Sheffield Children's Hospital and as part of this we are trying to gather related data.

One of our areas of interest is the children on high dose ICS managed in primary care. To this end we are conducting a survey of Sheffield GPs examining their awareness of adrenal suppression, prescribing practices and referral patterns. We would be most grateful if you could fill in this very short **anonymous** questionnaire regarding your own clinical practice when managing these children. It should take no longer than a few minutes. Additionally we hope you are happy for your Practice Manager to supply information regarding the number of asthmatic children in your practice and the proportion prescribed high-dose inhaled corticosteroids. We will endeavor to send you a copy of the survey results when it is completed.

Please return the questionnaire to us at your earliest convenience in the stamped addressed envelope provided.

If you have any questions about this work or require further information please do not hesitate to contact me on the following email. (Dr Razia Petkar: raziapetkar@gmail.com)

Many thanks in advance for your help,
Yours Faithfully

Dr Razia Petkar – Paediatric StR 4, Sheffield Children’s Hospital

Dr Charlotte Elder – Clinical Research Fellow in Paediatrics, University of Sheffield

Dr Neil Wright – Consultant Paediatric Endocrinologist, Sheffield Children’s Hospital

High dose inhaled corticosteroids in asthmatic children managed in primary care:

Thank you for taking the time to fill out this anonymous questionnaire. It should take no more than a few minutes to complete.

1) Are you a (please tick most appropriate answer using boxes provided)

GP Partner	π
GP Other	π
Asthma Nurse	π
Nurse (other)	π

2) Do you have a designated asthma lead at your practice?

Yes	π
No	π
Other (please specify)	

.....

3) Is there a policy in your practice for referring asthmatic children to secondary care?

Yes	π
No	π

If yes, please briefly detail the policy:

.....

.....

.....

4) If referring to secondary care, would you tend to refer to a:

General Paediatrician?	π
Respiratory Paediatrician?	π
It depends:	π (please

specify).....

.....

.....

.....

5) If referring to secondary care, what is most likely to prompt the referral?
(Please rank your answers from 1= most likely reason to refer, 2 = next most likely reason and rank all those that apply to your referral practice)

Increasing dose of inhaled steroids	Rank No
.....	
Poor compliance	Rank No
.....	
Persistent poor control	Rank No
.....	
Patient requiring multiple treatments	Rank No
.....	
Concern re medication side effects	Rank No
.....	
Young age of patient	Rank No
.....	
Multiple admissions	Rank No
.....	
Parental pressure to refer	Rank No
.....	
Other reasons: (Please specify)	
.....	
.....	
.....	
.....	

6) Are you aware of the association between high dose inhaled corticosteroids and adrenal suppression?

Yes	π
No	π

7) How often do you monitor height of children on high dose inhaled steroids?

Annually	π
Opportunistically	π
Never	π
Other (please specify)	
.....	
.....	
.....	
.....	

8) What dosage guidance do you use, if any, when prescribing inhaled corticosteroids to children?

BNF for children	π	
Committee on safety of medicines/DH		π
NHS National Prescribing Centre	π	
British Thoracic Society/SIGN		π
None of these	π	
Other (please specify)		
.....		

9) What is the maximum dose of Budesonide you would prescribe a patient of 6 years of age?

- 200 micrograms b.d π
- 400 micrograms b.d π
- 800 micrograms b.d π
- Other (please specify).....

10) What is the maximum dose of Beclomethasone dipropionate you would prescribe a patient of <2 years of age?

- 100 micrograms b.d π
- 200 micrograms b.d π
- 400 micrograms b.d π
- Other (please specify)

11) What is the maximum dose of Fluticasone propionate you would prescribe a patient aged 5 years?

- 100 micrograms b.d π
- 200 micrograms b.d π
- 400 micrograms b.d π
- Other (please specify)

To,
The Practice Manager

A group of paediatricians at Sheffield Children's Hospital are studying children who are prescribed steroid inhalers as part of their asthma treatment. There is increasing concern that some of these children may not be able to mount an adequate stress response due to the steroid medication in their inhalers (adrenal suppression). As part of a large program of work in this area the endocrinology department at SCH are conducting a survey of local GPs to find out more about their awareness of this subject, their prescribing practices and referral patterns. We would be most grateful if you could spare the time to inform us of the following things:

- i) The total number of children with a diagnosis of asthma at your surgery (0-15 years i.e. not anyone after their 16th birthday)
- ii) The numbers prescribed steroid inhalers stratified into the age group categories given in the table below.

We have also enclosed an anonymous questionnaire: one for a GP (whom ever you feel is most appropriate) and one for your practice nurse/asthma nurse (whom ever has the greatest role in your asthma service). We would be most grateful if you could distribute them, with the stamped addressed envelopes provided, and ask that they are returned to us. These questionnaires will only take your staff a few minutes to complete.

If you have any questions about this work please do not hesitate to contact us on the following email.

(Dr Razia Petkar: raziapetkar@gmail.com)

Many thanks for your help,

Yours faithfully,

Dr Razia Petkar – Paediatric StR 4, Sheffield Children's Hospital

Dr Charlotte Elder – Clinical Research Fellow in Paediatrics, University of Sheffield

Dr Neil Wright – Consultant Paediatric Endocrinologist, Sheffield Children's Hosp

	< 5 years of age	5-11 years of age	12-16 years of age
Total number of children with asthma registered at this GP practice			
Beclomethasone dipropionate Children on ≥ 400 mcg twice daily (≥ 800 mcg total daily dose)			

Table A (to be filled in by practice manager)

Fluticasone propionate Children on ≥ 200 mcg twice daily (≥ 400 mcg total daily dose)			
Budesonide Children on ≥ 400 mcg twice daily (≥ 800 mcg total daily dose)			
Mometasone furoate Children on > 800 mcg daily			

MANY THANKS FOR YOUR TIME AND EFFORT
Please return in the envelope provided or email to
raziapetkar@gmail.com

Emergency Standard Operating Procedure

NeSST STUDY

EMERGENCY Standard Operating

Procedure for:

SEVERE ALLERGIC REACTION

ANAPHYLAXIS

AND CARDIAC ARREST

IN ADULTS AND CHILDREN

Guidelines as per Resuscitation Council (UK)

2008

BASIC RULES:

- Read and familiarise yourself with this guidance
- Always be prepared
- Do not administer Synacthen until you:
 - Have completed the accompanying resuscitation form
 - Know they do not have a history of severe allergic reaction/anaphylaxis/previous allergy to Synacthen
 - Know how to access and use the resuscitation equipment
- If your subject becomes unwell follow this guidance
- If in doubt **CALL FOR HELP:**

If CHILD:

- Hospital Resuscitation Team call: Dial 2222 – stating “Paediatric resuscitation team to Clinical Research Facility, via surgical ward 3”

If ADULT:

- Hospital Resuscitation Team call: Dial 2222 – stating “Adult cardiac arrest, paediatric resuscitation team to Clinical Research Facility, via surgical 3”.
- If you then ring back on “0” switchboard will additionally connect you to 999.

This guideline should be left in the room with the subject at all times

Volunteer end of study questionnaires (NeSST and NeSST2 studies)

END OF NeSST STUDY QUESTIONNAIRE

Thanks again for taking part in the NeSST Study, we really appreciate it. As a last request we'd be grateful if you could spend a few minutes filling in this anonymous questionnaire about how you have found the study.

1. How did you find having the nasal spray administered? (please tick most appropriate answer)

- Very easy π
- Easy π
- OK π
- A little tricky π
- Difficult π
- Very difficult π

Comments:

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2. How did you find receiving the nasal Synacthen? (please tick most appropriate answer)

- No problem π
- Slightly unpleasant π

Unpleasant

Very unpleasant

Comments:.....

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3. Did you experience any unpleasant side effects from the nasal Synacthen?

(Please tick ALL that apply)

Stinging Soreness

Irritation Itching

Sneezing Nasty taste in mouth

Other (please state what).....

.....

.....

4. Overall how did you find the nasal Synacthen compared with the intravenous Synacthen (please tick the box of the most appropriate answer):

Much better

Better

Much the same π

Worse π

Much worse π

Please explain why:

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5. Do you have any general comments or suggestions about the conduct of the NeSST Study (e.g. recruitment, information leaflets, visits etc)

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Many thanks again – NeSST Study Team

END OF NeSST2 STUDY QUESTIONNAIRE

Thanks again for taking part in the NeSST2 Study, we really appreciate it. As a last request we'd be grateful if you could spend a few minutes filling in this anonymous questionnaire about how you have found the study.

1. How did you find having the nasal spray administered? (please tick most appropriate answer)

Very easy	π
Easy	π
OK	π
A little tricky	π
Difficult	π
Very difficult	π

Comments:

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2. How did you find receiving the nasal Synacthen? (please tick most appropriate answer)

No problem	π
Slightly unpleasant	π
Unpleasant	π

Very unpleasant π

Comments:.....
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3. Did you experience any unpleasant side effects from the nasal Synacthen?
(Please tick ALL that apply)

<input type="checkbox"/>	Stinging	π	<input type="checkbox"/>	Soreness
<input type="checkbox"/>			<input type="checkbox"/>	
<input type="checkbox"/>	Irritation	π	<input type="checkbox"/>	Itching
<input type="checkbox"/>			<input type="checkbox"/>	
<input type="checkbox"/>	Sneezing	π	<input type="checkbox"/>	Nasty taste in mouth
<input type="checkbox"/>			<input type="checkbox"/>	

Other (please state what).....
.....
.....

4. Overall how did you find the nasal Synacthen compared with the intravenous Synacthen (please tick the box of the most appropriate answer):

<input type="checkbox"/>	Much better	π
<input type="checkbox"/>	Better	π
<input type="checkbox"/>	Much the same	π
<input type="checkbox"/>	Worse	π
<input type="checkbox"/>	Much worse	π

Please explain why:

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5. Do you have any general comments or suggestions about the conduct of the NeSST2 Study (e.g. recruitment, information leaflets, visits etc)

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If you are willing for your comments to be published anonymously please sign and date below:

Print name:

Date:

Signature:

Many thanks again – NeSST2 Study Team