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***FREE CORTISOL IN HEALTHY INDIVIDUALS - COMBINING  
MICRODIALYSIS AND A NOVEL PORTABLE COLLECTION DEVICE FOR  
CONTINUOUS AMBULATORY SAMPLING***

**Dr Ragini Chandrashekhar Bhake**

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of PhD in the Faculty of Medicine and Dentistry, School of Clinical Sciences.

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## ***ABSTRACT***

Optimal concentration of glucocorticoids, principally cortisol is key to regulating vital physiological functions. Two proteins bind majority i.e. 94 % or more of cortisol in general circulation-corticosteroid-binding globulin and albumin. The biological rhythm of total cortisol (bound and unbound) in man is well established whereas much less is known about that of biologically active free cortisol. Microdialysis is a well-established technique that enables the measurement of the active free component of cortisol. Repeated blood sampling, although routine is best carried out at a clinical facility for safety. As a result, little is known about dynamic functioning of hormonal systems with ultradian (shorter than 24hour) rhythms requiring prolonged frequent sampling in the most prevalent, and perhaps more relevant, physiological setting of an individual's own environment.

In this thesis, I have described the validation of the technique of microdialysis for measuring free cortisol in healthy individuals followed by ambulatory sample collection using a portable automated microdialysate collector that was developed as a part of this project.

The profiles of free cortisol in two body compartments (subcutaneous tissue and intravenous) in response to Synacthen stimulation and dexamethasone suppression show good correlation with total cortisol, with no significant delay between the compartments. 24 hour measurements in healthy individuals confirmed the characteristic circadian variation in subcutaneous tissue similar to that of serum total cortisol. Furthermore, within an individual there was remarkable consistency of the circadian rhythm of subcutaneous free cortisol over 3 successive days even with dissimilar routines and activities during the three days. This is the first study whereby measurement of free cortisol continuously, including during sleep, up to 3 consecutive days in healthy people outside of a research facility setting, free to carry out their routine activities without major limitations has been reported.

## DEDICATION

For the unconditional love, support and faith of my parents and brother, and for my late grandfather who has been a silent inspiration.

They put in all the kosher ingredients and followed the instructions to the tee – not every recipe produces a ‘showstopper’ though!

## *ACKNOWLEDGEMENTS*

An aspiring young girl took the long road to the UK, full of dreams of a lucrative life. Immersing in research made her riches multiply, though not in monetary terms! Even her mentor, who guided the journey having (?mistakenly) left his door open 15 years ago (still happily fielding a multitude of queries), had been economical with this fact!

The journey of this PhD started with Colin Dayan wandering into the secretaries' offices late one evening and asking, "Have you ever thought about doing some research?"

Through this PhD, a collateral gain was acquiring the secret to eternal youthfulness, which I have learnt from my supervisor. Stafford has imparted knowledge and life-skills beyond the subject of this research, by just being himself! He has been a lighthouse and at times a rock through this process, which has seen a few storms. Astrid, my co-supervisor, has exemplified paying attention to detail.

My study participants generously gave a share of themselves – time, humour and useful tips and tricks. Their enthusiasm made the 36-hour shifts feel like a breeze.

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What lessons have I learnt through this journey? Its that no matter how far you run, a citizen and its birth country's economies could be like conjoined twins; that there is a strong correlation between research and wealth (positive for intellectual, negative for financial); that working beyond your 'banded' hours is good – never mind 'good time management' and, the most important one - that the values your parents endeavour to inculcate in you namely, hard work, perseverance, discipline and self-belief are key! I will be eternally grateful to them both!

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE:.....





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# CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

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The hypothalamic-pituitary-adrenal (**HPA**) axis is an important neuroendocrine system responsible for maintaining homeostasis. Glucocorticoids, principally cortisol in man and corticosterone in rodents, are the predominant product of the HPA axis that mediate biological effects attributed to this system. Optimal hormone concentration is key to regulating vital physiological functions in man - total lack of the hormone is incompatible with survival and excess levels result in morbid consequences. Of the circulating cortisol ('total' hormone) measured routinely in blood, the majority is bound to carrier proteins and only a small fraction is unbound or 'free'. It is the latter that is able to pass out of the circulation and bind glucocorticoid receptors in the cells within target organs to effect a hormone response.

The biological rhythm of total cortisol in man has been studied extensively and is well established whereas much less is known about the rhythm for free cortisol. Microdialysis is a well-established technique that enables the measurement of small (or larger, if desired) molecules in different compartments of the body including extracellular fluid. Importantly, it offers three significant advantages. First, it enables the measurement of the active free component of cortisol that is not bound to plasma or other proteins. It is also a minimally invasive technique, and relatively free of risk. Repeated blood sampling, the mainstay of human (HPA axis) hormone testing for decades, in contrast, although routine is best carried out at a clinical facility for safety. As a result, little is known about dynamic functioning of hormonal systems with ultradian (shorter than 24hour) rhythms requiring prolonged frequent sampling in the most prevalent, and perhaps more relevant, physiological setting of an individual's own environment. Finally, by virtue of its safety microdialysis presents itself as a potential method for ambulatory sampling.

In this thesis, I will describe the validation of the technique of microdialysis in measuring free cortisol in healthy individuals followed by ambulatory sample collection using a portable automated microdialysate collector that was developed as a part of this project.

## **1.1 HYPOTHALAMIC-PITUITARY-ADRENAL AXIS**

### **1.1.1 HPA AXIS ANATOMY**

The pituitary gland (or Hypophysis) situated in the saddle shaped 'sella turcica' of the sphenoid bone, lies inferiorly and in close proximity to the hypothalamus (1), an anatomical association of physiological significance. It has two anatomically and physiologically distinct parts – the adenohypophysis and neurohypophysis. The adenohypophysis is made up of three lobes - dominant anterior lobe (pars distalis), vestigial intermediate (pars intermedia) lobe and pars tuberalis. The neurohypophysis is composed of pars nervosa (posterior lobe), infundibular stalk, and median eminence. The infundibular stalk, surrounded by the pars tuberalis, forms the hypophyseal stalk which is connected with the median eminence of the hypothalamus and is rich in nerve endings. The hypophyseal-portal system is a complex vasculature, consisting of arterial tributaries around the median eminence that trail down the ventral hypophyseal stalk to merge with the rich fenestrated venous plexus surrounding the anterior pituitary (2). It serves as a conduit for hypothalamic releasing-factors that access the anterior pituitary. The hypothalamus is formed of many different groups of nuclei, but of particular importance to the HPA axis are the suprachiasmatic nucleus (**SCN**), which controls circadian variations, and the paraventricular nuclei, which produce corticotropin releasing-hormone (**CRH**) and Arginine Vasopressin (**AVP**) (1). Axons of the paraventricular nucleus project on to the median eminence (3). Thus, the hypothalamus and the pituitary form an integrated anatomical and physiological unit, and experimental or pathological separation of the two results in reduced hormonal output, among others of adrenocorticotrophic hormone (**ACTH**) (2), from the pituitary.

The two adrenal glands although at anatomically distinct sites, as suggested by their synonymous term supra-renal glands, respond synchronously with release of corticosteroids from the zona fasciculata of the adrenal cortex (4), upon receiving physiological signal from the pituitary gland in the form of ACTH. The steroid output from the other two zones of the adrenal cortex namely, zona glomerulosa and zona reticularis, comprises the mineralocorticoid aldosterone and adrenal androgens (dehydroepiandrosterone, dehydroepiandrosterone sulphate and androstenedione) respectively (5).



### **1.1.2 HPA AXIS PHYSIOLOGY**

Hypothalamic CRH via specific receptors on corticotrophs of the anterior pituitary (6,7), results in increased ACTH release (8,9) with its precursor, pro-opiomelanocortin (3). AVP like CRH is produced by the hypothalamic paraventricular nucleus cells, and is also a stimulus for ACTH secretion under certain conditions (10), although it acts on the corticotrophs via different set of receptors (11).

ACTH is trophic i.e. it maintains the size and structure in addition to regulating the function of the adrenal gland (12). Classically, ACTH stimulates the release of corticosteroids. Adrenal responsiveness to ACTH has been shown to be regulated to an extent by splanchnic innervation, which exerts an inhibitory effect during the circadian nadir phase in an unstressed animal although this effect can be overcome by certain stressful situations. Overall, adrenocortical secretory function may result from a complex interplay of influences other than or in addition to ACTH such as catecholamines (particularly epinephrine and norepinephrine), certain neuropeptides, cytokines, growth factors, and vascular endothelial molecules (4).

In summary, CRH (and AVP) acts on the pituitary gland to produce ACTH, which in turn results in synthesis and release of cortisol in man and corticosterone in rodents, in a sequential 'feed-forward' manner. Physiological levels of the secreted glucocorticoids exert 'feed-back' effect at the hypothalamus and pituitary, to regulate levels of CRH and ACTH respectively (13,14), and also at higher brain regions such as the hippocampus.

#### **1.1.2.1 RHYTHM OF THE HPA AXIS**

Maya Angelou, an accredited author famously said, "*Everything has rhythm.*" Indeed all living organisms, including relatively simple single celled organisms such as yeast (15) as well as highly complex mammals, exhibit temporal dependent behaviour of varying degrees. Two distinct types of rhythm operate to regulate HPA activity. The term circadian rhythm applies to a periodicity of approximately 24 hours i.e. a day (16). Although it is used synonymously with the term diurnal, strictly the latter encompasses day-time events in contrast with nocturnal, applicable to those occurring at night-time (17). Rhythms of duration shorter than 24 hours are ascribed the term ultradian, although it has been suggested but not widely accepted that the term be reserved for the shorter rhythms that are regular (18). Human beings as well as rodents exhibit both circadian and ultradian

rhythms. These rhythms also operate at the level of other hormonal axes e.g. insulin (19), but the pituitary-adrenal axis had been historically considered a representative model for these rhythms (20) and thus been subject to extensive exploration by the scientific community as discussed in this subsection. I have used the terminologies in this thesis as detailed above i.e. circadian for 24-hour and ultradian for shorter frequency rhythms.

Difference in urine steroid excretion between day and night was the first demonstration in 1943 of circadian adrenocortical function, although similar variations in other physiological functions in man had been known about for several decades prior to that (17). Urine samples collected over several hours reflect cumulative hormone secretion (having undergone metabolic process) during that period and values thus obtained are at best an approximation. Levels of 17-hydroxycorticosteroids in plasma (21) and of ketogenic steroids in urine (21,22) collected at more frequent intervals through 24 hours, have substantiated this pattern of corticosteroid secretion. This in turn is attributable to a similar circadian variation of ACTH (23).

Attempts to investigate the change in concentration of these hormones have led to the discovery of a distinct circadian pattern that is consistent across members of an individual species (24). Circadian rhythm appears to be intricately linked to the sleep-wake/activity cycle (25). In man, under normal circumstances, this rise begins during the latter half of sleep continuing into the early awake phase, sometimes until about noon, gradually declining thereafter to low levels during the hours surrounding sleep onset (24,26). In nocturnal species e.g. rodents, although the periods of activity (and inactivity) differ from man, the pituitary-adrenal rhythmicity is coordinated accordingly (27).

It is noteworthy that the circadian pattern of hormone levels is not smooth, but consists of pronounced secretory bursts (28). This ultradian rhythm is apparent on regular frequent sampling, usually with the sampling interval being no longer than 2-3 times the half-life of the hormone under investigation (29). For the ultradian pulsatile activity through the day, in most if not all individuals studied, historically three time periods are significant. The most striking change occurs during late sleep and early wake period when levels rise several-fold compared to very low levels in the preceding hours. The period surrounding the onset of sleep is characteristic for almost no or minimal ultradian activity of the axis with usually the lowest levels of day found at this time (30). Most individuals also show a post-meal surge at midday (31,32). Apart from these three consistent secretory components, the

remaining time-domains of a day reveal a relatively less reproducible pattern between individuals of some peaks late afternoon-early evening (33).

ACTH also displays circadian (23,25) and ultradian rhythmicity in man synchronous with cortisol, the latter lagging ACTH by minutes (24)(34,35). Similar ACTH and cortisol/corticosterone patterns and associations are well recognised in some primates (36) whose sleep wake cycle is more akin to man, as well as in rodents (37) albeit tailored to their natural nocturnal activity habit.

Integration of short periodic fluctuations into the circadian rhythm is complex. Change in cortisol levels throughout the day in man is primarily a dynamic interplay of amplitude variation of cortisol, and only about 30% is accounted for by change in frequency of these pulses (38). The same appears to be true for ACTH (35), a feature common to some pituitary hormones but not all (39). The average interval between successive secretory episodes is less than 80 minutes for cortisol and less than 40 min for ACTH, corresponding to around  $19 \pm 0.82$  and  $40 \pm 1.5$  pulses respectively during 24 hours (38,40). During these inter-pulse intervals, relatively low levels of hormone are achieved which could be potentially important to prevent receptor desensitisation (41). Corticosterone in rodents is also secreted in a pulsatile manner from the adrenal glands with a periodicity of 58-60 minutes (18). It is important however to note that the 'pulses' of hormone concentration measured in plasma are an amalgamation of secretory event and metabolic clearance (specific to a given individual) and not a simple indication of the former alone (38). Another noteworthy feature is that total secretory activity appears to occupy 6 (28) to 8 (38,40) hours with the adrenals remaining inactive for 75% of a day, this being explained as an economically efficient method by some (38). Perhaps the most important physiological feature of this dynamic pulsatile pattern is that it is not a mere phenomenon observed in blood levels, but that the same pattern is also translated into similar rhythms in tissue levels of glucocorticoid hormone (42,43) and the transcriptional response of target genes of corticosteroids (41).

SCN situated in the hypothalamus is the endogenous master pacemaker of circadian rhythm (44), although so called 'peripheral clocks' are also known to exist in various tissues including the adrenal glands (45). Circadian variation may be under some genetic influence (46). The SCN integrates various afferent stimuli to coordinate HPA activity in a circadian manner and in its absence circadian rhythm is lost, but not ultradian variability (47).

Ultradian activity seems integral to the pituitary-adrenal axis and critically dependent on the level of CRH drive, which within a certain range translates into pulsatile ACTH and corticosterone but outside of the range results in constant levels (37).

Abnormalities of cortisol and ACTH rhythm have been of great interest to clinicians and researchers alike. Measurements in older individuals aged >50 (48) and >80 (49), has confirmed persistence of the previously described rhythm, its features differ in some aspects. First, amplitude of its circadian variation appeared to be reduced and second, the morning elevation tended to occur earlier. Travel results in resynchronisation of the HPA axis in keeping with the new time-zone following a period of lag, which is greater after westward travel than eastward (50). Circadian rhythm may (51) or may not (52) be maintained in Addison's disease (cortisol deficiency state) as well as in Cushing's disease or syndrome (cortisol excess due to pituitary ACTH excess or independent of it, respectively) (53–55). Physiological deviation from normal rhythm, usually temporarily, is seen in individuals recovering from major surgery (56) but this relationship appears more complex (57). Other reported associations of abnormalities of the HPA axis rhythm include affective disorders (58) and diseases of the cardiovascular system (59).

In summary, the HPA axis has characteristic circadian and ultradian rhythms that appear to be intricately linked to the sleep-wake cycle. Apart from diseases originating in the HPA axis, abnormalities of circadian rhythm have associations with systemic diseases although the direction of causality remains to be ascertained.

### ***1.1.3 GLUCOCORTICOID SYNTHESIS AND METABOLISM***

Glucocorticoids, cortisol and corticosterone, being lipophilic in nature are not stored but synthesised de novo and released in the general circulation (60). Steroidogenesis is initiated in adrenocortical cells upon binding of ACTH to their surface receptor melanocortin-2 receptor (45) that trigger a cascade of events that eventually result in side-chain cleavage of cholesterol, the precursor of all adrenal steroids (12,60). A series of coordinated enzymatic reactions ultimately result in glucocorticoid production from the zona fasciculata (61).

Among the gamut of steroids synthesised by the adrenal gland, glucocorticoids are quantitatively the major physiological products with approximate total production of 5.7

mg/m<sup>2</sup>/day (62). The predominant active glucocorticoid in human is cortisol (63) (also known as hydrocortisone or compound F), whereas in rodents it is corticosterone (also known as compound B) (64) - the first steroid found to have typical adrenocortical activity (60).

The recognition towards the end of the last century that local glucocorticoid concentration, and its consequent action, was regulated in a tissue-specific manner has been a significant landmark. A revolutionary implication of this realisation is that levels in general circulation considered hitherto to reflect the 'functional state' in every individual tissue, may not entirely do so. Prior to this, the known metabolic fate of circulating glucocorticoids within various tissues was inactivation by two sets of enzymes located predominantly in liver and to some extent in kidneys - A-ring reductases generating 5 $\alpha$ - & 5 $\beta$ - tetrahydrocortisol and 5 $\beta$ - tetrahydrocortisone; and cytochrome p-450 microsomal enzymes generating polar steroids, cortols and cortolones (65). Ultimately, small amounts of hormone get eliminated unaltered in urine, which due to the nature of filtration at renal glomeruli tend to be hormone not bound to protein i.e. free hormone. Evolving knowledge about the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase (**11 $\beta$ -HSD**) (66), of which two iso-enzymes have so far been characterised – types 1 and 2 (67), has rekindled interest in the metabolic course of cortisol.

In view of heterogeneous expression of activating and inactivating enzymes in tissues, an important question about the nature of cortisol rhythm at the level of individual tissues remains to be answered adequately in man. There are challenges to elucidating it, particularly in vital organs - the evidence to date, especially in rodents is discussed later in this chapter.

#### ***1.1.4 GLUCOCORTICOID ACTION***

The term Glucocorticoids relates to their effects on carbohydrate metabolism (68), and it includes a repertoire of adrenal cortical steroids including cortisol and corticosterone. In general, the metabolic effect of these classic compounds is one of increasing ambient glucose levels in the serum not only by reducing uptake in muscle and fat but also by increasing their production from carbohydrate and non-carbohydrate sources. Thus, protein and fat are broken down and diverted towards glucose production and where possible used

as energy source in favour of glucose. The net result is hyperglycaemia (which can normally be antagonised by Insulin), muscle wasting and lipolysis. Glucocorticoids serve an important physiological function, of impairing certain immunologic responses and inflammatory reactions, which form the cornerstone of some cancer and anti-inflammatory therapy. However, major disadvantages of the latter effects are susceptibility to infection and poor wound healing. They also have detrimental effects on bone formation and blood pressure, particularly when in found in excess (69). The effect on blood pressure, and salt and water retention, are a manifestation of the mineralocorticoid action of glucocorticoids, which is weak at physiological levels (68) yet may be potentiated at high concentrations. Finally, corticosteroids are known to affect higher functions of the central nervous system including, cognition, mood and memory (68,70,71).

Adrenal corticosteroid effects are mediated by two mechanisms – classical genomic mechanism, that involve receptor-mediated gene transcription (transactivation and transrepression) and de novo protein synthesis usually taking place over a period of hours and, non-genomic mechanisms with rapid action occurring within seconds to minutes of binding to receptors but devoid of genomic processes (72). The essential step towards mediation of these effects is corticosteroid binding to widely expressed low-affinity glucocorticoid receptors (**GR**), and high-affinity mineralocorticoid receptors (**MR**) (58,73). The former are ubiquitously expressed (69), including in the brain (73), as compared to the latter.

Glucocorticoids are involved in processes of cell differentiation and development at the foetal stage including myelin formation, surfactant formation in lungs, specific protein formation in the developing retina, pancreas and mammary tissues (68). Excess cortisol may have teratogenic effect and the foetus is protected by high expression of 11 $\beta$ -HSD type 2 in the foeto-placental unit capable of inactivation of endogenous and some synthetic corticosteroids (66).

## **1.2 FREE HORMONE**

Elegant literature from previous decades responsible for current knowledge about the rhythm of HPA axis is based on frequent blood sampling. Glucocorticoids circulate in blood bound to proteins in a high proportion, compared to that which is unbound or 'free'. The concept of 'free hormone' is integral to this thesis, a brief overview of which is

presented here. The fundamental concept linked to this concept is that biological activity of a hormone is determined by its free or unbound fraction promulgating a view that plasma levels may be an index of adrenocortical activity (74).

### **1.2.1 FREE HORMONE HYPOTHESIS**

Recant and Riggs' suggestion (75), formalised as the free hormone hypothesis in 1957 by Robbins and Rall (76) originally related to thyroid hormone, was gradually accepted in principle to hold true for other hormones including corticosteroids. Bound cortisol administered to adrenalectomised mice was shown to be biologically inactive, in contrast to unbound hormone (77). Observation of 'normal' free hormone levels in individuals with binding protein abnormalities and *in vivo* kinetics studies have further supported this theory (78).

Corticosteroids secreted by the adrenal gland are not bound to protein (Murray 1967). Two proteins bind majority i.e. 94 % or more of cortisol in general circulation (79). Corticosteroid-binding globulin (80) (**CBG**) or Transcortin (74) and albumin bind cortisol in a ratio of 1:1 (81). Both these proteins are capable of binding several other hormones (82,83) but at physiological concentrations of hormones CBG is very cortisol specific, unlike albumin. The precise purpose of protein-hormone association apart from facilitating transport in general circulation of hydrophobic cortisol (78) is unclear. It may have more than one function purported amongst which are, its role as a buffer mitigating tissue effects of extreme fluctuations in circulating hormone levels thus protecting tissues from exposure to high hormone levels; targeted hormone delivery to tissues; reducing elimination either by conjugation in liver for excretion or by filtration of the free hormone at the glomeruli (84).

CBG, an alpha-globulin (74,80) is a serpin of molecular weight 52 Kilo Daltons (**kDa**) (81) and is synthesised predominantly by the liver (85), while the physiological significance of small amounts of extra-hepatic synthesis is unknown (86). Physicochemically, CBG has high affinity but low capacity for cortisol (74,80) and ranges between 23 and 45 milligrams (**mg**) /100 millilitres (**ml**) in circulation (81). Within the normal range of CBG, the proportion of free hormone is determined by ambient temperature. Above the normal range there is a greater increase in free fraction (83,87,88). A rise in temperature from 35 degrees Celsius (**°C**) to 42°C reduces the affinity of CBG for cortisol sixteen fold *in vitro* which can have remarkable implications *in vivo* (83). In addition, the proteinase neutrophil elastase released

at sites of inflammation destroys the steroid-binding site of CBG irreversibly (89) resulting in potentially increased local corticosteroid levels. Oestrogen excess, physiological e.g. in pregnancy or pharmacological e.g. contraceptive administration, increase CBG levels possibly in a dose-dependent manner above a certain threshold (87), although free cortisol appears to be unaffected (81). Other conditions known to affect CBG levels without conclusive evidence of impact on free fraction include cirrhosis, nephrotic syndrome and thyrotoxicosis (87). The steroid-binding site, one per CBG molecule, is capable of binding substances other than cortisol namely, progesterone, cortisone, corticosterone, deoxycorticosterone and, 11-deoxycortisol (90), but ordinarily these substances are present in small amounts (except progesterone in late pregnancy) and unlikely to affect cortisol-binding in human circulation (83). Finally, reduction in steroid binding affinity or complete absence of CBG may result from rare inherited abnormalities in the CBG gene (89). In animal studies, the profile of free corticosterone response to stress appears to be modulated by stressor-specific changes in CBG levels (42,91). It is of interest to note that Prednisolone is the only synthetic glucocorticoid that binds CBG (affinity 50% that of cortisol) significantly, while the remaining steroids of this class barely do so (87).

Another piece in the free hormone jigsaw is the 69 kDa protein albumin (82), which has high capacity but lower affinity for cortisol (74,80). Despite the several fold higher concentration of albumin in serum of 5 grams (**g**) /100 ml (81) compared to CBG, albumin-bound cortisol accounts for only 14% of the total circulating cortisol whereas 80-90% is CBG-bound cortisol (92). The role of albumin assumes greater significance when CBG is indisposed either quantitatively or qualitatively (92), and individual thresholds have been suggested for low, intermediate and high levels of CBG, beyond which the relative proportion of bound and unbound cortisol changes significantly (83). Albumin binds to cortisol with about 6000 times less affinity than CBG (74) and cortisol bound to it is more readily available compared to that bound to CBG. Of note, the binding of albumin to cortisol is independent of temperature but acidotic pH reduces the affinity of albumin binding site for cortisol (83). Situations when albumin itself is reduced may have more significant impact on the changes in free cortisol fraction than in normoalbuminaemia (88).

Biological effects of corticosteroids according to the free hormone hypothesis are determined by free rather than protein-bound hormone. However, dynamics of cortisol



binding to circulating binding proteins are no less important as demonstrated above and can have significant impact on free hormone available for cellular action. (**Figure 1.1**).

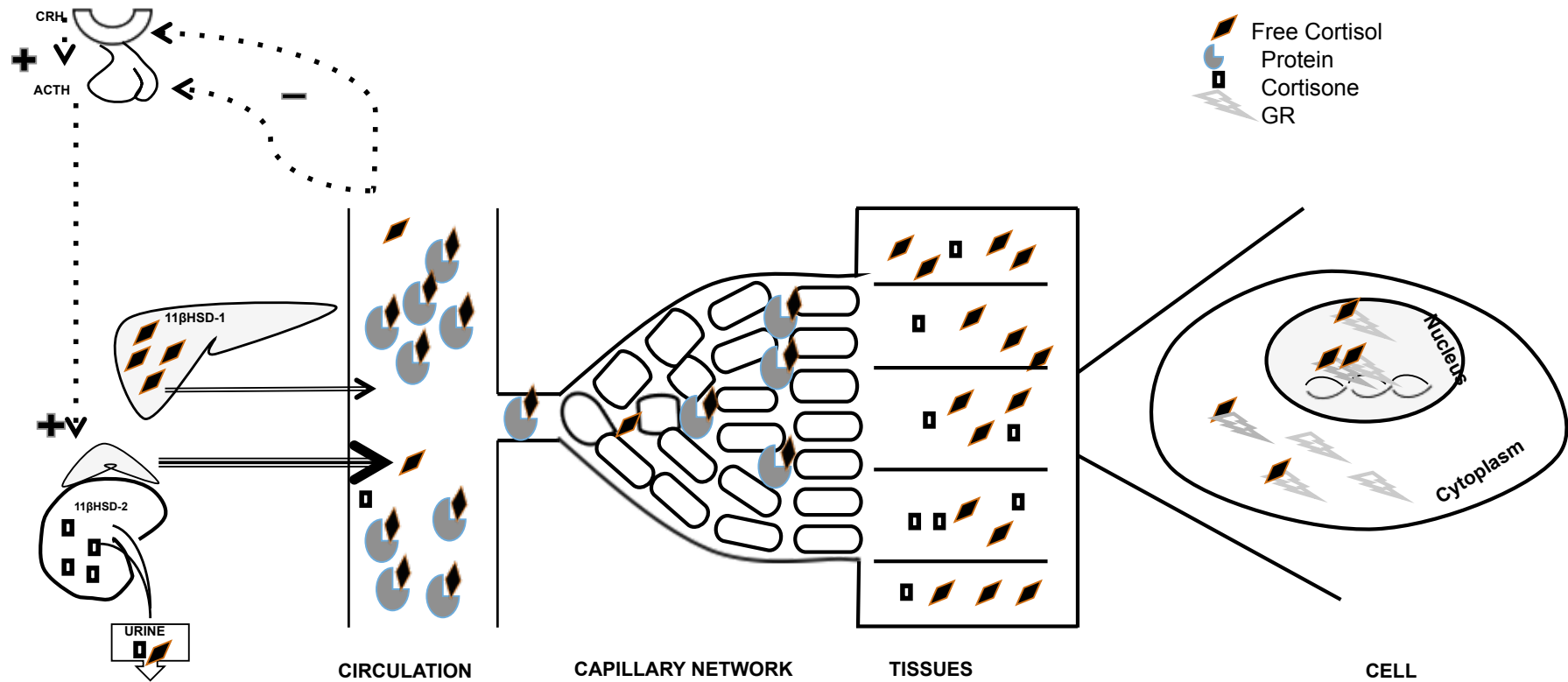


Figure 1.1 HYPOTHALAMIC-PITUITARY-ADRENAL-TISSUE AXIS.

CRH released from the hypothalamus, stimulates ACTH release from the anterior pituitary which results in cortisol production from the adrenal glands. Free cortisol thus released in the vascular system circulates as the protein-bound fraction ( $\approx 95\%$ ) and the free fraction ( $\approx 5\%$ ). Free hormone enters tissues, then into each cell where it binds its receptors (only GR represented in this figure). Hormone-receptor complex translocates from the cytoplasm to the nucleus, attaches to the glucocorticoid response element on the DNA triggering a cascade of events leading to hormone actions.

### **1.2.2 FREE HORMONE TRANSPORT HYPOTHESIS**

Although there is some evidence for membrane associated glucocorticoid receptors, the classic effects of corticosteroids depend on entry into cells, in particular its nucleus, in order for them to bind to intracellular receptors, which in turn bind to cellular DNA. The relatively large size of CBG only allows the lipophilic free cortisol to cross cell membranes out of capillaries and into cells. This theory is also known as the 'free hormone transport hypothesis' (78). Demonstration of this phenomenon *in vivo* is not without difficulties but observed tissue uptake of hormone has been commensurate with spontaneous dissociation of protein-hormone complex and has been cited in its support.

Since its first isolation to location of its human gene, understanding of the function of CBG has evolved to be more complex as perhaps could have been predicted. Its function as a transport protein is now considered by some to be a more active role, modifying local availability of hormone in certain target tissues perhaps specific to context (93). This free hormone transport theory has been challenged by the presence of CBG on the cell membrane (94) and within certain cell types (95), however lack of further evidence has weakened the proposal that CBG has intrinsic biological activity.

### **1.2.3 FREE CORTISOL**

Urine and saliva, uncontaminated with blood, contain unbound cortisol (96,97). Urine analysis for free corticosteroids has been in use for over half a century (98), previously by historical assays but recently with modern qualitatively superior analytical methods e.g. tandem mass spectrometry. Cortisol is filtered at the glomeruli and its concentration in urine is affected in suboptimal renal function (99). Passive diffusion of cortisol into saliva without adverse effect of rate of flow of saliva (96,100) offered a novel non-invasive method of assessment of corticosteroid status, and its use has proliferated since the availability of modern accurate laboratory methods. Free hormone levels are measurable in various body fluids in man but have not achieved diagnostic utility.

Ascertaining the free fraction in serum has been challenging however. Indirect methods such as calculation using Coolens' method (101), or more direct but technically demanding procedures such as ultrafiltration (82) & equilibrium dialysis (79) that separate the bound and unbound fractions *in vitro* using a semi-permeable membrane, have limitations (82). Measurements of the free fraction obtained from latter two methods can improve in accuracy with modern sensitive analytical techniques e.g. ultra-high-performance liquid chromatography – tandem mass spectrometry method, and are clinically comparable (102). The calculation method requires measurement of at least total cortisol and CBG; and physical separation methods estimate the free fraction based on an equation incorporating components measured individually. More recently, a formula accounting for both intact and neutrophil-elastase cleaved CBG has been proposed (103). Ultimately, all these methods are however indirect.

Microdialysis offers a minimally invasive, technically relatively simple procedure to collect interstitial fluid samples for a prolonged period. Despite significant refinement since its use in the early 1970s (104), it remains a technique sparingly used in clinical environment. The inspiration for this thesis was derived from this potential application, and from recent demonstration of free glucocorticoid rhythms in freely behaving rodents under physiologically relevant conditions (42,43,105). The salient mechanical aspects of microdialysis as a technique therefore merit attention, a brief overview of which is outlined below.

#### **1.2.3.1 MICRODIALYSIS**

The term microdialysis originates from the 1950s when it was used to describe simultaneous dialysis and extraction of compounds from blood plasma volume of less than 1ml (106). It has been used for *in vivo* sampling of exogenous substances (e.g. drugs) or endogenous substances from the interstitial fluid of selected tissues (104). Direct local administration of drugs into tissues (104,107,108) is perhaps its most innovative use. The first published application of microdialysis in humans was for glucose measurement in the interstitial fluid (109).

It is important to note some of its distinct advantages. It allows direct sampling from interstitial fluid of most body compartments, including the vascular compartment (43,110), which is relatively easily accessible compared to the brain (42,105,111). Interstitial fluid is devoid of high molecular weight proteins and with its close proximity to cells of a tissue is a promising alternative to blood to examine tissue *milieu*, which is the ultimate aim of measuring biological substances of interest. By preventing large molecules from diffusing through its membrane, microdialysis obviates the need for complex sample preparation procedures to separate free from bound components, as well as protecting certain molecules from enzymatic degradation after collection. It is a minimally invasive technique that facilitates repeated sampling over several consecutive hours or days after a single penetration of the tissue without the need to draw any blood. Fluid loss can be a disadvantage of blood sampling over prolonged periods and is negligible with this method (106,112).

The system consists of a pump, a catheter or probe, and a sample collector. A microdialysis probe has three basic components, an inlet tube, semi-permeable membrane of variable length where exchange takes place and, an outlet tube. The probe design is of two types, concentric and linear (112). A concentric probe consists of a shaft with the inlet and outlet tubes emerging from one end with the membrane at the other end. The inner diameter of inlet tube ranges from 0.15-0.3mm (Figure 1.2)

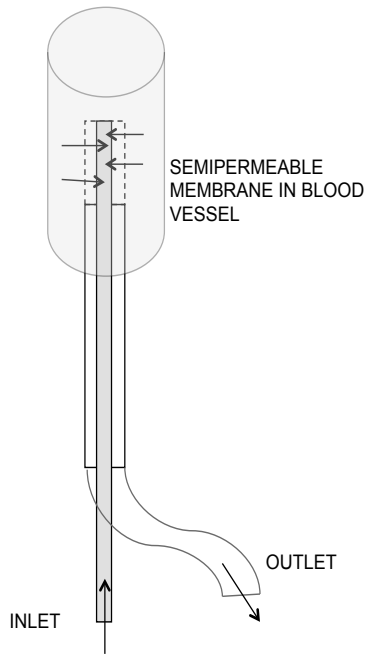


Figure 1.2. CONCENTRIC CATHETER WITH TIP IN A BLOOD VESSEL.

The shaft of the catheter contains an inlet tube within the outlet tube with the semipermeable membrane at one end and the two tubes emerging at the opposite end.

A linear probe, as indicated by its name, has its inlet and outlet at opposite ends with the membrane located along the length of the catheter closer to its outlet (Figure 1.3).

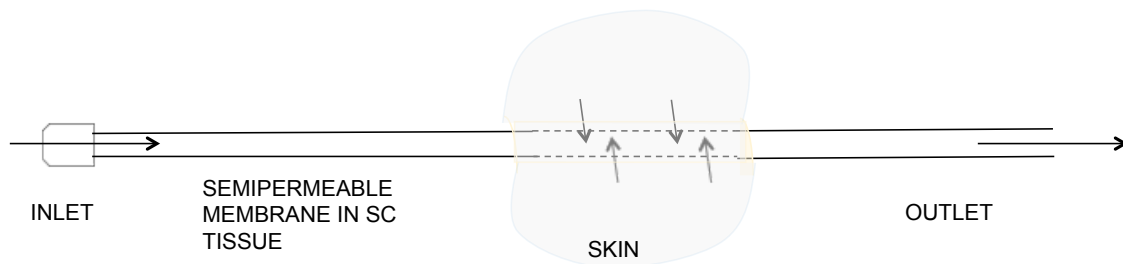


Figure 1.3. LINEAR CATHETER WITH SEMIPERMEABLE MEMBRANE UNDER THE SKIN.

The inlet and the outlet tubes emerge at opposite ends to each other with the semipermeable membrane located along the length of the catheter, closer to the tip of the outlet tube.

Clinical probes tend to be flexible and their membranes, where dialysis or exchange takes place, are usually made of polycarbonate, polyethylsulfone or cuprophan (regenerated cellulose), polyamide, or polyacrylonitrile AN69 (113). The molecular weight cut-off (in KDa) specific to each probe is the key determinant of its

use in measuring the substance of interest. It is defined as the average point where 80-90% of molecules with a nominal size will be retained by the membrane. Larger molecules including proteins are unable to pass into the dialysate (Plock 2005). In essence, it mimics passive function of a capillary blood vessel in the implanted tissue (112).

A microdialysis pump pushes perfusion fluid or perfusate, isotonic to the composition of the surrounding medium or matrix, through the catheter at a fixed rate, resulting in a constant flow of the 'dialysate' containing the analyte. 'Ultrafiltration', a technique in its own right, resulting from transport of solvent/water across the membrane (113) can cause loss of sample volume or alter the amount of substance recovered (114) must be avoided during microdialysis.

'Recovery' of a substance may be relative, i.e. percentage of the concentration in the surrounding medium or absolute, i.e. the total amount of substance recovered during a defined time period, expressed as moles/litre (112). If perfusion conditions are maintained at a steady level, *in vivo* relative recovery is constant, but absolute recovery of a substance varies with its production/release in the tissue. Relative recovery depends on the membrane length and other membrane characteristics including molecular cut-off and material, flow rate of the perfusate, speed of diffusion of analyte through the extracellular fluid, tortuosity of the extracellular matrix, and analyte characteristics. In general, a higher flow rate increases absolute recovery and lower flow rate increases relative recovery, however in practice flow rate is usually commensurate with the analytical technique's sensitivity to the volume of a sample. Recovery is dependent on diffusion and may therefore be influenced by (large) changes in temperature.

### 1.2.3.2 CURRENT LITERATURE ON FREE CORTISOL

One of the major advances in HPA physiology, made possible by microdialysis, was the elegant demonstration of circadian and ultradian rhythm of free corticosterone in the hippocampus (42,43) and the subcutaneous tissue of freely behaving rats. The rhythm of free hormone in an individual compartment was synchronous with that of plasma total corticosterone when measured simultaneously

in the same animal under resting conditions. Circadian and ultradian rhythms of free corticosterone in the circulation and that in target tissues were synchronous and both have similar characteristics. The only difference found was slightly (15-20%) lower levels of free hormone in target tissues between 15:00 and 21:00hrs. Following forced swim manoeuvre, peak free hormone level is achieved in the brain 20 minutes later compared to that of plasma total hormone, with concurrent fall and hence reduced exposure of the brain to corticosteroids. This phenomenon is not restricted to brain and is evident in the free hormone response in the intravascular compartment as well as in subcutaneous tissue (91). The acute stress-induced rise in CBG levels, as found after moderate to strong forms of stress, may be an explanation for this temporal lag.

In man, free cortisol percentage in serum measured by ultrafiltration, appears to have a similar circadian rhythm as that of total cortisol, and excursions of the former occur in close correlation to the latter during both spontaneous and exercise-induced peaks (115). The increase in percent free cortisol relative to that of total cortisol was 1.5 times greater during these peaks. Using the same method, serum free cortisol increment from baseline (morning before operation) in response to major stress (coronary artery bypass grafting) is significantly more than that in serum total cortisol (88). This difference is maintained in resolution of the levels a day after surgery in the same cohort. Free cortisol, as a percentage of total cortisol, was  $9.1 \pm 3.0\%$  at baseline and  $13.1 \pm 3.9\%$  immediately after major stress in this study. In an earlier study involving critically ill patients due to various medical and surgical conditions, free cortisol percentage was nearly three times as high as that in healthy volunteers (116). Reduced cortisol metabolism in patients in intensive care has been suggested to be a contributory factor in the elevated free cortisol levels found in these patients (117), but reduced levels of CBG in chronically ill patients (116), reduced levels of albumin (88) and dilution by infusion of intravenous fluids undoubtedly contribute as well.

Vast amounts of data reporting salivary cortisol measurements in disease states have been published and its discussion is out of the remit of this thesis. Studies with multiple measurements during a day attempting to investigate the nature of the rhythm are mentioned briefly here. Of particular note is a recent study of healthy male



participants in whom simultaneous blood and saliva samples were collected every fifteen minutes for 8 hours during the day (118). Using deconvolution analysis, a validated and widely used mathematical approach for the analysis of hormone pulsatility, a high concordance (70-84%) was observed between majority of the pulses in the two body compartments, leading to the authors' suggestion of saliva sampling being a suitable alternative to blood sampling for ultradian rhythm analysis. They however obtained shorter inter-pulse interval and a shorter half-life for both total and free cortisol in comparison to previous literature and therefore more evidence is required to support their proposition. In a meta-analysis (119) of patients with depression compared to healthy individuals a small but statistically significant difference in morning and evening saliva cortisol levels is apparent, though there was no evidence corroborating the long-held belief of an abnormal rhythm in this condition. Flatter slope of diurnal salivary cortisol was associated with coronary artery calcification in a small study (120). Among diseases originating in the HPA axis e.g. Cushing's syndrome, although late night salivary free cortisol alone has been recommended as an additional screening test (121), demonstration of lack of/abnormal diurnal rhythm was investigated (122) but not yet widely accepted. A person's timed saliva measurements can indicate the underlying rhythm to be both 'dynamic' and 'adynamic', depending upon the samples being taken along different time points during a pulse.

First reports of human studies utilising microdialysis for free cortisol estimation examined the role of  $11\beta$ -HSD in adipose tissue (70,123), and glucocorticoid production rate in obese patients. In patients treated for severe burns, a single sample of microdialysate from each of burn and non-burn sites in an individual, showed overall higher levels than in healthy controls (124). Eight hourly free cortisol measurements from the brain interstitial tissue of patients with traumatic brain injury suggest a lack of circadian rhythm in these patients (111). In a septic cohort, there was moderate correlation between subcutaneous adipose free cortisol and serum total and free cortisol (125). These studies were not focussed on studying the endogenous rhythm of free cortisol, and used samples collected at several hours intervals.

### **1.3 CONTINUOUS AMBULATORY MEASUREMENT OF HORMONES**

Under the auspices of the Society for Ambulatory Assessment “Understanding behaviour in context”, by definition ambulatory assessment comprises the use of field methods to assess the ongoing behavior, physiology, experience and environmental aspects of humans in naturalistic or unconstrained settings (126). In order to examine the perspective of understanding biological processes as they naturally unfold in time and importantly in context, invasive methods (most commonly, blood sampling) and their use in artificial clinical laboratory settings are obsolete. For endogenous substances, especially hormones like cortisol, with a circadian as well as an ultradian rhythm frequent continuous sampling outside the medical environment can be of immense clinical and scientific value – a feat that has yet to be attempted.

With regard to cortisol measurement, the methods that potentially qualify for ambulatory assessment are assessed below. Urinary cortisol has carved its niche in clinical endocrinology with its use spanning well over half a century. Nevertheless, it requires due diligence on the part of the individual who is usually confined indoors for the duration of collection for convenience. It must also be remembered that it is a collective measure of overall cortisol production during a given period. Approval of salivary cortisol in 1985 for standard use was therefore welcomed as it offered a non-invasive and relatively easy to handle alternative (97). It can be and has indeed been used for frequent measurements but it can become cumbersome if several frequent samples are collected over a prolonged period. The most significant drawback it has is the inability to collect samples during sleep, without waking an individual. The importance of nocturnal levels (particularly in context of day-time levels) has been emphasised previously in this chapter. More recently, cortisol has been measured successfully in human hair and purported to reflect an aggregate level for the past six months (127). This method is at present in its infancy stage and yet again may be a good collective measure of cortisol status over an even longer duration than that in urine.

The technique of microdialysis, in our opinion offers itself as a unique tool for ‘ambulatory assessment’, not just for cortisol although that is the focus of this

research, in further understanding tissue physiology 'in context' as outlined here. It thus lends itself to the fields of clinical research, clinical diagnostics as well as possibly therapeutics.

#### **1.4 SUMMARY, AIMS AND HYPOTHESIS**

The circadian and ultradian rhythm of cortisol in blood has been extensively researched and is well established. Cortisol measured in blood is a combination of >90% hormone attached to binding proteins (CBG and albumin) and approximately 5% as the free fraction. It is the latter that is able to negotiate capillaries reaching end organs and their individual cells. Free glucocorticoids interact with their receptors, GR and MR, translocate to the nucleus of cells initiating a cascade of events leading to their ultimate physiological effects. Therefore, free cortisol is deemed to be the biologically active moiety.

The mainstay of clinical investigation remains blood sampling, which is safe only in a medical facility, making it an impractical candidate for real-time monitoring in an individual's sphere of life. Foray into ambulatory sampling in an attempt to understand this physiological system outside of artificial laboratory settings is an inevitable next step to further our knowledge about it.

Microdialysis as a method for reliable measurement of free glucocorticoid hormones has been demonstrated, but its validity in being able to reflect the well-described rhythm of cortisol has not yet been demonstrated to our knowledge.

The first aim of this thesis was to develop a technique that would allow us to measure tissue levels of free cortisol. This necessitated the validation of the microdialysis technique itself and its correlation (or otherwise) with serum total cortisol levels in the basal state and following stimulation or inhibition of the HPA axis.

My second aim was to develop an automated portable collection device for use with commercially available microdialysis catheters to allow frequent microdialysate sampling for prolonged periods automatically and safely in an individual ambulatory within their own environment.

## CHAPTER 2 GENERAL MATERIALS AND METHODS

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This section describes the microdialysis equipment, *in vitro* tests and laboratory assays used in this project. Human automated blood sampling equipment (**HABS**) and its peripherals were used as described by Henley et al (40). Research protocols specific to individual studies are included in the relevant chapters.

### 2.1 MICRODIALYSIS EQUIPMENT AND IN VITRO TESTS

#### 2.1.1 EQUIPMENT

A portable CMA 107 microdialysis pump powered by a 3-volt battery was connected to sterile subcutaneous (**SCC**) and intravenous (**IVC**) microdialysis catheters. Each catheter, also called probe, had a luer connection that fitted on to a sterile CMA 106 pump syringe (2.5 ml capacity) housed within the pump. Sterile isotonic solution within the syringe was pumped into the probes. CMA 66 linear microdialysis probe with polyarylethersulphone membrane of 30 millimetres (**mm**) length and 0.5 mm diameter, and molecular cut-off of 20 KDa was used. Its inlet and outlet tubes, made of polyurethane, were 400 mm and 100 mm long respectively, and had 0.5 mm diameter. Perfusion fluid T1, specific for peripheral tissue use, was used to perfuse the SCC. Its composition was: 147 millimoles/litre (**mmol/l**) sodium, 4 mmol/l potassium, 2.3 mmol/l calcium, and 156 mmol/l chloride ions giving it an osmolality of 290 milliosmoles/litre (**mOsm/l**). All the products described above were manufactured for commercial use, and used as recommended, by CMA Microdialysis AB, Stockholm, Sweden.

MicroEye PME011 (Probe Scientific, The Venture Centre, Sir William Lyons Road, Coventry, CV4 7EZ, UK) concentric probe had a polymer membrane of 15 mm exposed length, 0.2 mm diameter; inlet and outlet tubes made of PA6228 of lengths 400 mm and 200 mm respectively, and molecular cut-off of 9 KDa. It was inserted via 18-G B-Braun intravenous catheter as per manufacturer's recommendation. The perfusion fluid was a mixture of 2 ml of 0.9% saline (containing 154 mmol/l each of sodium and chloride, osmolality 300 mOsm/l) and 0.5 ml Arixtra (Fondaparinux Sodium

marketed by GlaxoSmithKline). Arixtra is an anticoagulant (factor Xa inhibitor) used routinely in clinical practice. Prior to insertion, the outlet tube of each catheter was truncated to 100 mm (to match the length of SCC) with a clean blade and its membrane was dipped in Arixtra to avoid microclots blocking the membrane. The latter was done in accordance with the manufacturer's instructions.

When the CMA 107 pump lid is closed, 5-minute flush cycle (15  $\mu\text{l}/\text{min}$ ) is followed by an automatic decrease to the pre-set operating rate (128). For the experiments in chapters 4 and 5, flow rate of 2 microlitres/minute ( **$\mu\text{l}/\text{min}$** ), whereas 1  $\mu\text{l}/\text{min}$  for that in chapter 6 was used from the range (0.1 to 5  $\mu\text{l}/\text{min}$ ) available on the pump. When samples were collected manually, fresh-labelled collection tubes were replaced for each ten-minute sample. When the novel collection device (described in chapter 3) was used (in chapters 5 and 6), the connections were made into a closed system before starting the pump. Care was taken to discard the first 75  $\mu\text{l}$  to account for the flush cycle (15  $\mu\text{l}/\text{min}$  for 5 min), prior to decanting samples from the collection device.

As blood sampling is instantaneous and microdialysate sampling continuous, the latter reading was considered to represent the midpoint of a given sampling frequency e.g sample timing of dialysate obtained between 10:00 and 10:10 was 10:05 and so on. According to the product description supplied by the two companies delay in obtaining dialysate at the tip of the outlet tube length of 100mm was less than a minute for both SC and IC, hence no additional time adjustment was made to match the timing of corresponding blood sample.

## 2.1.2 IN VITRO TESTS: MICRODIALYSIS PUMP AND CATHETERS

### 2.1.2.1 MICRODIALYSIS PUMP

Having allowed a stabilisation period of at least 30 minutes, ten minute samples were collected in pre-weighed vials that were re-weighed with the perfusion fluid. The weight, taken as the volume, of successive samples was calculated for each of the 14 samples (

**Table 2.1).** The average weight was 0.01919 g (range 0.01892 – 0.02008 g) representing a volume of 19  $\mu$ l.

TABLE 2.1 TESTING CMA 107 PUMP FLOW RATE		
WEIGHT OF VIAL (gram)	WEIGHT OF VIAL +DIALYSATE (gram)	DIFFERENCE (gram)
0.34542	0.36538	0.01996
0.34463	0.36365	0.01902
0.34328	0.36258	0.0193
0.34479	0.36487	0.02008
0.34337	0.36229	0.01892
0.34404	0.36369	0.01965
0.34171	0.36172	0.02001
0.34459	0.3638	0.01921
0.34254	0.36236	0.01982
0.34412	0.36403	0.01991
0.34408	0.36333	0.01925
0.3449	0.36474	0.01984
0.34558	0.36531	0.01973
0.34134	0.36053	0.01919

**Table 2.1** TESTING CMA 107 PUMP FLOW RATE.

Weight of each vial (g) was recorded first without and then with the perfusion fluid for ten consecutive samples. The flow rate was set at 2  $\mu$ l/min and ten minutely samples were collected.

### 2.1.2.2 MICRODIALYSIS CATHETERS

This study aimed to establish the *in vitro* recovery of cortisol across the membranes of SCC and IVC. The systems for both were set up as described previously

and allowed to stabilise for 30 min before each experiment. Due care was taken to ensure that the membrane of each catheter was immersed fully in the solution and as little a membrane area touching the wall of the container as possible. The prepared solution was suspended in a waterbath for 30 min at a temperature of 37°C prior to starting the experiment. Cortisol assay optimisation was taking place simultaneously, the details of which are discussed later in this chapter. Radioimmunoassay (**RIA**) and Netria saliva cortisol enzyme-linked immunosorbent assay (**ELISA**) assays were ultimately abandoned (For details see appendix I). IBL saliva cortisol ELISA (Hamburg, Germany) was developed and used for the entire study.

For all experiments, recovery was calculated by dividing the dialysate concentration by the concentration of the solution in which it was immersed, and expressed as percentage. Samples collected from all experiments were stored at -20°C and analysed at a later date.

#### **2.1.2.2.1 RESPONSE TO CHANGE IN CONCENTRATION OF CORTISOL**

For all the experiments in this sub-section, the flow-rate was set at 2 µL/min. Three cortisol solutions [low - 1.38, medium - 6.9, and high - 34.5 nmol/L] were prepared. A waterbath was set to the temperature of 37°C. Each solution was suspended in the waterbath 30 minutes prior to sampling and temperature was maintained throughout the duration. All samples were collected and stored at -20°C for analysis at a later date, using IBL saliva cortisol ELISA kit.

For the first set of experiments (**Table 2.2**), SCC membrane was immersed for 90minutes in each of the prepared solutions (in the order low, medium and then high). Half hourly dialysates with samples from the immersed solution at the end of half hour were collected.

TABLE 2.2. SC RESPONSE TO CHANGE IN CONCENTRATION						
	Experiment 1		Experiment 2		Experiment 3	
30 min	DIAL /SOL (nmol/L)	REC %	DIAL /SOL (nmol/L)	REC %	DIAL /SOL (nmol/L)	REC %
1.5 L	4.32/1.11	389	4.17/1.30	321	1.27/1.35	94
	1.06/1.16	91	0.94/1.10	85	1.13/1.57	72
	1.29/1.29	98	1.74/1.27	137	1.68/1.54	109
1.5-3.0 M	6.24/7.71	81	5.85/7.01	83	6.65/7.70	86
	6.75/7.30	92	8.25/7.59	109	7.67/7.92	97
	6.97/7.66	91	6.38/7.53	85	7.67/7.92	97
3.0-4.5 H	27.34/31.68	86	26.39/28.21	94	28.35/31.60	90
	29.23/31.35	93	27.63/28.95	95	30.97/32.15	96
	29.31/39.39	74	30.53/31.27	98	32.24/33.48	96
<b>AVERAGE RECOVERY</b>		<b>122</b>		<b>126</b>		<b>93</b>

**Table 2.2.** SC RESPONSE TO CHANGE IN CONCENTRATION (three experiments).

The first column in each experiment depicts actual concentration of a thirty minute dialysate (DIAL) and is divided by the concentration of solution (SOL) it was immersed in. Samples from the solution were collected at the end of the thirty-minute period. The second column shows the recovery (REC). Average recovery for the entire experiment is stated in the last row. (L=low conc; M=medium conc; H=high conc)

The results are tabulated in Table 2.2. For experiments 1 and 2, the very first dialysate concentrations were unexpectedly high. The variation in second dialysate values from low concentration (more than in other concentrations) is likely due to assay error. The remaining values were consistent with each other. There was good response to a change in concentration throughout all three experiments, with the first dialysate from the fresh solution yielding recovery values of 80% or more. The average values per experiment, excluding the two outliers (first samples in experiments 1 and 2), were 88, 98 and 93% respectively.

The following sets of experiments were carried out with both types of catheters, SCC and IVC. A shaking waterbath with a shaker setting of 75/min and at



temperature of 37°C was used for this experiment. Solutions were prepared using ringer for SCC and saline for IVC in keeping with the perfusion fluid recommendation for the individual catheter types. A catheter was immersed for an hour each in three solutions (in the order L, M, H then L). Frequency of dialysate sampling was every ten minutes, and samples from the immersed solution were taken at the beginning and end of the hour. In order to calculate recovery, dialysates from the first half hour were each divided by the measured concentration of the solution at the beginning of the hour. The dialysate values obtained during the remaining half hour were each divided by the concentration of the solution measured at the end of the hour. Values higher or lower than the detection limits of the assay were recorded as the respective limits.

Table 2.3 shows the results obtained from three individual experiments carried out with SCC.

TABLE 2.3 SCC RESPONSE TO CHANGES IN CORTISOL CONCENTRATION						
HR	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
10 min	DIAL/SOL (nmol/L)	REC %	DIAL/SOL (nmol/L)	REC %	DIAL/SOL (nmol/L)	REC %
1 L	5.6/5.3	106	9.91/8.86	112	6.71/7.42	90
	4.75	90	10.57	119	8.34	112
	4.97	94	10.27	116	6.71	90
	4.39/5.85	75	10.96/8.91	123	8.50/6.65	128
	5.11	87	9.99	112	5.66	85
	4.86	83	10.43	117	5.82	88
2 M	27.21/29.61	92	35.13/39.33	89	25.75/28.62	90
	26.25	89	41.01	104	28.90	101
	25.53	86	39.88	101	26.77	94
	23.79/26.63	90	41.32/37.23	111	26.94/27.74	97
	23.49	89	40.46	109	27.43	99
	24.51	93	39.80	107	26.55	96
3 H	90.42/113.71	80	103.17/117.74	88	85.67/101.32	85
	94.61	83	129.91	110	102.23	101
	98.34	86	118.98	101	96.49	95
	113.24/107.78	105	118.35/118.98	100	92.10/88.34	104
	98.34	91	136.15	114	92.49	105
	109.10	101	121.55	102	92.49	105
4 L	21.83/4.28	510	29.59/10.96	270	22.05/7.51	294
	4.20	98	11.98	109	7.98	106
	6.62	155	11.04	101	6.96	93
	5.05/5.22	97	11.37/9.77	116	7.07/6.07	116
	4.55	87	10.82	111	6.40	106
	6.46	123	10.29	105	4.86	80
<b>AVERAGE RECOVERY</b>		<b>112</b>		<b>115</b>		<b>107</b>

**Table 2.3.** SCC RESPONSE TO CHANGES IN CORTISOL CONCENTRATION (four experiments).

The first column in each experiment depicts actual concentration of a ten minutely dialysate (DIAL) and is divided by the concentration of solution (SOL) it was immersed in. For the first 30 minutes, each of the dialysates was divided by the concentration of the solution as measured at the beginning of the hour. For the remaining part of the hour (HR), the dividing concentration was that obtained at the end of the hour. The second column shows the recovery (REC). Average recovery for the entire experiment is mentioned in the last row. (L=low conc; M=medium conc; H=high conc).

First of all, the concentrations of the prepared solutions measured at the beginning and end of the hours were fairly consistent (variation of 10% or less in most pairs). In all experiments, there were good recovery rates through every hour as well as when the surrounding concentration changed. However, the first dialysates of the fourth hour return an unusually high recovery value and later settle to more expected values of 80% and above consistent previous experience. Similarly, when the membrane is moved from lower to higher concentration surrounding, the first ten-minute value is the lowest of the hour. Both these phenomenon can be explained by experimental error.

When average recovery values were calculated for each hour from the above experiments (Table 2.4), they were 89% and above for the first three hours (excluding experiment 1). The fourth hour values, skewed by those obtained in the first 10 minutes if excluded, the average per experiment was at least 90% and the average per hour across all experiments was at least 87%.

<b>TABLE 2.4 AVERAGE HOURLY RECOVERY (%) OF SCC OVER THREE EXPERIMENTS</b>				
<b>HOURLY (Conc)</b>	<b>Experiment 1</b>	<b>Experiment 2</b>	<b>Experiment 3</b>	<b>Hourly Average of 3 Experiments</b>
1 (Low)	89	117	99	<b>101</b>
2 (Medium)	90	104	96	<b>97</b>
3 (High)	91	103	99	<b>98</b>
4 (Low)	178	135	133	<b>148</b>
<b>Average per Experiment</b>	<b>112</b>	<b>115</b>	<b>107</b>	

**Table 2.4.** AVERAGE HOURLY RECOVERY OF SCC (3 EXPERIMENTS).

Averages were calculated for each hour of each experiment, and for the respective hours of all experiments. Low, medium and high relate to the concentration of the solutions of cortisol.

The conclusion drawn from the above three experiments is that the SCC is reliable in detecting a change in concentration – immediately (next 10 minutely

sample) when concentration rises, but with a delay of at most 10minutes when the concentration suddenly drops.

Table 2.5 show the results obtained from experiments carried out in exactly the same manner as above, using IVC this time.

TABLE 2.5 IVC RESPONSE TO CHANGES IN CORTISOL CONCENTRATION						
HR	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
	DIAL/SOL (nmol/L)	REC %	DIAL/SOL (nmol/L)	REC %	DIAL/SOL (nmol/L)	REC %
1 L	No sample	----	3.01/10.49	29	3.23/9.69	33
	2.70//7.07	38	7.62	72	4.91	51
	4.61	65	9.33	89	7.70	79
	7.56	107	7.84/10.74	73	8.91/9.49	94
	12.59	178	7.65	71	5.16	54
	11.92	169	8.42	78	7.70	81
2 M	5.63/43.58	13	8.25/40.3	20	6.71/39.63	17
	27.60	63	26.00	64	23.82	60
	No sample	----	34.33	85	28.95	73
	25.56/39.41	65	30.88/37.29	83	36.13/46.75	77
	27.96	71	29.59	79	33.75	72
	25.28	64	30.66	82	32.02	68
3 H	23.13/113.19	20	26.22/138	19	32.46/138	24
	89.23	79	98.81	72	59.56	43
	88.54	78	108.44	79	73.28	53
	91.49/133.39	69	119.78/138	87	53.49/137.59	65
	99.06	74	120.28	87	Battery error	---
	92.43	69	122.30	89	Battery error	---
4 L	92.65/7.26	1276	125.41/9.33	1344	94.01/11.95	786
	34.42	474	24.54	263	32.65	273
	6.87	95	9.83	105	11.45	96
	4.06/9.33	43	10.18/10.29	99	10.71/11.87	90
	3.89	42	9.77	95	10.21	86
	3.48	37	9.25	90	10.57	89
<b>AVERAGE RECOVERY</b>		<b>140</b>		<b>136</b>		<b>103</b>

**Table 2.5.** IVC RESPONSE TO CHANGES IN CORTISOL CONCENTRATION (four experiments).

The first column in each experiment depicts actual concentration of a ten minutely dialysate (DIAL) and is divided by the concentration of solution (SOL) it was immersed in. For the first 30 minutes, each of the dialysates was divided by the concentration of the solution as measured at the beginning of the hour. For the remaining part of the hour (HR), the dividing concentration was that obtained at the end of the hour. (Exception: First hour of experiment 1 only the former value was used as the latter value (41.07) was erroneous). The second column shows the recovery (REC). Average recovery for the entire experiment is mentioned in the last row. Two dialysates at the end of the third hour in experiment 3

were not collected as the pump ceased to work due to battery error. (L=low conc; M=medium conc; H=high conc)

First of all, the concentrations of the prepared solutions measured at the beginning and end of the hours were fairly consistent (variation of 7% or less in most pairs). A definite pattern is evident here such that, the first dialysate from the fresh solution is extremely low for the first three hours – when the preceding concentration is lower. In contrast, the first two dialysate values were extremely high for hour 4. During hour 1 of experiment 1, pump/catheter malfunctioning was suspected as no dialysate was collected for the first 10 minutes and the last 3 were progressively higher. Similarly, for hour 3 of experiment 3 there was a problem with the battery. Apart from those two hours, the average hourly recovery (Table 2.6) for the first 3 hours was between 55 and 72% individually and between 60 and 71% collectively. The fourth hour values are too high to be practically possible. Without the fourth hour, the average recovery per experiment was 77, 70 and 58%, respectively (not in table 2.6).

<b>TABLE 2.6 AVERAGE RECOVERY OF IVC OVER THREE EXPERIMENTS</b>				
<b>HOUR (Conc)</b>	<b>Experiment 1</b>	<b>Experiment 2</b>	<b>Experiment 3</b>	<b>Hourly Average of 3 Experiments</b>
1 (Low)	111	69	66	<b>82</b>
2 (Medium)	55	69	61	<b>62</b>
3 (High)	65	72	46	<b>61</b>
4 (Low)	328	333	237	<b>299</b>
<b>Average per experiment</b>	<b>140</b>	<b>136</b>	<b>103</b>	

**Table 2.6.** AVERAGE HOURLY RECOVERY OF IVC (3 EXPERIMENTS).

Averages were calculated for each hour of each experiment, and for the respective hours of all experiments. Low, medium and high relate to the concentration of the solutions of cortisol.

**DISCUSSION OF RESULTS:** The implication of these experiments is that the subcutaneous probe is able to reflect a change in composition of surrounding medium

promptly, especially if the direction of change is from low to high, but when it is in the opposite direction there is a delay of at most 10 minutes.

In general, the recovery values of IVC are lower than those for the SCC. This is not entirely unexpected as the membrane length of the former (2.5 cm) is half that of SC (5 cm) consequent to which the surface area available for the exchange to occur is lower.

Delay of twenty minutes in the IVC dialysate reflecting the surrounding matrix/solution was apparent. This is likely due to the IVC outlet tube length (200 mm, volume 12.3  $\mu$ l) being twice that of SCC. When the first sample of every hour was eliminated to allow for the longer outlet tube, the recovery was largely stable for the remaining part of the hour and, the average hourly recovery for all 3 experiments improved to between 69 and 81% (Table 2.7). Consequently, for all *in vivo* studies the length of each IVC outlet tube was cut down by 170 mm with the volume of the remnant being reduced to 1.6  $\mu$ l, comparable with the outlet tube volume of SC.

<b>TABLE 2.7 AVERAGE RECOVERY OF IVC OVER THREE EXPERIMENTS EXCLUDING THE FIRST SAMPLE OF EVERY HOUR</b>				
<b>HOUR (Conc)</b>	<b>Experiment 1</b>	<b>Experiment 2</b>	<b>Experiment 3</b>	<b>Hourly Average of 3 Experiments</b>
1 (Low)	111	77	72	<b>75</b>
2 (Medium)	66	79	70	<b>81</b>
3 (High)	74	83	54	<b>69</b>
4 (Low)	138	130	127	<b>180</b>
<b>Average per experiment</b>	<b>97</b>	<b>92</b>	<b>81</b>	

**Table 2.7.** AVERAGE RECOVERY OF IVC OVER THREE EXPERIMENTS EXCLUDING THE FIRST SAMPLE OF EVERY HOUR.

Averages were calculated for each hour of each experiment, and for the respective hours of all experiments. Low, medium and high relate to the concentration of the solutions of cortisol.

In addition to the delay accounted for by the long outlet tube of IVC, ten-minute delay was detected between dialysates reflecting the surrounding matrix, particularly

when the concentration of the cortisol solution dropped precipitously (e.g. from 138 to 6.9 nmol/l). This was true for both catheter types. The most likely explanations for this is part of the residual solution from the previous hour remaining on the membrane and/or within the outlet tube. There are certain inherent limitations in an ex-vivo setting. A shaking waterbath although able to reproduce core body temperature, is an artificial system that cannot accurately reflect the dynamic flow state within tissue compartments. The agitation setting on the waterbath had to be stable enough to avoid spillages of the solutions contained within it. In our opinion, blood flow within the tissue compartments tested in human subjects, i.e. peripheral intravenous and subcutaneous, would avoid the likelihood of some carry over of the preceding higher concentration for a given ten minute time period as seen in this *ex-vivo* experiment. The velocity of blood flow in capillaries, which would abound in subcutaneous tissue, is expected to be 0.5 mm/second and (129) although the exact velocity is not known. This effect was not that apparent when a membrane was transferred from a lower to a higher concentration, evidently due to the former adding relatively little to the prevailing higher strength solution. We note however that this a potentially significant factor to consider when evaluating the timing of a 'change' in concentration particularly in comparison with the serum and, where available saliva, values. The limitation therefore in being to accurately able to identify the delay between serum total and SC free cortisol down to a minute may not be possible within this project.

## **2.2 ASSAYS**

### **2.2.1 CORTISOL ELISA FOR DIALYSATE**

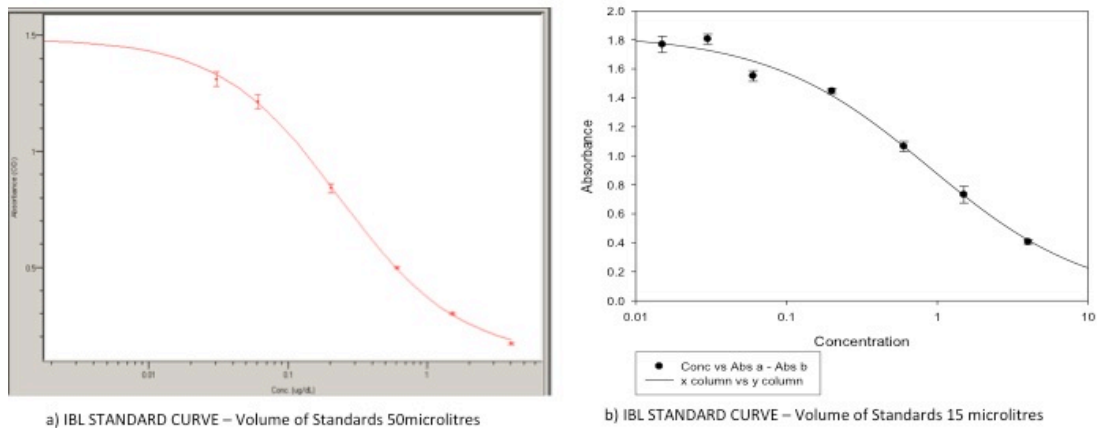
#### **2.2.1.1 ASSAY DEVELOPMENT:**

A sensitive assay was needed to measure the very low concentrations of free cortisol expected in the dialysate. This has been achieved in rodents using a commercially available RIA kit. Cortisol RIA, DSL-2000 (Diagnostic Systems Laboratories, Texas, USA) was tested but the lack of sensitivity (0.11 µg/dl or 3.03 nmol/l) prevented its further use. (Please see annexe I for further explanation).



We attempted to optimise Netria Free cortisol ELISA kit (Immunometrics Ltd, 50 Bronsart Road, London SW6 6AA). We were not convinced by the reproducibility and consistency of this product, as well as the quality of reagents supplied on more than one occasion. It is no longer commercially available. Most of the early in vitro experiments with the SCC were analysed with this particular assay (annexe I), and some repeated with the final assay we homed in on - IBL ELISA kits for salivary cortisol (IBL, Hamburg, Germany).

The basic principle of the IBL (as well as that of Netria) ELISA assay is that an unknown amount of antigen in the dialysate and a fixed amount of enzyme labelled antigen compete for the binding sites of the antibodies coated onto the wells (IBL ELISA KIT INSERT - (130)). The test procedure recommended pipetting 50  $\mu$ l each of sample i.e. dialysate, and standards & controls per well, and to determine samples in duplicates to identify potential pipetting errors. As per our study protocol, the sample size for a given time period was no more than 20  $\mu$ l and 15  $\mu$ l was considered to be the optimal volume per sample for the assay. A series of assays were performed where the amount of one of the kit reagents, including enzyme conjugate, substrate and stop solutions was reduced at a time while keeping the remaining reagent volumes as per product literature. The configuration of the standard curve obtained following the exact recommendation by the manufacturer was reproducible only when reagents were used without any alterations in their amounts keeping the sample (i.e. dialysate, standard, control) volume of 15  $\mu$ l (Figure 2.1). This was true for several experiments. Therefore, in the final assay protocol, there were two deviations from manufacturer's instructions - in the volume of sample, standards and control (15  $\mu$ l instead of 50  $\mu$ l) and, in that samples were not determined in duplicates (standards and control solutions were). It is important to note that dialysate samples we obtained using the membrane cut-off of 20 KDa were believed to be free from larger molecules and so were used directly without the any further processing.



**Figure 2.1.** IBL STANDARD CURVES USING 50 $\mu$ L (A) AND 15 $\mu$ L (B) OF STANDARD VOLUME.

Absorbance is displayed along the Y-axis and log of concentration along the X-axis. Each point on the curve represents an individual standard in duplicates (closed circle=mean). The plate reader software changed during optimisation process and hence the graphs appear different.

T1 perfusion fluid used with SCC was similar in chemical composition to ringer solution (Sodium 147 mmol, Potassium 4 mmol) apart from Chloride (155.6 instead of 156 mmol) and Calcium (2.3 instead of 2.25 mmol) content giving T1 an osmolality of 290 mosm/l (Ringer 300 mosm/l). 0.9% saline solution was recommended for use with IVC. IBL ELISA kit was primarily marketed for analysis of saliva samples (although also recommended for diluted serum) and so the slimy matrix of the kit standards reflected its consistency. In order to avoid analysis errors due to the different perfusion fluids in our experiments, ten standard cortisol concentrations were prepared by adding cortisol stock to ringer solution and to saline.

Cortisol stock solution was prepared by adding 0.05 g of hydrocortisone MR, molecular weight 362.47 (Sigma-Aldrich, 3050 Spruce St., St. Louis, USA) to 10 ml of absolute Ethanol. It was stored at -20°C and used for up to a year. Ringer and saline standards (including those used in the previous subsection) were prepared using this stock solution as follows:

- Solution A (1380 nmol/l): 100  $\mu$ l stock (138,000 nmol/l) + 9.9 ml ethanol (100%)
- Solution B (13.8 nmol/l): 100  $\mu$ l solution A + 9.9 ml Ringer/Saline
- Standard 1 (1.38 nmol/l): 20  $\mu$ l solution B + 180  $\mu$ l Ringer/Saline

- Standard 2 (27.6 nmol/l): 40 µl st1 + 160 µl Ringer/Saline
- Standard 3 (13.8 nmol/l): 100 µl st2 + 100 µl Ringer/Saline
- Standard 4 (6.9 nmol/l): 100 µl st3 + 100 µl Ringer/Saline
- Standard 5 (2.76 nmol/l): 80 µl st4 + 120 µl Ringer/Saline
- Standard 6 (1.38 nmol/l): 100 µl st5 + 100 µl Ringer/Saline
- Standard 7 (0.69 nmol/l): 100 µl st6 + 100 µl Ringer/Saline
- Standard 8 (0.14 nmol/l): 40 µl st7 + 160 µl Ringer/Saline
- Standard 9 (0.06 nmol/l): 100 µl st8 + 100 µl Ringer/Saline
- Standard 10: Ringer/Saline alone as a zero

The IBL kit standards that were ready to use had the following concentrations: 110.4, 41.4, 16.56, 5.52, 1.66, 0.83, and 0 nmol/l. The lowest concentration (0.83 nmol/l) was diluted with equal amount of sterile water providing eighth standard (0.41 nmol/l) the purpose of which was to observe the sensitivity of the assay at lower level of the range. For analysis of SC dialysate samples ringer standards were used in addition to kit standards to construct two calibration/standard curves. In addition to these two, a saline standard curve was used when analysing samples from IVC.

The following protocol was used for each assay:

1. Mix IBL kit reagents without foaming (manually). Standards (Std) come ready to use. Add 15 µl of IBL standards and controls per well in duplicates, moving downwards.
  - a. Std A= 0 µg/dl
  - b. Std B/2 = 0.015 µg/dl (50 µl sterile water + 50 µl std B)
  - c. Std B = 0.03 µg/dl
  - d. Std C = 0.06 µg/dl
  - e. Std D = 0.2 µg/dl
  - f. Std E = 0.6 µg/dl
  - g. Std F = 1.5 µg/dl
  - h. Std G = 4 µg/dl

(Cortisol nmol/l = µg/dlx27.6. Values in nmol/L for IBL standards mentioned in text above)

2. Add 15  $\mu\text{l}$  of each of the T1 (or ringer) standards 1 to 10 in duplicates
3. For IVC samples, add 15  $\mu\text{l}$  of each of the saline standards 1 to 10 in duplicates
4. Add 100  $\mu\text{l}$  enzyme conjugate to each well.
5. Cover with supplied adhesive foil and agitate for 2 hours at room temperature, at 400-600rpm. Used 300 rotations per min as that is the max our shaker goes to.
6. Ascertain the amount of wash solution required and make it up in a 1:10 ratio (10 ml of wash concentrate + 100ml double distilled water). Mix well.
  - a. Lower meniscus should correspond to the desired volume mark.
  - b. Wash each well x4 i.e.  $250\ \mu\text{l} \times 4 = 1000\ \mu\text{l}/1\text{ml}$  needed per well. So, amount to make up is no. of wells x 1ml
7. Tip off and then wash x4 with wash solution as follows - Add 250  $\mu\text{l}$  to each well with multichannel pipette, tip in sink, blot, add, etc.
8. Add 100  $\mu\text{l}$  TMB substrate per well.
9. Agitate for 30 minutes at room temperature.
10. Stop the reaction by adding 100  $\mu\text{l}$  TMB stop solution. Shake briefly.
11. Read plate with a photometer at 450 nanometres wavelength, within 15 minutes.

Every plate was read in a plate reader with the help of Microplate manager version 6.0. In general, higher the concentration, lower the absorbance value. The optical density of the standards was plotted linearly on the Y-axis. The X-axis had logarithmically transformed values of the concentration. The curve was fitted with 4 parameter logistics curve as recommended in the product literature. Occasionally, when there was an outlier within the standard curve, one or both of the duplicate values, was omitted.

All three standard curves were similar in configuration and the range of absorbance levels achieved matched that with the IBL standards (Figure 2.2). The upper part of the sigmoid curve for the standards made by us was flat therefore limited in discriminating values below 0.69 nmol/l. SCC dialysate samples were read from the ringer standard curve in the first pharmacological manipulation experiment in

Chapter 4 (SCC alone) and from T1 standard curve in the second pharmacological manipulation experiment in Chapter 4 (SCC and IVC) as well as in Chapters 5 and 6. This was because the Ringer solution manufacturer stopped production of that particular solution. Samples from IVC were read from saline standard curve.

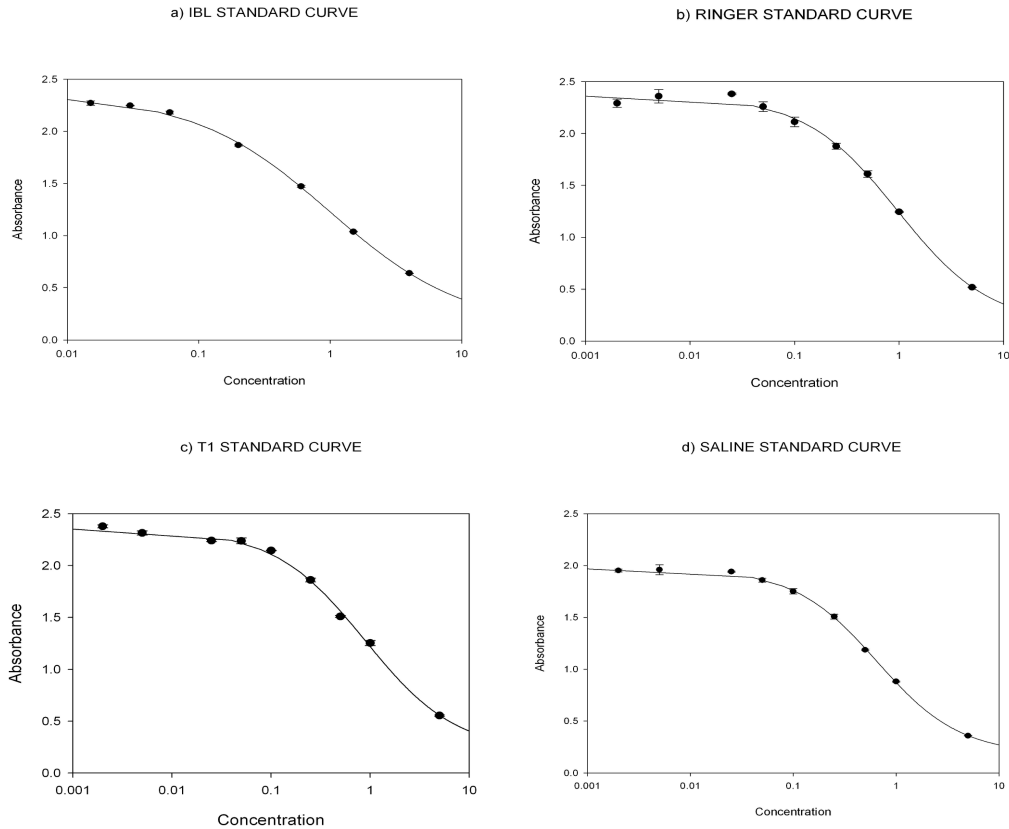


Figure 2.2. STANDARD CURVES OBTAINED WITH STANDARDS IN IBL KIT (a) and those prepared in house as described in the text using ringer solution (b), T1 perfusion fluid (c), or saline (d). Absorbance values are represented on the Y-axis and X-axis shows log transformed values of standards. Each point on the curve represents an individual standard in duplicates (closed circle=mean).

The intra-assay precision (or repeatability) for saliva concentrations of 7.452 and 64.58 nmol/l had coefficients of variation (**CV**) of 7.3 % and 3.1 % respectively. The inter-assay/intermediate precision for saliva concentrations of 14.904 and 64.86 nmol/l had CV of 8.8 % and 6.4 % respectively.

Overall, the results with this ELISA kit showed good reproducibility and sensitivity.

### ***2.2.2 SERUM CORTISOL ASSAY***

Blood samples were collected in BD Vacutainer® SSTTM Advance tubes, 3.5mL, with inert gel barrier and clot activator coating (Becton Dickinson, Oxford, UK) and then stored at -80°C after centrifugation. Cortisol concentrations were measured by electrochemiluminescent immunoassay (Cobas® e601 immunoassay analyser, Roche Diagnostics, Burgess Hill, UK). The assay is based on chemiluminescence (emission of light resulting from a chemical reaction) produced as a result of electrochemical reactions (Knight 1999 Trends in Analyt Chem), a combination that results in high sensitivity and specificity. These assays were performed, at cost, by the department of Clinical Biochemistry at the University Hospitals Bristol NHS Foundation trust, Bristol, United Kingdom. Intra-assay precision for serum concentrations of 208, 561 and 1268 nmol/l had CV of 1.3, 1.3 and 1.1 % respectively. The inter-assay precision for the same serum cortisol concentrations had CV of 1.6, 1.5, and 1.6 % respectively.

### ***2.2.3 SALIVA CORTISOL ASSAY***

Saliva samples were collected in Salivette® Cortisol and stored at -80°C after centrifugation. Salivary cortisol and cortisone were analysed with an in-house assay developed by the department of Clinical Biochemistry, University Hospitals Bristol NHS Foundation trust, Bristol, United Kingdom using UPLC®-tandem mass spectrometry (Waters Acquity- Premier XE, 34 Maple Street, Milford, MA 01757 USA). Tandem mass spectrometry is a structural assay based on identifying a specific fragment of the analyte of interest. The inter-assay CV for cortisol were 17.6 % and 9.9 % for 2 nmol/l and 46 nmol/l respectively, whereas those for cortisone were 6.7 % and 10.4 % for 4 nmol/l and 94 nmol/l respectively. The intra-assay CV for cortisol were 18.3 % and 8.8 % for 2 nmol/l and 46 nmol/l respectively, whereas those for cortisone were 10.2 % and 8.0 % for 4 nmol/l and 94 nmol/l, respectively.

## CHAPTER 3      NOVEL PORTABLE AUTOMATED COLLECTION DEVICE

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### 3.1 INTRODUCTION

Living on a planet which rotates on its axis every 24 hours results in life forms that have evolved to regulate their activities into daily periods of rest and wakefulness. The HPA axis is a major regulator of metabolic and cognitive function and is also a critical system for the homeostatic regulation of the stress response. As discussed in chapter 1, the circadian rhythm of glucocorticoid hormone secretion is actually made up from an underlying approximately hourly ultradian rhythm, which is important for optimal gene transcription and brain and metabolic function (131).

In order to understand the importance of both circadian and ultradian rhythms in man, both in health and disease, it is essential to be able to obtain multiple samples over extended periods. In order to get over the problems of the stress associated with multiple episodes of venous sampling, especially during the hours of sleep, Henley et al reported a Human Automated Blood Sampling (**HABS**) system which could be used in the setting of a clinical investigation unit (40). They also went on to show how this system could be used to provide resolution of clinical research problems that had not been amenable to previous studies using more limited sampling techniques.

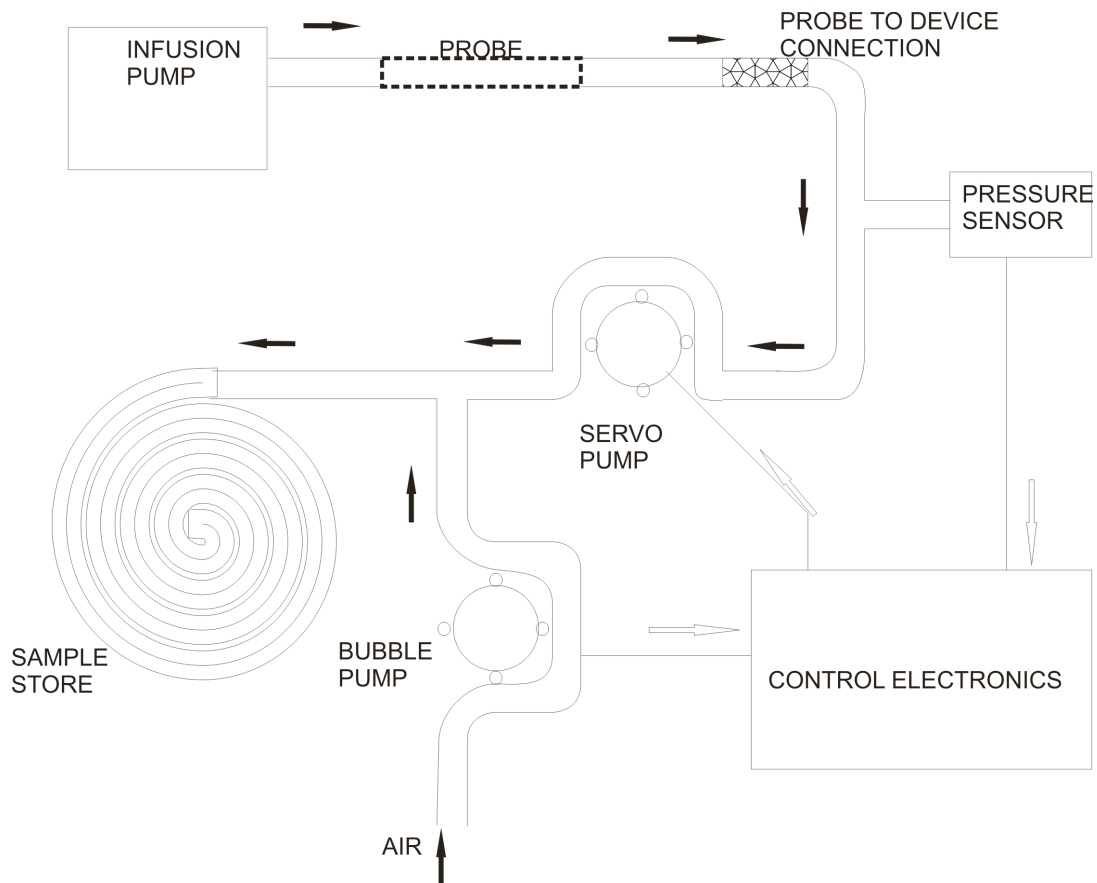
One major drawback of the HABS system however, is that it relies on blood samples and is therefore limited to use within a medical facility. This prevents its use for continuous monitoring in a subject's home setting which is, for most diagnostic and scientific questions, the most meaningful physiological setting to look at homeostatically important hormones. A second disadvantage is that in whole blood approximately 90% of cortisol is inactive and bound to carrier proteins (92). Since only the free unbound cortisol has access to the tissues, total blood levels may not accurately estimate the levels of hormone found in the extracellular space where they have access to tissues and their receptors. Levels of free cortisol in human tissues can, however, be measured using the technique of microdialysis (70,111,124,125).

In this chapter, a novel miniaturised sampling system that allowed us to combine the technique of microdialysis with the ability to collect multiple samples automatically over 24 hours is described. The automated sampler is carried on a pouch attached to a belt around the subject's waist, allowing the individual to be free to go about his or her normal everyday activities. We have linked this compact sampling unit to a microdialysis system which does not need venous access.

### **3.2 MATERIAL AND METHODS**

A commercial microdialysis system was set up as described in Chapter 2. The membrane of a linear catheter was inserted subcutaneously into the interstitial compartment of the anterior abdominal wall. The flow rate was set at 1 (chapter 5) and 2 (Chapter 4) microlitres/min. The microdialysate was collected in our automated collection system consisting of a length of polytetrafluoroethylene (**PTFE**) tubing, wrapped concentrically around a spool. The layout of this equipment is shown in the block diagram Figure 3.1.





**Figure 3.1.** BLOCK DIAGRAM OF THE COLLECTION DEVICE.

Perfusion fluid is pumped by the microdialysis ‘infusion pump’ which is connected to a syringe within it. Exchange takes place at the membrane or ‘probe’ of the catheter. The dialysate from the distal end of the catheter flows through a segment of FEP tubing attached to the catheter at one end (via MAB 8 connector) and to the collection device at the other end (using a pink adapter). The device contains a pressure sensor, a servo pump that propels dialysate, a bubble pump that inserts air bubble at set intervals, all controlled by the electronics board and a sample store.

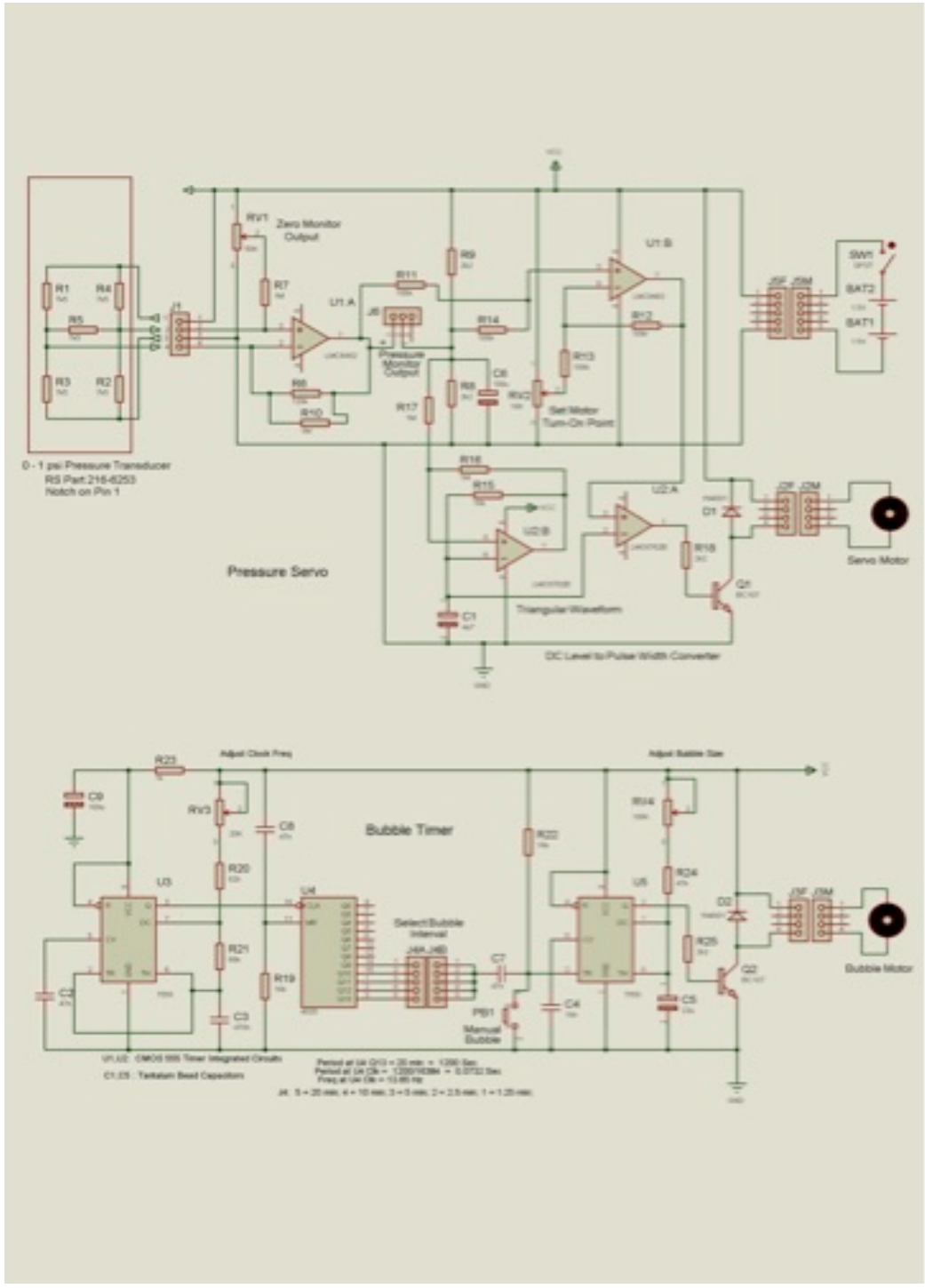
### **3.2.1 SAMPLE COLLECTION**

The division of samples generated at different times is achieved by the injection of an air bubble at predetermined intervals. These bubbles separate individual samples, which then move along the tubing as new samples are introduced into the collecting system. This is achieved by a special peristaltic pump which injects bubbles through a T-junction. The bubble pump needed to be small in size, light weight and use low current consumption, which was realised using a pump with overall dimensions 28 mm X 28 mm X 52 mm (length x width x height).

### **3.2.2 MAINTENANCE OF PRESSURE ACROSS MICRODIALYSIS MEMBRANE**

As the number of samples in our collecting device increases, there is an increase in the pressure needed to move them along the PTFE tubing. For instance, this pressure increased to 0.5 atmospheres when there were 72 samples in the tube. In order to maintain normal pressure in the microdialysis probe, pressure is detected by a sensor between the microdialysis probe and the tubing store. This controls a second peristaltic pump, the servo motor, identical to the bubble pump, which provides the pressure to move the samples along the collecting line. The sensor itself has semi-conductor piezo resistive elements in a Wheatstone bridge arrangement.

The alphanumeric characters in this and the following section relate to Figure 3.2. The output from the bridge is fed into a high gain differential stage using U1A. RV1 is used to offset any bridge output present when the pressure is zero. The servo pump motor is driven from the pressure signal using pulse width modulation. This is necessary to maintain a high torque at low running speeds. The op-amp U2B is an oscillator which generates an approximately triangular waveform across C1. This signal is fed into the –ve input of U2A, which is used as a comparator. The +ve input is fed with the pressure signal after adding a preset DC level using op-amp U1B as a unity gain differential amplifier. The output from the comparator is fed into transistor Q1 which switches the current to the servo motor between full current and zero. As the pressure increases the full current dwell time increases driving the motor harder and thus reducing the pressure. RV2 sets the probe line pressure by determining the point where the motor starts to get driven. This servo system keeps the pressure constant to within about 10 cm of water pressure.



**Figure 3.2.** CONTROL ELECTRONICS INSIDE THE COLLECTION DEVICE.  
See text for details.

**3.2.3 BUBBLE TIMING**

The frequency of sampling is determined by the periodicity of the bubbles inserted in the microdialysate column by the U3 complementary metal-oxide

semiconductor (**CMOS**) 555 timer (Figure 3.2. – includes alphanumeric characters in this section). This runs as an astable multivibrator to give a clock frequency of 13.65Hz, pre-set using RV3. This clock signal is fed to U4 which is a type 4020 multi stage binary divider. The output from a range of stages in U4 determines the frequency of sampling (e.g stage 12 has a period of 10 minutes) which then triggers U5. This is another CMOS 555 timer, this time running in monostable mode, generating a pulse of around two seconds duration, pre-set by RV4. This pulse turns on transistor Q2 which powers motor for the air bubble insertion pump.

### **3.2.4 COMPLETE SYSTEM**

The dimensions of the final collection system are 152 mm x 84 mm x 52 mm (length x width x height respectively), and the weight is 400 grams (Figure 3.3). The inner components of the compact device, which is made of plastic, are accessible through its removable lid. Access to the device is needed routinely for the replacement of batteries and PTFE tubing. The system will run for over 24 hours on two AA size cells, which should be of the lithium iron disulphide type.

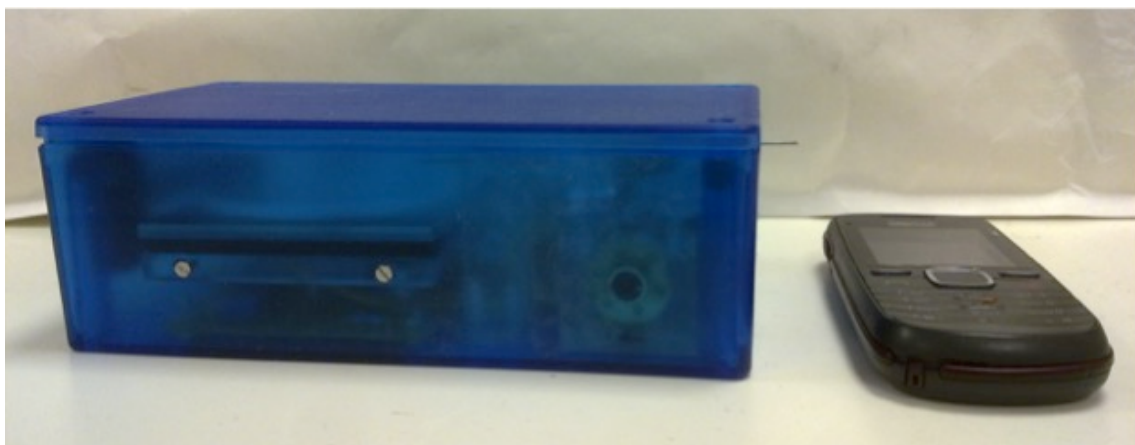


Figure 3.3. PHOTOGRAPH OF THE COLLECTION DEVICE next to a mobile phone.

The distal end of a linear microdialysis probe is connected to the collection system using a 15-20 cm section of fluorinated ethylene propylene (**FEP** – Linton Instrumentation) tubing, and commercially available tubing connectors. The two types of tubing connectors namely, pink adapter (connects FEP segment to device inlet) and MAB-8 connector (connects probe outlet to FEP segment) is immersed in absolute

ethanol for at least ten minutes prior to connection, as per manufacturer's recommendation (Royem Scientific limited, Luton, Bedford UK). For the system, the most convenient site of insertion for the microdialysis probe is the lower abdominal wall. Each sterile probe is inserted subcutaneously using standard aseptic precautions recommended for an invasive procedure in a hospital setting. As is standard for all cannulations, probes are never left in place for more than 3 days. With these precautions we have encountered no problems with inflammation or infection.

When sampling is complete, individual samples are decanted manually into marked tubes and stored at -80°C for later laboratory analysis.

### **3.2.5 CHALLENGES IN DEVELOPING THE SYSTEM**

There were many challenges in the development of our sampling system. An inherent problem with all microdialysis studies i.e. low sample volumes with low concentration levels was encountered in our study too. Development of a highly sensitive ELISA for this purpose has been described in Chapter 2.

A potential safety concern would be the risk of cross infection related to successive use of the device. For each individual patient, a sterile catheter is connected to the device using a fresh set of tubing and connectors. The device is programmed to allow liquid/air movement in a single direction i.e. away from the subject and the device inlet. In case of unexpected mechanical failure we also add a disinfecting cycle between studies by flushing with chlorhexidine acetate solution (0.05%) before insertion of a new spool.

Furthermore, in miniaturising the device, it was important to ensure the highest possible level of safety features. Electronic circuits and batteries were of low voltage (maximum 3 volts) and a fuse was specially designed to eliminate unexpected problems.

Since it is crucial to collect dialysate at reasonably frequent time intervals for a period of at least 24 hours, we needed a low power consumption unit to ensure that we had the capacity for the system to work on battery power only for prolonged periods.

The separation of samples by air bubbles was itself very effective but did provide problems with maintaining a normal pressure gradient across the microdialysis membrane which we needed to compensate with an appropriate servo system to prevent ultra-filtration (see Chapter 1). Despite the use of such a system, fragmentation of the air bubbles and consequently the sample columns was observed infrequently. This is further explained in appendix II.

Within the collection device is a section of dead space, from the point of sample entry into the device to the point of sample entry into the sample store, which effectively contains about forty microlitres of sample volume. For future development of the device, especially for frequent sampling protocols, reducing the dead space to a bare minimum would be advantageous.

### **3.3 VALIDATION OF THE DEVICE**

Ethical approval for this study was obtained from the Bath Research Ethics Committee. Two male healthy volunteers (age 18-24) gave their informed consent. The study was carried out at the local clinical research facility where the participants spent the duration of sampling. The microdialysis components were set up and connected to the collection system as described above. The entire system was set up at least 80 minutes before sampling, between 1700 and 1740 hours, when the collection device and microdialysis pump were placed in a commercially available waist-bag. Microdialysate samples were collected at 10-minute intervals for 24 hours and later analysed using ELISA (Saliva cortisol ELISA, IBL, Hamburg, Germany), as described in Chapter 2.

### **3.4 RESULTS AND DISCUSSION**

All results are discussed in detail in the following chapters. These data clearly show that this novel automated sampling system can collect timed samples of microdialysis fluid over a full 24 hours in ambulatory humans going about their normal everyday activity – including the very important period during the sleeping hours.

We have previously shown that the HABS system allows automated frequent, undisturbed sampling of blood in an individual which is a significant step towards establishing diurnal and ultradian rhythms of hormones (40). Human beings adapt remarkably well to novel environments, yet a clinical facility necessary for the application of HABS system, may introduce an element of artificial stress (132). It is an environment alien to the individual. Hence, the next logical step in understanding hormone rhythms is to measure hormone levels in ambulatory individuals in their own environments. Salivary cortisol measurements, relatively simple to administer and non-invasive in nature, have been widely used in recent years (133). Unfortunately however, frequent saliva collection, each requiring the ritual of fasting 30 minutes prior to collection is impractical. Moreover, it is not possible to collect saliva during sleep.

There is a large literature not only on altered levels of cortisol in endocrine conditions such as Cushing's disease, but also during psychiatric disorders such as depression and other stress related psychiatric disorders. Many of these studies have been bedevilled by an inability to measure cortisol at genuine nadir periods during early sleep and this new technique which allows continuous sampling to take place painlessly without the awareness of an individual, within their own home environment, should not only provide improved research information but also allow the development of one-stop clinics for the diagnosis of conditions such as Cushing's disease.

# CHAPTER 4      VALIDATION OF FREE CORTISOL MEASUREMENTS BY MICRODIALYSIS – PHARMACOLOGICAL STUDIES

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## 4.1 INTRODUCTION:

In man there have been recent publications of free cortisol measurement in healthy controls and cohorts of patients undergoing medical (not on intensive care) and surgical stress (elective coronary artery bypass graft) (88), of critically ill patients (116) including those in septic shock (125,134), and cohorts of burns patients (124). Free cortisol was measured by equilibrium dialysis (88,116) or ultrafiltration (134) in blood, by microdialysis in the subcutaneous adipose tissue (125), dermis (124) and brain (111). However, none of these studies were dynamic, looking at multiple samples over a short period of time to examine changes over time.

Although the plasma responses of total cortisol to stimulation with synthetic ACTH or suppression by exogenous dexamethasone are very well established and indeed much used as diagnostic tests in clinical practice, there is very little information as to the effect of the agents on free cortisol levels in extracellular fluid. Microdialysis has been used to measure free cortisol in the subcutaneous (**SC**) tissue in man as mentioned previously, but had not been systematically validated for the purpose, which was the aim of this study. These studies were designed to investigate the relationship between total cortisol levels observed in blood and free cortisol in SC tissue of the upper arm and anterior abdomen, and in the intravenous compartment.

These experiments were conducted under two physiological situations. In the first set of experiments (this chapter), changes in response to pharmacological manipulations were studied and in the next (Chapter 5), 24-hour endogenous cortisol profiles were investigated.



## 4.2 SUBJECTS AND METHODS

Non-smoking male volunteers of normal BMI, aged 18 to 24 years, were recruited as per local ethical committee regulations (Ref 08/H0101/16). They had no known medical conditions and were on no regular treatment. There was no history of corticosteroid use. They arrived at the clinical research unit an hour or more in advance, and intravenous cannulae and microdialysis catheters were inserted and set up at least 45 minutes before beginning the experiment. A flow rate of 2  $\mu$ l/min for the perfusion of the dialysis probe was used for all experiments in this chapter (Chapter 2 contains the detailed set-up of the microdialysis system).

Two sets of experiments (results in section 4.3.1 and 4.3.2) were conducted, the details of which are in the following paragraphs and are depicted in Figure 4.1 and Figure 4.5. The two experiments were different in two ways, in that the second experiment had additional intravenous microdialysis catheter (4.3.2) and that the timing of the dialysates in relation to blood sampling was different in the two experiments (described later in this subsection).

Intravenous cannula for blood sampling was inserted in the left ante-cubital fossa. In experiment 4.3.1, a linear microdialysis catheter (described in chapter 2) was inserted subcutaneously in the middle part of left upper arm. In 4.3.2, the site chosen was lower anterior abdomen and in addition, a concentric microdialysis catheter (described in chapter 1) was inserted intravenously in the right ante-cubital fossa (arm opposite to that used for blood sampling). Microdialysates were collected manually in 300  $\mu$ l polypropylene vials (Royem Scientific limited, Luton, Bedford UK). Both serum and microdialysate samples were collected at ten-minutely intervals.

The sampling clock-periods for the individual experiments were as follows - experiment 4.3.1 - 10:00 to 15:00 (n=6); experiment 4.3.2 - 09:55 to 15:05 (n=8). As blood sampling is episodic but microdialysate sampling continuous, the sample reading of the latter was considered to represent the midpoint of a given sampling duration e.g. sample timing of 10:05 was for dialysate obtained between 10:00 and 10:10 and so on. In all experiments, serum samples were collected at 10:00, 10:10 etc. The

dialysate samples in 4.3.1 correspond to 10:05, 10:15 and so on, and in 4.3.2, the sample timings coincide with that of blood, exactly.

Saliva samples were collected every thirty minutes during both sets of experiments using Salivette® synthetic swab (Sarstedt, Nümbrecht, Germany) starting at 10:00. Cortisol samples were processed as described in chapter 2.

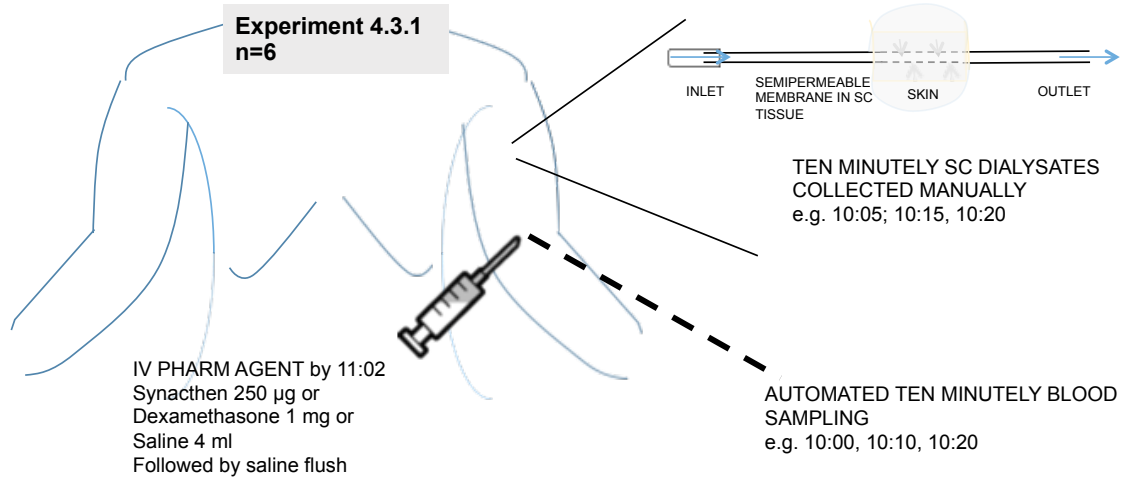
In both experiments, one of the pharmacological agents [Synacthen 250 µg (Ciba-Geigy, Basel, Switzerland), dexamethasone 1mg (1ml of 3ml saline mixed with 1ml (4mg/ml) dexamethasone, Organon laboratories, Cambridge UK), Saline (Sodium chloride 0.9%, Pfizer Ltd, Kent UK)] was administered by 11:02.

The participants remained seated in a chair or reclining on a bed throughout the duration of sampling apart from during comfort breaks, when microdialysis sampling was not interrupted. Standard meals were served at midday for all experiments. Subjects were allowed to carry out work-related activities on their personal computers.

All analyses were performed with Stata 13 (Stata Corp, College Station, TX, USA). The only assumption made was that the direction of correlation was from serum to SC tissue i.e. the pattern in the former would precede the latter. Correlations among serum, SC, and saliva values were estimated using Spearman tests. For each experiment, correlation coefficients were reported in tables and graphs for each subject and summarised as median and interquartile ranges (**IQR**). Median and IQR were computed as the data was not normally distributed. Correlation coefficients were also estimated between cortisol values in various compartments for different time lags. For section 4.3.1, the range of time lag was from 5 to 125 minutes (i.e., values of serum cortisol at time zero were correlated with successive 10-minute values of SC free cortisol up to 125 minutes). Due to the design of the experiment, there was a minimum of five minutes difference between successive serum total and SC free cortisol measurement. For section 4.3.2, the time lag range was from isothermal measurements (lag zero) to lag 120 minutes. A limit of either 120 or 125 minutes was decided as there were no further values in the time series to examine the relationship.

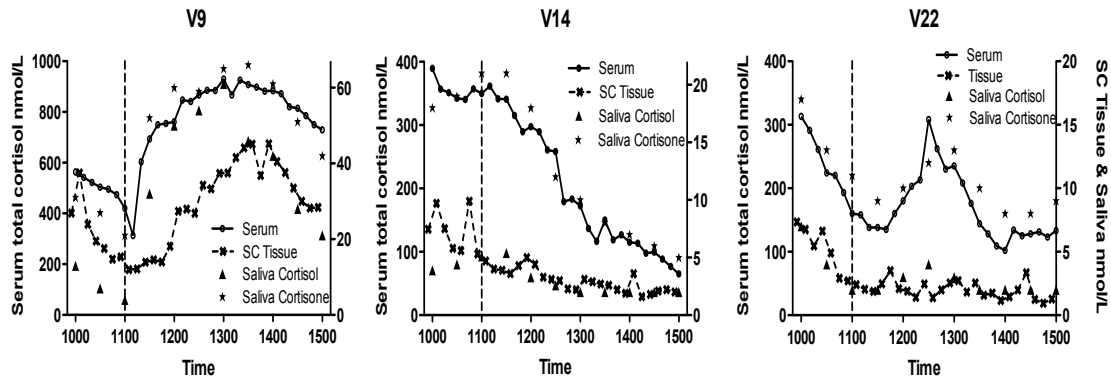
## 4.3 RESULTS OF PHARMACOLOGICAL MANIPULATION ARM

### 4.3.1 FREE CORTISOL IN THE SC TISSUE COMPARTMENT



**Figure 4.1.** SCHEMATIC OF EXPERIMENT 4.3.1.

Blood sampling site was left ante-cubital fossa, and subcutaneous microdialysate sampling site was left upper arm. Sampling period was from 10:00 to 15:00 during which ten minute samples were collected corresponding to sampling times of 10:00, 10:10 etc. for serum and 10:05, 10:10 etc. for SC free cortisol. Lunch was served at 12:00 (sandwich, orange juice, banana/apple). A pharmacological agent (250 µg Synacthen, 1 mg Dexamethasone or 4 mls saline) was injected intravenously by 11:02. No. of participants in each group =6.



**Figure 4.2.** TYPICAL PROFILES OF CORTISOL IN SERUM (TOTAL), SC TISSUE (FREE) & SALIVA (FREE CORTISOL AND CORTISONE).

250 µg Synacthen (participant identification V9), 1 mg dexamethasone (participant identification V14) and saline/placebo (participant identification V22) were administered by 1102 hours. Sampling commenced at 1000 hours, standard lunch was served at midday. Ten minutely samples were collected e.g. at 1000, 1010 etc. from serum and at 1005, 1015 etc. from the SC tissue. Saliva samples were collected every half an hour starting at 1000. Dashed line at 1100 denotes the time of administration of the pharmacological agent.

Between 1000 and 1100, before the administration of a pharmacological agent, the trend for cortisol is downwards, as expected at this time of the day. Following stimulation by Synacthen, there is a marked rise in cortisol levels almost instantaneously and in all compartments (Figure 4.2 participant identification V9). Peak levels are achieved between 2 and 3 hours following the agent, with a gradual decline in the final hour of sampling. The median serum total cortisol level immediately before Synacthen was 321 nmol/l (range 188-773) and at peak was 973 nmol/l (range 778-1369) between 2 and 3 hours post injection. The median SC tissue free cortisol level pre-Synacthen was 11.55 nmol/l (range 2.26-38.53) and at peak was 67.38 nmol/l (18.66-101.49) between 1 and 3.25 hours post injection.

The downward trend in pre-dexamethasone levels in both serum and SC tissue continued post-dexamethasone but was more marked in serum (Figure 4.2 participant identification V14). Median serum total cortisol level pre dexamethasone was 265 nmol/l (range 169-434) and nadir level post dexamethasone was 66 nmol/l (range 34-78). For SC tissue free cortisol values, median pre dexamethasone level was 6 nmol/l (range 3.17-24.4) and nadir 2 nmol/l (range 0.88-4.4). Although in the placebo group the general trend for cortisol levels was downward, compared to the former group, there was a clear pulsatile activity around lunch-time (midday) in all participants (Figure 4.2 participant identification V22). This pulse was prominent in serum but less so in SC tissue. During this pulse, peak level was achieved between 30 and 60 minutes after lunch was served, and had returned to baseline (pre-lunch levels) latest by 2 hours post-lunch.

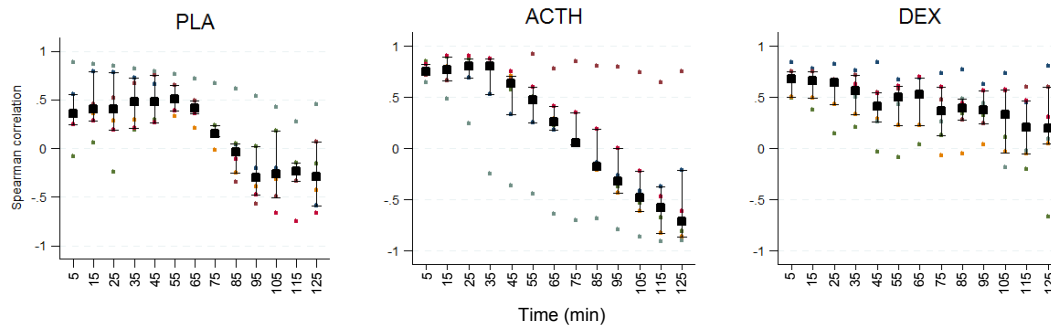
Spearman correlation coefficients between serum and SC tissue measurements were calculated for the entire group in each treatment arm (Table 4.1). The strongest correlation was in the ACTH group and weakest in the placebo group.

TABLE 4.1. CORRELATION BETWEEN SERUM AND SC CORTISOL AT TIME LAG 5 min		
TREATMENT (n=6)	SPEARMAN CORRELATION COEFFICIENT	p VALUE
ACTH	0.75	<0.0001
DEXAMETHASONE	0.68	<0.001
PLACEBO	0.36	0.102

**Table 4.1.** CORRELATION BETWEEN SERUM AND SC CORTISOL AT TIME LAG 5 min (minimum time difference in this study)

The next step was to address the existence of any delay between serum and SC levels i.e. would the correlation be better between serum value at a given time and SC value at a later time? A maximum limit of 125 minutes was considered as that was the last sample available in the series to test the relationship. Spearman correlation coefficient values (median and IQR) were computed (

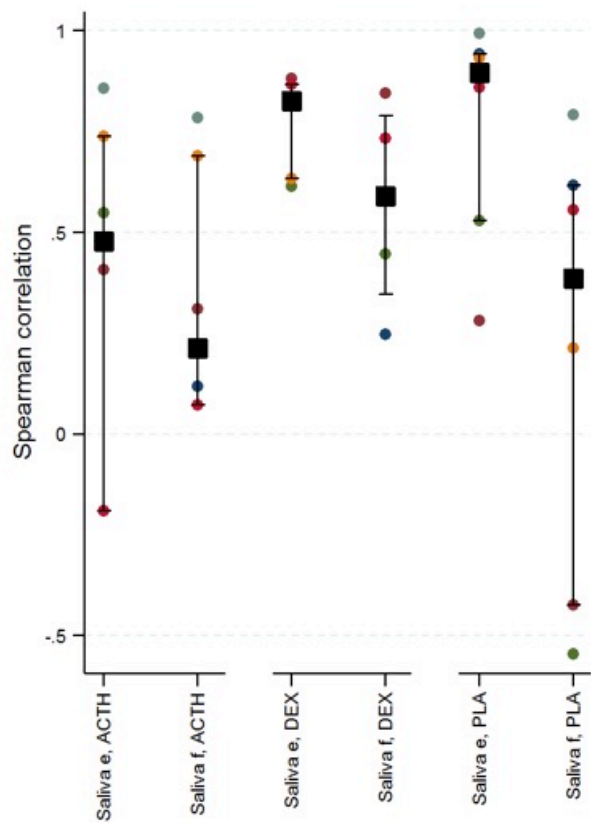
**Figure 4.3**) at successive 10-minute time delay such that SC would lag behind serum from a minimum of 5 to maximum of 125 minutes. There was best positive correlation at a delay of 5 minutes, indicating against a significant lag between a change in serum level to be reflected in the SC tissue of the upper arm. For ACTH group however, there was a linear trend such that the median correlation value at 35minutes (+0.75) was nearly equally inverse of that at 125 minutes (-0.71). This suggests that 125 minutes after a secretory pulse of cortisol SC value would decrease.



**Figure 4.3.** SPEARMAN CORRELATION COEFFICIENT VALUES (Y AXIS) BETWEEN SERUM AND SC AT SUCCESSIVE 5 MINUTE TIME DELAY FROM 5 TO 125MINUTES (X-AXIS).

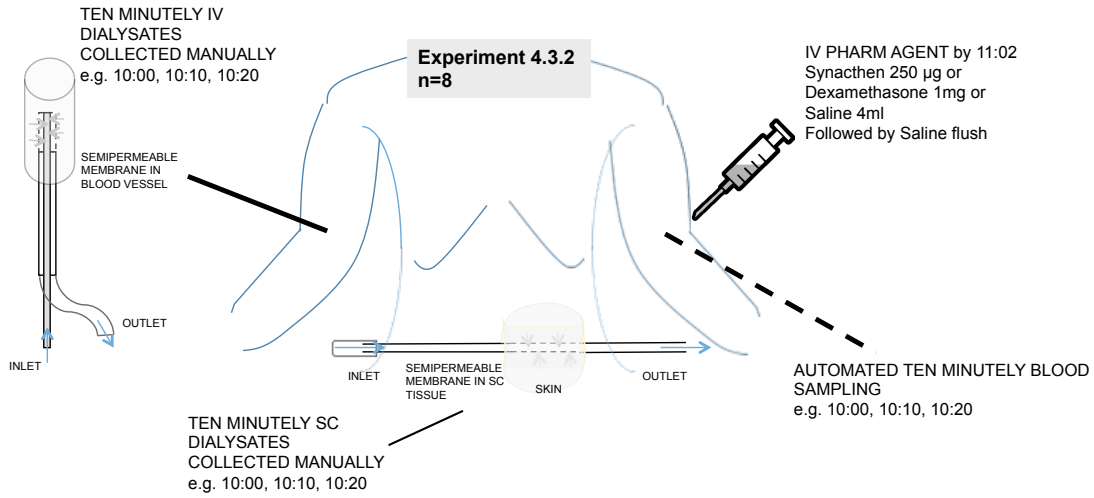
Squares indicate median values, vertical bars the interquartile range and dots are individual participant values. There is no significant trend in placebo and dexamethasone groups, with the best positive correlation between 35-45minutes delay. For Synacthen group, there is a linear trend such that the median correlation value at 35minutes (+0.75) is nearly equally inverse of that at 125minutes (-0.71).

Finally, the relationship between SC tissue free cortisol and that of saliva free cortisol and saliva cortisone was examined. Spearman correlation coefficients were calculated (median and IQR) as in Figure 4.4. For each of the three pharmacological agent groups, the median correlation between saliva cortisone and SC is better (+0.5, +0.83 and +0.9 for the Synacthen, dexamethasone and placebo groups respectively) than that between saliva free cortisol and SC (+0.21, +0.6 & +0.4 respectively). At baseline, cortisone levels were higher than cortisol levels in saliva and the increase in the latter following Synacthen was more than that in the former fraction.



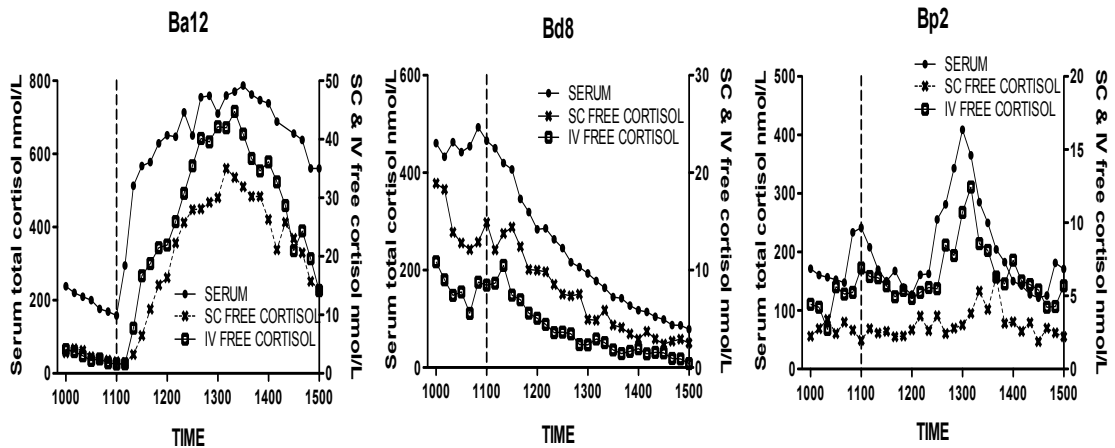
**Figure 4.4.** SPEARMAN CORRELATION BETWEEN SC AND EITHER SALIVA FREE CORTISOL (f) OR CORTISONE (e) FOR EACH OF THE TREATMENT GROUPS (SynACTHen stimulation, DEXamethasone suppression or PLAcebo. Squares indicate median values, vertical bars the interquartile range and dots denote individual participant values.

### 4.3.2 FREE CORTISOL IN THE SC AND INTRAVENOUS COMPARTMENTS



**Figure 4.5.** SCHEMATIC OF EXPERIMENT 4.3.2.

Blood sampling site was left ante-cubital fossa, intravenous (IV) microdialysate sampling site was right ante-cubital fossa and subcutaneous microdialysate sampling site was lower anterior abdominal wall. Sampling period was from 09:55 to 15:05 during which ten minutely samples were collected corresponding to sampling times of 10:00, 10:10 etc. Lunch was served at 12:00 (sandwich, orange juice, banana/apple). A pharmacological agent (250 µg Synacthen, 1 mg dexamethasone or 4 mls saline) was injected intravenously by 11:02. No. of participants in each group=8.



**Figure 4.6.** TYPICAL PROFILES OF SERUM TOTAL CORTISOL AND FREE CORTISOL IN SC TISSUE & BLOOD.

250 µg synacthen (Participant identification Ba12), 1 mg dexamethasone (Participant identification Bd8) and saline/placebo (Participant identification Bp2) were administered by 1102 hours. Sampling commenced at 1000 hours, standard lunch was served at midday. Serum and SC dialysate samples were collected at 1000, 1010 etc. Saliva samples were collected every half an hour starting at 1000.



As in the earlier study, between 1000 and 1100 before the administration of a pharmacological agent, the trend for cortisol is downwards (Figure 4.6 Participant identification Ba12). Following stimulation by Synacthen, there is a marked rise in total serum cortisol levels as well as free cortisol in blood and SC tissue. Maximum levels are achieved and maintained between 2 and 3 hours following the stimulus, with gradual decline in the final hour of sampling. The median serum total cortisol level immediately before Synacthen was 238 nmol/l (range 95-375) and at peak was 872 nmol/l (range 600-960) between 110 and 150 minutes post injection. The median SC tissue cortisol level pre-Synacthen was 6.07 nmol/l (range 0.61-16.75) and at peak was 44.55 nmol/l (28.81-95.69) between 120 and 190 minutes post injection, and those for intravenous free cortisol were 2.69 nmol/l (range 0.61-16.75) before, and 53.6 (range 29.34-101.68) after the agent between 120 and 160 minutes respectively.

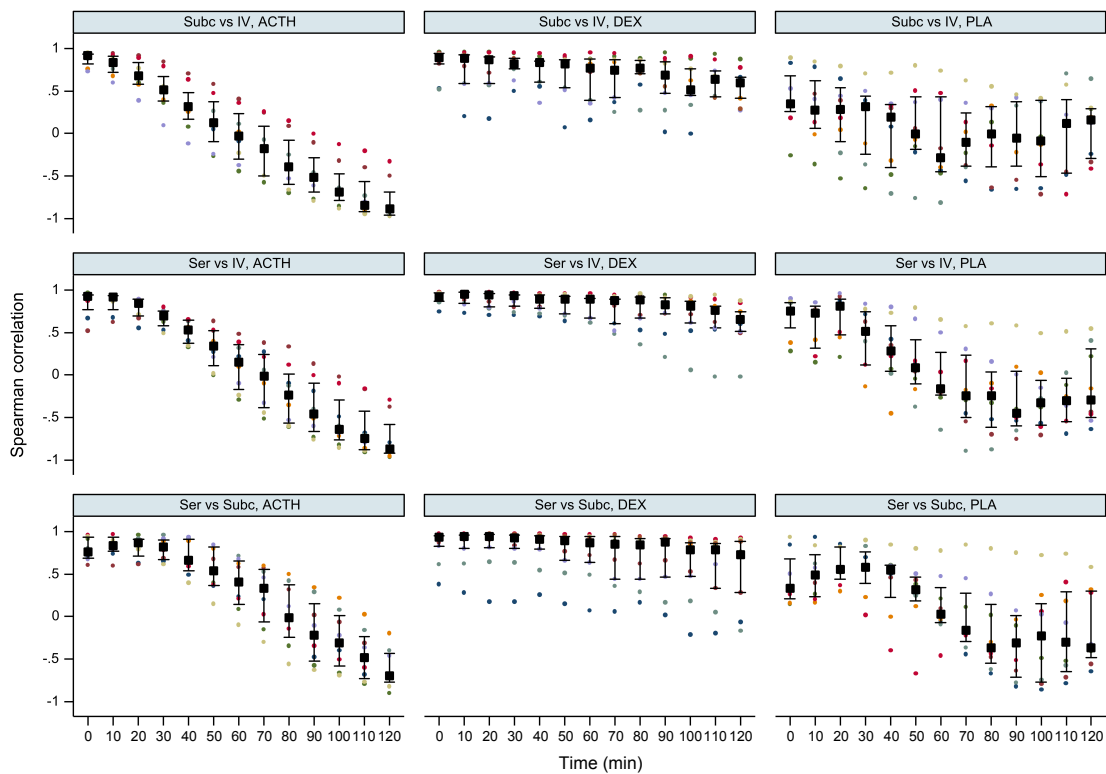
The downward trend in pre-dexamethasone levels in both serum and SC tissue continued post-dexamethasone (Fig 4.6. Participant identification Bd8) as in the previous experiment. Median serum total cortisol level pre dexamethasone was 265 nmol/l (range 144-475) and nadir level post dexamethasone was 62.59 nmol/l (range 37.26-122.8). Median pre dexamethasone level for SC tissue free cortisol was 5.52 nmol/l (range 3.17-11.84) and nadir 0.61 nmol/l (range 0.13-1.76) whereas those for intravenous free cortisol were 5.80 nmol/l (range 1.66-9.27) and 0.52 nmol/l (range 0.29-1.32), respectively. As in the former group, the difference between placebo and dexamethasone groups was the clear peak around lunch-time (midday) in all participants on a background of declining cortisol levels for the duration of sampling (Figure 4.6 Participant identification Bp2). In keeping with the above experiment, this activity was prominent in serum total cortisol levels and in intravenous free, but less so in SC free cortisol levels.

Spearman correlation coefficients were calculated for paired combinations of serum total, SC tissue free and intravenous free cortisol for each treatment group (Table 4.2). Once again, the weaker correlation was for all in the placebo group but the strongest correlation was for all 3 relationships in the stimulation and suppression groups.

TABLE 4.2. CORRELATION COEFFICIENTS AT TIME LAG 0			
TREATMENT	SERUM & SC Free	SERUM & IV Free	SC Free & IV Free
ACTH	0.68 (p=0.001)	0.76 (p<0.001)	0.82 (p<0.001)
DEXAMETHASONE	0.82 (p<0.001)	0.86 (p<0.001)	0.82 (p<0.001)
PLACEBO	0.21 (p=0.322)	0.55 (p=0.008)	0.25 (p=0.309)

**Table 4.2.** CORRELATION COEFFICIENTS AT TIME LAG 0.

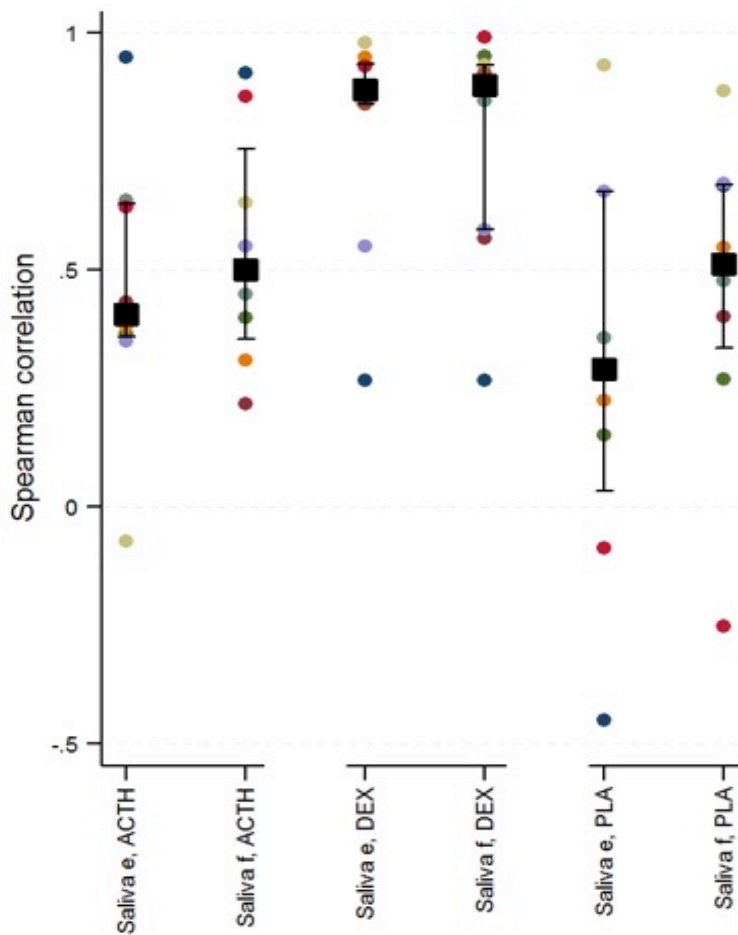
The next step was to address the existence of any delay between serum and SC; serum and intravenous free levels and between SC and intravenous free levels. Spearman correlation coefficient values (median and IQR) were computed for time-lag range of 0 to 120 minutes (Figure 4.7) at successive 10-minute time delay such that SC would lag behind serum from a minimum of 10 to maximum of 120 minutes. There was no significant trend in placebo and dexamethasone groups, with the best positive correlation at time 0. For Synacthen group however, there was a linear trend such that the median correlation value at 35 minutes (+0.52) is nearly equally inverse of that at 120 minutes (-0.58). This suggests that 120 minutes following a pulse of cortisol, SC value would be expected to decline. These results are again in keeping with those found in the earlier study, pointing against it being a chance finding.



**Figure 4.7. CORRELATION COEFFICIENT**

Spearman correlation coefficient values (y axis) between serum and sc (bottom row); serum and intravenous (iv) free (middle row) and, sc and iv free (top row) cortisol at successive 10-minute time delay from 0 to 120 minutes (X-axis). SynACTHen group results are in the left column, DEXamethasone in the middle column, and PLACebo in the right column. Squares indicate median values, vertical bars the interquartile range and dots are individual participant values. There is no significant trend in placebo and dexamethasone groups. For ACTH group, there is a linear trend such that the median correlation value at time 0 is nearly equally inverse at 120minutes.

Finally, the relationship between SC tissue free cortisol and saliva free cortisol and saliva cortisone was examined. Spearman correlation coefficients were calculated (median and IQR) as in Figure 4.8. The strongest median correlation was found for the dexamethasone group (0.88), and it was true for both saliva fractions. The relationships for the other two treatment groups were weaker (0.3-0.5) for both saliva fractions. At baseline, cortisone levels were higher than cortisol levels in saliva and the increase in the latter following Synacthen was more than that in the former fraction.

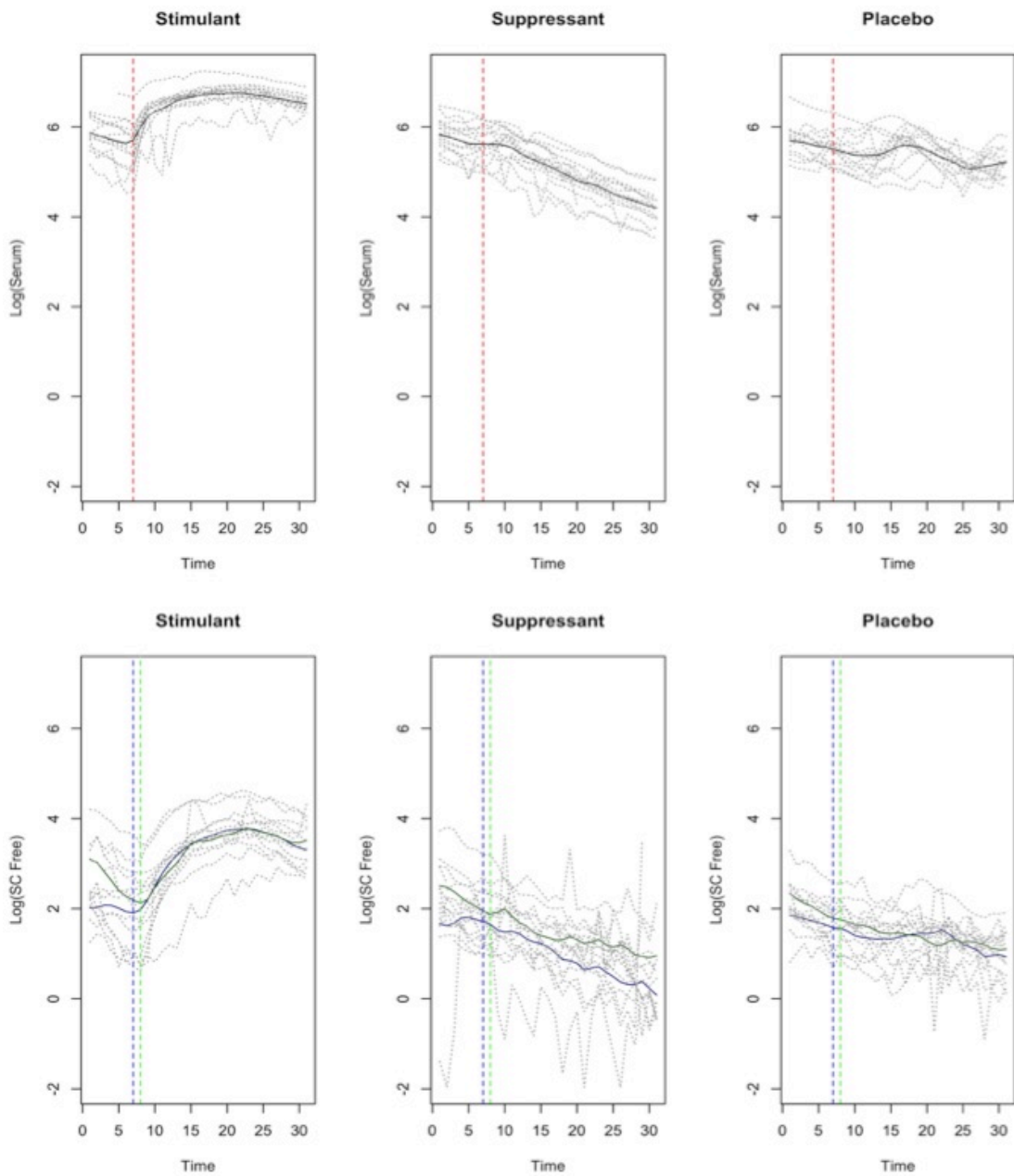


**Figure 4.8. SPEARMAN CORRELATION**

Values (Y-axis) between SC and either saliva free cortisol (f) or cortisone (e) per treatment groups (ACTH stimulation, DEXamethasone suppression or PLAceto. Squares indicate median values, vertical bars the interquartile range and dots denote individual participant values.

In order to further examine the relationship between serum total and SC free cortisol, data from the two experiments were combined. Log transformation of this entire data set was carried out and smoothed time-wise mean of these log-transformed values were plotted using the statistical package R (Figure 4.9). There is clear effect of treatment in each of the three groups. This is less clear in the free cortisol values between dexamethasone and placebo groups. In the dexamethasone group, the mean values from both the experiments continue to decline throughout the experiment like in the placebo group, apart from the mealtime peak in the latter

group. The mean SC free cortisol levels appear to be different (lower) in the second experiment, more noticeable in the hour prior to administration of the pharmacological agent. Despite the difference, the mean peak values from the two experiments following Synacthen overlap suggesting maximum stimulation at two hours (regardless of baseline levels). They maintain segregation in the dexamethasone group, however in the placebo groups there is a hint of an upward deflection in the second experiment set.

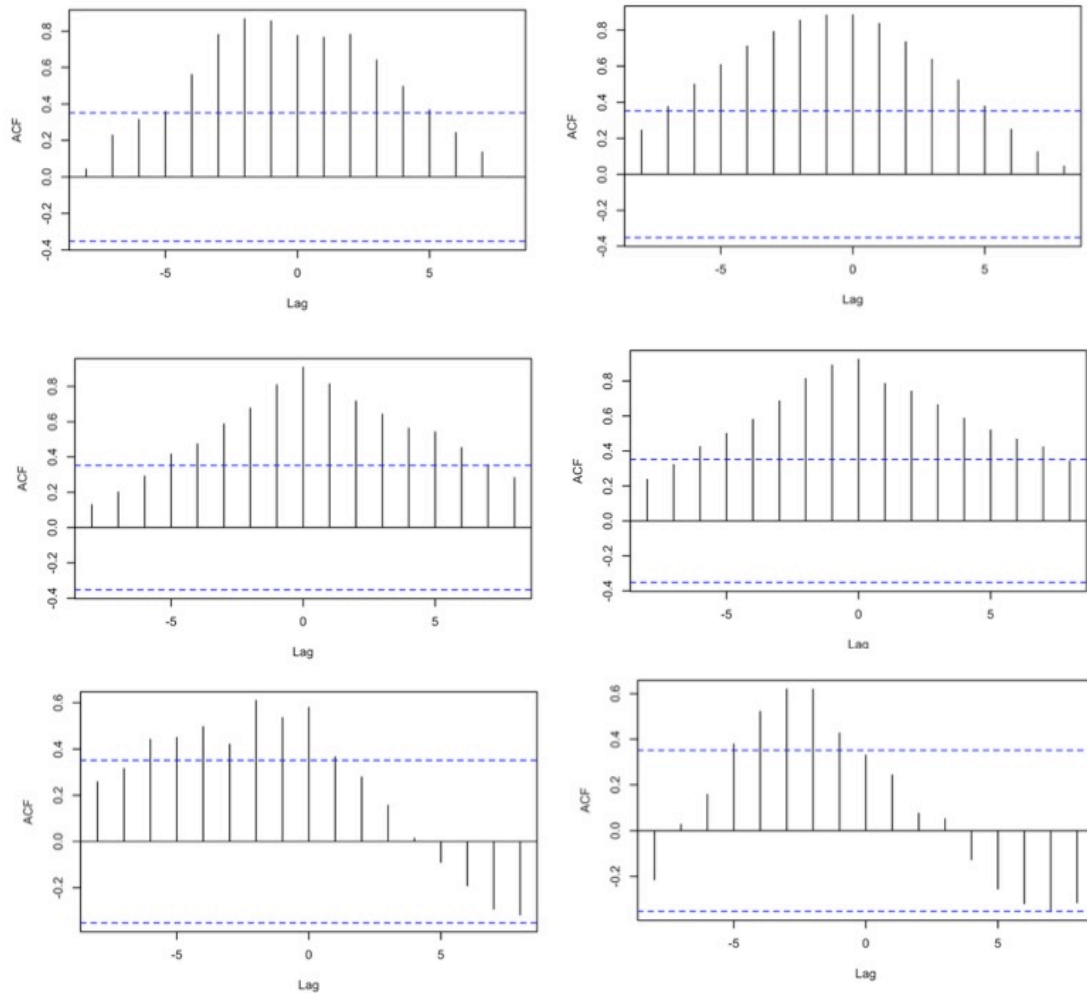


**Figure 4.9.** LOG TRANSFORMED MEAN VALUES OF SERUM TOTAL (UPPER PANEL) AND SC FREE CORTISOL (LOWER PANEL) FROM THE TWO PHARMACOLOGICAL MANIPULATION EXPERIMENTS.

In the bottom panel, green lines=experiment 4.3.1 and blue lines=experiment 4.3.2. Dotted vertical lines (two in the bottom panel to indicate the difference in sample timing in relation to the intervention) indicate time of pharmacological intervention e.g. Synacthen, Dexamethasone or placebo. X-axis displays time in minutes, such that 1=10minutes.

Cross-correlation plots of serum total and SC free cortisol for each individual in each of the treatment arms of the two studies were created using the statistical

package R (Figure 4.10). This data confirmed previous analysis by demonstrating significant positive cross-correlation between serum and SC values across all participants in the Synacthen and dexamethasone groups. However, there was a lot of variability in the placebo group with poorer cross-correlation across the group.



**Figure 4.10. CROSS-CORRELATION**

Cross-correlation plots of log transformed serum total and sc free cortisol for representative individuals from the two experiments described in the text. ACF (autocorrelation factor) is on Y-axis, time lag on X-axis (lag 1=10min). Plots on the left are three typical individuals per treatment group from experiment 4.3.1 and those on the right from 4.3.2. The top row=Synacthen group, middle row=dexamethasone group and the bottom row=the placebo group. Blue dotted lines indicate confidence intervals. Where vertical lines exceed blue dotted line (top two rows), there is a significant cross correlation.

#### 4.4 DISCUSSION

The only studies which have compared the dynamic responses of both blood and tissue glucocorticoid levels have been performed in the rat. A landmark series of studies of free corticosterone rhythms in different body compartments, has been performed in this animal using a stress paradigm in which animals are forced to swim in water at 25°C. In these studies peak free hormone levels in the intravascular compartment, SC tissue (91) and in the brain (42,43) were found 20 minutes later than the peak of total hormone in the plasma.

Rather than using a stress manoeuvre we used the Synacthen test, first described by Wood, used clinically to ascertain adrenocortical reserve (135) and which can be performed at any time of the day (136). It has been shown to correlate well with response to major surgical stress (88). There is a large literature relating to serum total cortisol levels at time 0 and 30 (and or 60) minutes after Synacthen, but little is known about the effect on the pattern of free cortisol levels in a tissue, other than saliva but again these are not multiple continuous samples (133).

A plot of the raw data of individual measurements of free cortisol in intravenous and subcutaneous tissue compartments appear to follow serum total cortisol closely, with the expected lower levels of free hormone at all time points (Figure 4.2, Figure 4.6). The profile of Synacthen test is entirely different from the remaining groups, which are similar with a gradual decline throughout the duration of sampling. Dexamethasone is a substrate for both the 11  $\beta$  HSD isoenzymes, but the effect as measured in serum is small (137), although tissue-specific effects - the hallmark of these enzymes - are as yet unclear.

There was good correlation between serum total and free cortisol (SC and intravenous) in the Synacthen and dexamethasone groups, with no discrimination between compartments. The relationship between serum total and free cortisol (SC and intravenous) is not as strong in the placebo group, which is expected in a short sampling duration due to individually distinct secretory pulse patterns. With stimulation and suppression, the axis was entrained resulting in better correlation,



whereas in the placebo group due to inter-individual variation in pulses is likely to have affected the correlation values for this group.

There was evidence of some secretory activity around noon in the placebo group – the only difference between placebo and dexamethasone groups. This clearly visible peak in serum was not discernible in the SC tissue in the majority, but was visible in the intravenous free fraction. This was especially true when the serum total cortisol peak was 400 nmol/l or more and, when serum total cortisol was at or above this level a change in SC levels was more noticeable (Figure 4.6 Participant identification Bp 2). One of the major regulators of the ratio of free to total cortisol is CBG. Changes in the level of CBG or its affinity for cortisol via neutrophil elastase or changes in temperature can alter free cortisol fractions relatively more than that in serum total cortisol (138). Similar evidence for a drop in albumin levels affecting free cortisol levels is not as strong (88). In our cohort of normal individuals, there is no reason to expect abnormalities of albumin or CBG levels. It has been demonstrated *in vitro* that at body temperature, CBG binding capacity is exceeded when total cortisol exceeds 400-500 nmol/l (83). I did not monitor body temperature but change in core and surface temperature was not expected through the duration of sampling.

A (supra-physiological) pulse of total cortisol resulted in a rise in free cortisol as expected. An interesting prediction however was that in both SC and intravenous compartments, between 120 to 125 minutes after a concurrent rise in free fraction levels would fall. This effect was limited to the group stimulated with Synacthen. Pharmacodynamics of Synacthen (1-24) are somewhat different to endogenous ACTH (1-39), with high levels evident up to 60 min following an intravenous injection of high dose Synacthen (139). Serum total cortisol levels post 250m µg of Synacthen continue to increase at least until 75 minutes. Therefore, an endogenous cortisol pulse of a shorter duration and consequent drop to low levels (38,40), with no further hormone secretion would lead to drop in free cortisol levels in the SC tissue. This phenomenon may be related to the saturation kinetics of CBG. Total and free cortisol remain in equilibrium in the serum, beyond which excess free unbound cortisol would readily permeate various tissues resulting in a rise in tissue levels soon after a pulse. With no

further adrenal secretion of cortisol there would likely be no surplus to reach a tissue like SC tissue. The half-life of free cortisol is not known but would be expected to play a role in determining this relationship.

Methodological factors may be responsible for some of the results obtained. Both the free cortisol fractions were assayed in the same batch by the same method, and hence this would be a true physiological phenomenon rather than an assay variation. The membrane length of the SC and intravenous microdialysis probes was different (described in Chapter 2), which may have resulted in universally lower absolute free cortisol levels intravenously, as the membrane area was half that of the SC probe. This was not the case. And as demonstrated in Chapter 2, the difference in recovery was minimal (about 10%). This was not enough to warrant an adjustment for membrane length. Additionally, there were some individuals in whom intravenous free levels were higher than SC levels and vice versa. In other words, there was no indication of a methodological error. All of our participants received a standard lunch (commercially available 'healthy' range sandwich, orange juice and piece of fruit), although a complex and not yet fully understood mechanism of the midday/meal related peak is noted (32,140,141).

The relationship between saliva cortisol and cortisone with SC free cortisol was not the primary focus of this study. Infrequent (half hourly) sampling and differential (unidentical) timing in relation to dialysates in the two experiments meant that pooling the data was of no benefit (unlike the pooling of serum total and SC free cortisol). Cortisone appeared to be a better correlate of SC free cortisol. Indeed it has previously been shown to be superior to saliva cortisol as a predictor of serum free cortisol in a study that confirmed the importance of 11- $\beta$  HSD enzymes regulating tissue levels of cortisol (142). However, the strength of the association in this study was generally poorer, not allowing insightful conclusions. If both cortisol and cortisone values were available for the two body compartments, and had the timing of samples been consistent, it may have helped understand the role of the 11- $\beta$  HSD enzymes in the SC tissue.

The overarching aim of this study was to look at the profiles of free cortisol in the two body compartments (SC tissue and intravenous) in response to Synacthen stimulation and dexamethasone suppression, which show good correlation with total cortisol. I have found no significant delay between the changes being cascaded down to the compartments studied, although with this data it was not possible to detect a delay of less than 5 minutes. In summary, I have successfully demonstrated that major secretory activities as well as switching off of endogenous secretory activities are accurately reflected in the free cortisol profiles measured by our technique, hence validating the novel method. However, smaller endogenous pulses appeared to not be readily detected in the SC tissue. I then set about investigating the relationship between serum total and SC free cortisol when native endogenous secretory activity is undisturbed by pharmacological manipulation (following chapter).

## CHAPTER 5      CIRCADIAN FREE CORTISOL PROFILES

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### 5.1 INTRODUCTION

The next step in validation was the use of subcutaneous microdialysis for measurement of cortisol profile over a period of 24 hours using the portable automated collection device described in chapter 3. In pursuit of this, two separate studies were performed. In the first (this chapter), simultaneous sampling of total serum cortisol and SC free cortisol was carried out at a research facility. In the second study (Chapter 6), the rhythm of cortisol over three consecutive days in volunteers free to go about their day-to-day activities was recorded.

Cortisol has a characteristic circadian and ultradian rhythm over a given 24-hour period and is intricately linked to the sleep-wake/activity cycle (25,143). In man, under normal circumstances, this rise in cortisol begins during the latter half of sleep continuing into the early awake phase, sometimes until about noon, gradually declining thereafter through the day to lowest levels during the hours surrounding sleep onset (24,26). In nocturnal species e.g. rodents, peak corticosterone secretion also occurs around the beginning of their active period, which is of course during the dark period of the day (27). There are various methods of statistically defining 'normal' cortisol circadian periodicity (24,33,144,145), with no one that is preferred over the other.

The period surrounding the onset of sleep is characterised by minimal ultradian activity of the axis and the lowest levels of the day are found at this time (30,38). This is truly a quiescent period when both cortisol and ACTH are maintained at very low constant levels, certainly during the first two hours of sleep (146) - random activity may be present before onset of sleep in some individuals. There is a moderate inhibitory effect from the SCN on cortisol levels at the onset of sleep period (147). Within a circadian cycle the most striking change occurs during late sleep and early wake period when cortisol levels rise several-fold above the low early sleep time levels. The majority, about 75% (33), of total daily pulsatile release is observed during this period. Most individuals also show a post-meal surge at midday (31,32). During

this period, on a background of progressive morning decline cortisol levels rise sharply coincident with food intake achieving peak level 45 (31) to 60min (148) later. Knowledge of its relationship to activity/meal-timing/sleep schedule; diet composition (Krieger 1971, van Cauter 1992) and its interaction with  $11\beta$ -HSD enzymes (Stimson 2014), and consumption (or not) of a meal (Follenius 1982) is in evolution. Omitting a habitual midday meal may delay this peak by an hour (Follenius 1982). In contrast, the same meal consumed in the evening produces a variable response, with much greater inter- and intra-individual variability compared to that consumed around noon (32). The overarching impression of these experiments is that meal ingestion around midday augments the natural secretory burst of cortisol seen at this time. The remaining time period has more inter-individual variability and is less well understood.

Although the timing of individual ultradian peaks varies greatly, the 24-hour pattern in general is remarkably consistent, with an acrophase (peak levels of a day that start to rise before waking) followed by a gradual decline through the day, until its culmination in a nadir at night (lowest levels of the day). It is a robust rhythm independent of light perception on the basis of its demonstration in blind individuals (21) and in fully sighted individuals working in complete darkness for long periods (149,150). The sleep-wake transition i.e. awakening (morning or nocturnal) period is associated with elevated cortisol levels (48). Complete reversal of sleep pattern artificially or voluntarily as in shift-workers, after a time gap, leads to realignment of the circadian rhythm with the new schedule (21,26,30).

Until a few decades ago, equilibrium between bound and unbound free cortisol in serum was believed to be the prime determinant of the functional state of the HPA axis. The metabolic fate of the steroid is now known to be more than simple inactivation/degradation and elimination.  $11\beta$ -HSD enzyme system mediates the only reversible pathway of cortisol metabolism known to date. Type 1 is widely expressed in liver, adipose tissue, lung, skeletal muscle, vascular smooth muscle, anterior pituitary, brain and adrenal cortex and has comparatively lower affinity for cortisol (65). Although this enzyme is bidirectional, possessing both reductase (activating cortisone to cortisol) and dehydrogenase (inactivating cortisol to inert cortisone)

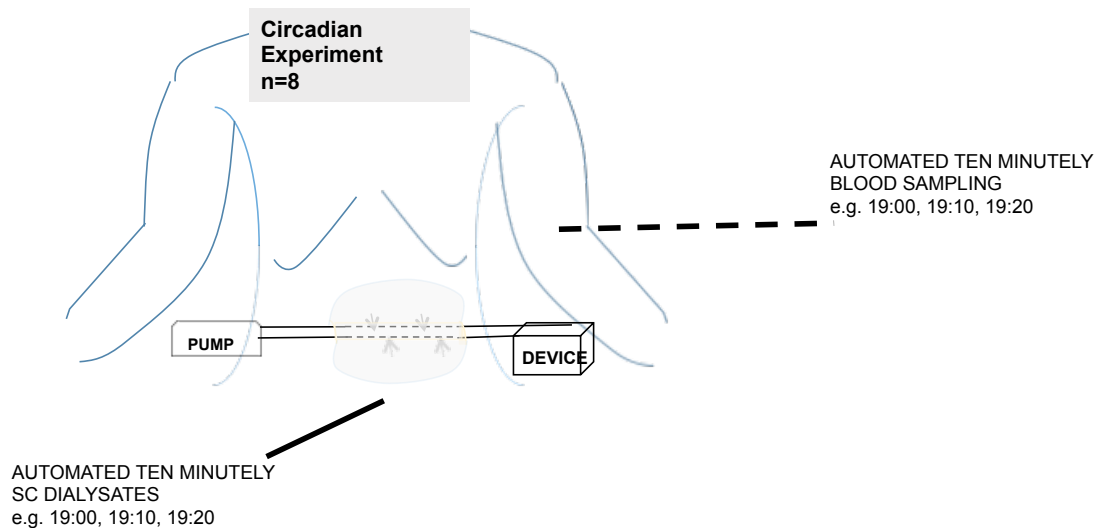
activities, it predominantly functions in vivo as a reductase. Type 2 HSD has high affinity for cortisol and is widely distributed in distal nephron in the kidney, sweat and salivary glands, colonic mucosa in adults and in placenta and foetus up to mid gestation. The distribution pattern of type 2 enzyme appears to be related to MR rich areas suggesting a protective role of this enzyme towards these tissues from inappropriate access by active cortisol.

## **5.2 METHODS**

The details in the following paragraphs are depicted in Figure 5.1

Non-smoking male volunteers aged 18 to 24 years were recruited as per local ethical committee regulations in Bristol (Ref 08/H0101/16). They had no known medical conditions and were on no regular treatment. There was no history of corticosteroid use. They arrived at the clinical research unit at least an hour in advance, and intravenous cannulae and microdialysis catheters were inserted at least 45 minutes before beginning the experiment.

An intravenous cannula for blood sampling was inserted in the left ante-cubital fossa. A linear microdialysis catheter (described in chapter 1) was inserted subcutaneously in the lower anterior abdomen, and connected to microdialysis pump 107 set at a flow rate of 2  $\mu\text{l}/\text{min}$ . An assembly of a 15 cm FEP tubing was prepared in advance on the day and connected to the collection device with a pink adaptor prior to catheter insertion. Once the catheter was inserted, outflow from the catheter observed, then using a MAB-8 connector FEP tubing was connected to device inlet creating a closed system. Samples were collected using the novel automated sampling system (described in Chapter 3), decanted within 24 hours in polypropylene vials and stored at  $-80^{\circ}\text{C}$ . In order to account for the in-built flush cycle of 5 minutes at the rate of 15  $\mu\text{l}/\text{min}$  prior to establishment of the desired flow rate, the first 75  $\mu\text{l}$  were discarded prior to storage. Both serum and microdialysate samples were collected at ten-minute intervals. Cortisol samples were processed as described in chapter 2.



**Figure 5.1.** SCHEMATIC OF EXPERIMENT 5.1.

Blood sampling site was left ante-cubital fossa and SC microdialysate sampling site was lower anterior abdominal wall. Sampling period was from 18:55 on day 1 to 19:05 on day 2 when ten minute samples were collected corresponding to sampling times of 19:00, 19:10 etc. Lights were switched off between 23:00 and 07:00 and meals were served as follows: breakfast at 07:00 (milk, cornflakes, banana/apple); lunch at 12:00 (sandwich, orange juice, banana/apple) and hot supper (ready meal) at 18:00. Snacks were allowed between meals and were sparingly consumed by a few.

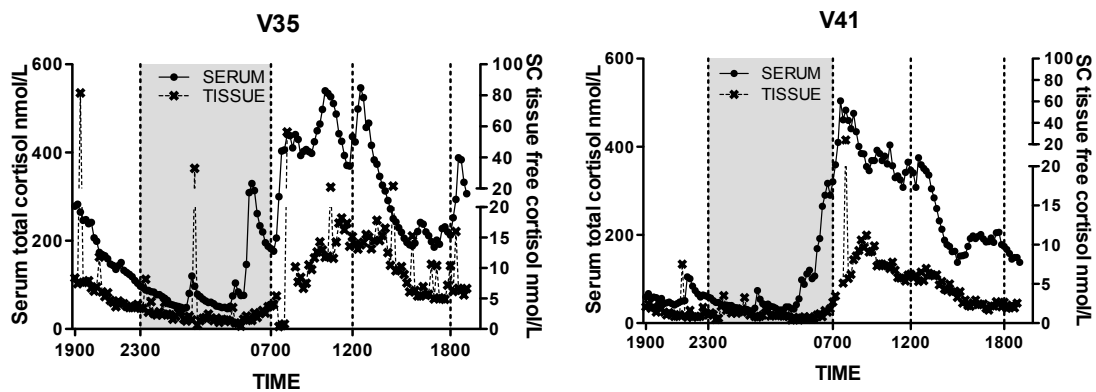
The sampling was carried out from 17:55 on day 1 to 19:05 on day 2 (n=8). As blood sampling is episodic and microdialysate sampling continuous, we chose to register the timing of the microdialysate samples to represent the midpoint of the duration of a given sample e.g sample timing of dialysate obtained between 09:55 and 10:05 was 10:00 and so on. Serum and dialysate samples were collected at 10:00, 10:10 etc.

The participants remained seated in a chair or reclining on a bed throughout the duration of sampling apart from during comfort breaks, when microdialysis sampling was not interrupted. Breakfast was served at 0700 (milk, cornflakes, banana/apple), lunch at midday (sandwich, orange juice, banana/apple) and hot ready meals at 1800 hours. Lights were switched off between 2300 and 0700 hours. Subjects were allowed to carry out work-related activities on their personal computers when awake.

### 5.3 STATISTICS AND RESULTS

Statistical analyses were performed using the command based program R (151).

On visual inspection of the circadian profiles, the characteristic circadian profile is evident in both compartments. The lowest levels are achieved and maintained in the hours surrounding sleep onset. Sustained rise from such low levels leading to peak levels begins prior to lights being switched on, although peak levels are achieved in every individual after waking – for most (n=5) within an hour of waking but later (up to an hour after lunch) in the remaining few (n=3). All participants showed a degree of response to the midday meal. Two profiles depicting the two patterns described above are shown below Figure 5.2.

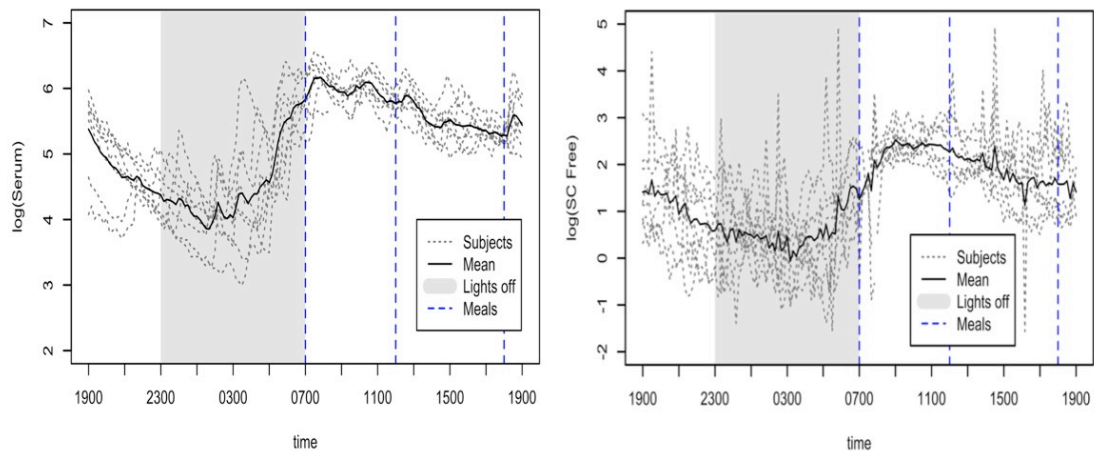


**Figure 5.2.** CIRCADIAN PROFILE OF SERUM TOTAL AND SUBCUTANEOUS FREE CORTISOL IN TWO HEALTHY INDIVIDUALS.

Clock time is displayed along the X-axis and serum total cortisol on left and SC free cortisol on the right Y-axes. Shaded area represents the lights-off period. Meal times are represented by dotted lines at 0700, 1200 and 1800.

A plot was created of the raw data of log transformed serum and SC levels with a point-wise mean (Figure 5.3).

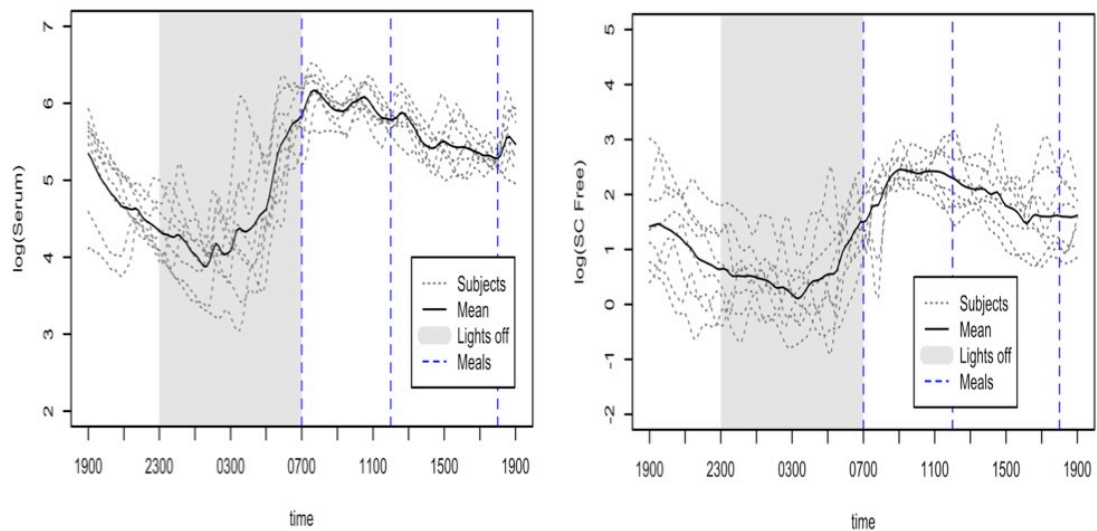




**Figure 5.3.** PLOT OF LOG TRANSFORMED SERUM TOTAL (LEFT plot) AND SC FREE (RIGHT plot) CORTISOL LEVELS.

Time is displayed along the X-axis. Shaded area denotes the lights off period. Dotted lines indicate meal times (breakfast at 07:00, sandwich lunch at 12:00 and hot meal at 18:00). Bold line is the average of point-wise mean for 8 individuals and dotted lines show individual profiles.

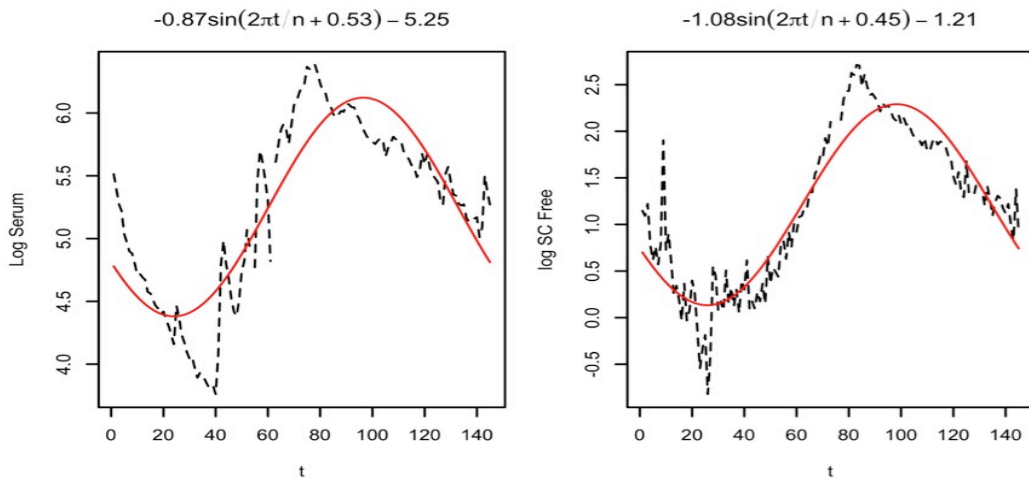
The SC time series had short bursts of single readings not corresponding to the activity in serum, such that SC baseline was significantly more unstable than the smoother profile of serum values. Hence the SC series was thought to have more ‘noise’. A simple plot of the kernel-smoothed log transformed serum and SC was created to account for temporal noise (Figure 5.4). For Kernel smoothing, time window of half an hour on each side of an observation was created and point-wise mean calculated. These plots display the circadian rhythm in both serum and SC series.



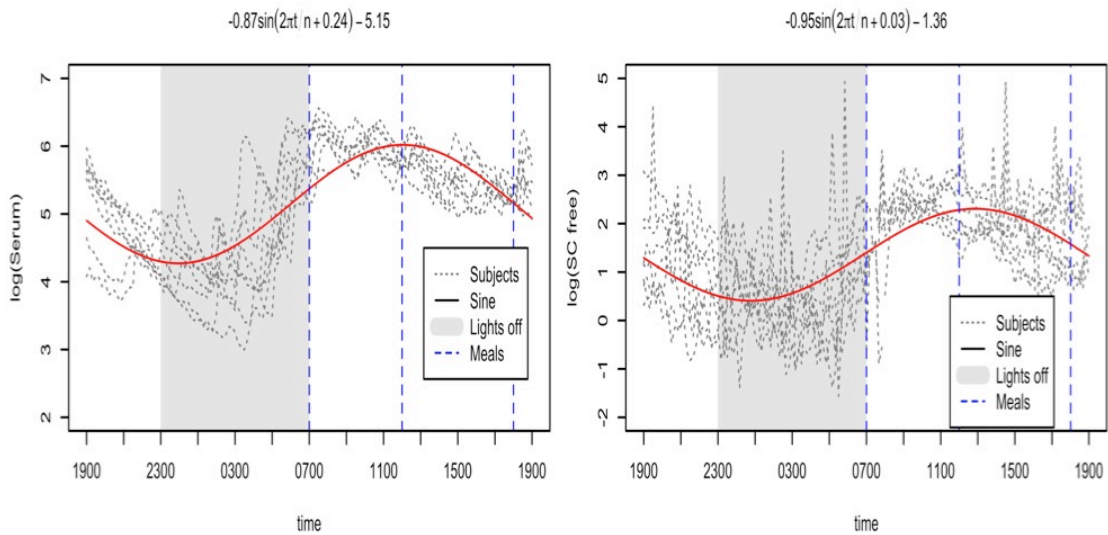
**Figure 5.4.** PLOT OF THE KERNEL-SMOOTHED LOG TRANSFORMED SERUM TOTAL (LEFT plot) AND SC FREE (RIGHT plot) CORTISOL LEVELS.

Time is displayed along the X-axis. Shaded area denotes the lights off period. Dotted lines indicate meal times (breakfast at 07:00, sandwich lunch at 12:00 and hot meal at 18:00). Bold line is the average of point-wise mean for 8 individuals and dotted lines show individual profiles.

Using sine-wave fitting (one method of detecting circadian rhythm), circadian rhythm was evident in both body compartments i.e. serum and SC tissue in each individual, a typical example of which is depicted in Figure 5.5. The pattern of the two measurements is the same but that of SC is of lower amplitude, as evidenced by the value of A in the sine wave function equation for the group (-0.87 for serum and -0.95 for SC - **Error! Reference source not found.**) and for a typical individual (-0.87 for serum and -1.26 for SC – Figure 5.6).



**Figure 5.5.** SINE WAVE FITTING TO LOG OF SERUM TOTAL CORTISOL (LEFT plot) AND LOG OF SC FREE CORTISOL (RIGHT plot) IN A SINGLE INDIVIDUAL.



**Figure 5.6.** SINE WAVE FITTING TO LOG OF SERUM TOTAL CORTISOL (LEFT plot) AND LOG OF SC FREE CORTISOL (RIGHT plot) IN ALL INDIVIDUALS (n=8).

In order to quantify how well the sine wave fits the data, the variance of the residuals for the sine model was calculated using the formula:  $1 - \text{Var}[\text{sine model residuals}] / \text{Var}[\text{intercept model residuals}]$  (Table 5.1). This indicates that although the sine wave model is not ideal for either time series, it is a better fit (maximum values

closer to 1) for log of serum values than for SC, a fact also reflected by goodness of fit values (sine serum model=0.603 and sine SC model=0.440)

TABLE 5.1. TABLE OF THE RATIO BETWEEN THE VARIANCE OF THE RESIDUALS FOR THE SINE MODEL BY PARTICIPANT		
PARTICIPANT ID	SERUM	SC
V31	0.703	0.579
V35	0.712	0.563
V36	0.631	0.568
V38	0.733	0.349
V40	0.598	0.691
V41	0.803	0.598
V42	0.683	0.737
V43	0.786	0.859

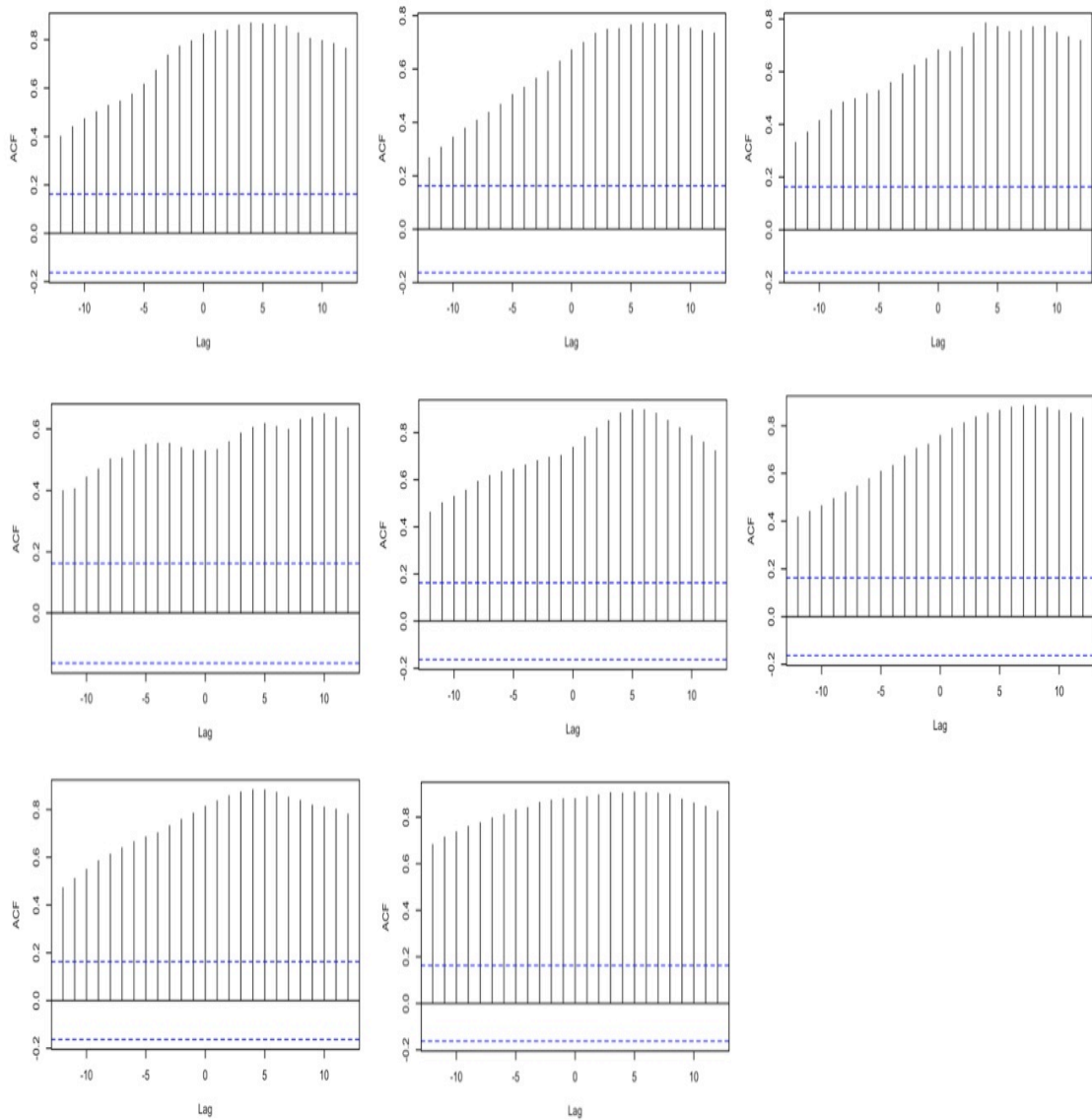
**Table 5.1.** RATIO BETWEEN THE VARIANCE OF THE RESIDUALS FOR THE SINE WAVE MODEL FOR SERUM AND SC TIME SERIES

To use another model for the relationship between serum and SC free cortisol, a repeated measures, mixed effects, generalised linear model was used. While ascertaining the most important components of the model, time was undoubtedly an important feature, which, due to the circadian rhythm was a cubic orthogonal polynomial, of which linear and cuboid component of the polynomial time best describes the relationship (Table 5.2). The time lag that best captured the relationship between the two was that of 50 minutes (log SC free at time lag 5 in model). Random intercept to account for differences over subjects was also included. The intercept represents the mean log serum level across time, which was not zero. So, the null hypothesis test of whether the intercept is significantly different from zero was satisfied. However, the programme was unable to produce an estimate of random slope over time.

TABLE 5.2. TABLE OF FIXED EFFECTS FOR THE GENERALISED LINEAR MODEL OF THE RELATIONSHIP BETWEEN SERUM AND SC FREE LEVELS OVER A 24-HOUR PERIOD				
COVARIATE	VALUE	STD. ERROR	t-VALUE	p-VALUE
Intercept	5.121	0.103	49.699	<0.001
Log SC Free 5	0.020	0.008	2.435	0.015
Time (poly1)	17.638	2.631	6.703	<0.0001
Time (poly2)	1.805	1.836	0.983	0.325
Time (poly3)	-12.133	1.406	-8.628	<0.001

**Table 5.2.** GENERALISED LINEAR MODEL OF RELATIONSHIP BETWEEN SERUM AND SC FREE CORTISOL

Next, to examine the correlation between the two measurements, the data is assumed to be stationary (i.e. constant mean and variance). Differencing the data (previous time's observation from the current time) helps stabilise the mean and variance and thus induce stationarity, which was carried out for the data series. The cross auto-correlation function (**cacf**), which is the correlation between the differenced log serum and differenced log SC values, was estimated for each individual. There was strong cross-correlation between the two series in every individual (Figure 5.7). As each process is an autoregressive process, there is a strong correlation between the observation at a particular time point and those preceding and following it (explaining the cross correlation at negative values in Figure 5.7).



**Figure 5.7.** CROSS AUTO-CORRELOGRAM OF SERUM AND SC FREE CORTISOL FOR EACH INDIVIDUAL PARTICIPANT (n=8).

The values of cacf are along the Y-axis and time lag along the X-axis. Time lag of +1 is an interval of 10 minutes. The values of each correlogram at all time points exceed the confidence interval (dotted line) suggesting a strong correlation between the two profiles in every individual.

## 5.4 DISCUSSION

There have previously been no studies validating 24 hour measurements of SC free cortisol in healthy individuals. All of the study participants displayed the characteristic circadian variation in both body compartments i.e. serum (total) as well as SC tissue (free cortisol). The rise in levels started before waking and peak levels were achieved soon after waking or later in the morning. All participants' profiles had a pulse following the consumption of lunch. The lowest levels of a 24 hour period were observed during the hours following lights switch-off (assumed sleep onset). It is important to note that all of the participants appeared to have slept undisturbed through the night, with the collection device in the travel bag around their waist. The most active period of cortisol release into circulation was from about 05:00 to 14:00. From 14:00 HPA activity was variable between individuals - some showed consistent decline until 19:00 and others had apparent release of cortisol. All participants were University of Bristol students who had no entrainment schedule prior to study day. They kept themselves busy during the awake period with a combination of activities including, coursework, watching movies, listening to music and speaking to friends and family on the phone.

Two methods, sine wave and generalised linear mixed model were used to detect circadian rhythm in these participants, neither of which was adequate for the purpose. The sine wave was better for comparing the two profiles of an individual. Overall, however it was a better fit for serum values and not for SC values. The latter method was employed to increase the generalizability of the model. It was inadequate as well, however it could be improved by adding other parameters such as BMI.

Secretory peaks and hence ultradian activity was clearly discernible visually and detected objectively in the serum compartment but not in the SC tissue compartment, using two standard techniques namely, Pulsar and Deconvolution analyses. Both were unsuccessful in detecting equal number of pulses in the SC tissue (data not included). A number of considerations are likely to influence this key finding as discussed below.

The nature of sampling with microdialysis is continuous, however a timed reading is an aggregate of the dialysate collected over the period, in this case over 10

minutes. This may potentially dampen a pulsatile component. The half-life of free cortisol is not known, but that of serum total cortisol is 68 (28) to 82.8 minutes (152). Although binding to CBG may reduce the available fraction and therefore increase the elimination of free cortisol, it is unlikely to be shorter than 10 minutes.

Whether there was a contribution of  $11\beta$ -HSD enzyme system to the SC free cortisol profiles needs consideration. The SC tissue of the human abdomen is anatomically divided into three parts, superficial and deep adipose layers separated by a membranous layer (153). Great care was taken during the insertion of the subcutaneous probe to avoid the adipose layers, such that catheter was placed in between two adipose planes. However, I did not employ direct visualisation to ascertain the exact position of the catheter or blood flow measurement techniques (107,154) and hence cannot be absolutely certain of the catheter position. The purpose in avoiding placement of the catheter into the deep adipose tissue was due to the presence of the enzyme  $11\beta$ -HSD-1 in the adipose tissue, which is known to generate cortisol from inactive cortisone in vivo (65,70,155).  $11\beta$  HSD-1 is a bidirectional enzyme and hence can serve to regenerate cortisone (156) under certain conditions but this action is less predominant in vivo. The most important difference in these studies is that they did not measure endogenous compounds, but measured metabolites of labelled cortisol and cortisone. Cortisone is present in much lower quantities in serum than in saliva (133,157), but little is known of it in SC tissue. It would have been highly desirable to measure both cortisol and cortisone in the dialysates however I did not have sufficient sample volumes to do so, and using a technique like mass spectrometry for all the samples was not financially or technically feasible. And on the homogeneity of SC tissue in various body parts, Dube *et al* showed that SC tissue of the abdomen has higher  $11\beta$  HSD-2 activity than that of the leg in lean individuals.  $11\beta$  HSD-2 has been found in the human epidermis and can be induced on injury (158), but its role in modulating the pulses of cortisol in skin is not yet known. This study was not designed to address this aspect.

The nature of the SC tissue itself may have diffusion kinetics that does not readily transmit ultradian activity present in the serum. As cortisol is lipophilic it may



exist as a 'depot' in the SC adipose tissue with 'slow release' of free hormone over time. The persistence of circadian rhythm would suggest that there are other factors associated with the dynamic component to the slow release mechanism, if this indeed is true. In rodent studies (43), there is clear demonstration of simultaneous circadian and ultradian pulsatility of free corticosterone in both SC tissue and intravenous compartments. The only difference they found was slightly (15-20%) lower levels of SC tissue free hormone between 15:00 and 21:00hrs, when the levels are rising to the acrophase prior to their activity phase. The explanations for this may relate to increased clearance of free corticosterone as the authors suggest or may also be a property of the rat SC tissue. The SC tissue of rat is highly vascular, unlike that of man and so synchronous rhythms in the former are not a surprise. Difference in vascularity however is unlikely to play a role in the lack of the same in man as Qian *et al* found similarly low levels of free corticosterone in the brain. I may have been able to explore the differential profiles of free cortisol in the intravenous and SC tissue compartments, had I measured both in this study. However, this was not possible due to limited availability of automated collection device and even if the facility was available, it was likely to have interfered with the participants' sleep quality due to the multiple cannulations and devices attached. Nevertheless, as alluded to in the previous chapter, there is a suggestion that pulses of free cortisol are more readily detectable in the intravenous compartment, and these furthermore likely to be transmitted to the SC tissue where the threshold for CBG is reached or exceeded.

The fact that pulsatility was evident in serum and not in the SC tissue could be attributable to different assay techniques. The RIA used for serum samples had superior sensitivity and specificity to the ELISA used for dialysates. Due to the small volumes obtained during our studies samples were analysed in singlicates, which would have prevented detection of analytical errors. Through the optimisation procedure, there were no compromises on the quality and performance of each assay. However, due to the small size of the samples, and lower concentration of free cortisol in the small samples the signal to noise ratio may not have been adequate to detect pulses in the dialysates. The most important difference between serum and SC levels

was the absolute concentration of cortisol. Serum values were much higher and in contrast, dialysate values were relatively very low which pushed the sensitivity of the ELISA assay. It is possible that this was a factor in the lack of demonstration of an ultradian rhythm in SC tissue.

This is the first study whereby measurement of free cortisol continuously has been made possible by the use of a novel automated collection device, which paves the way for ambulatory sample collection in individuals who are free to go about their day to day activities. In addition, undisturbed sampling throughout the duration of sleep can now be achieved.

## CHAPTER 6 DAY-TO-DAY VARIABILITY OF FREE CORTISOL PROFILES

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Having successfully validated the new technique of microdialysis and the novel automated collection device, the next step was to employ it in the evaluation of active free cortisol profiles in individuals going about their day-to-day activities in their own surroundings. Research facilities are unnatural environments which not only can affect levels of hormones that respond to stress, like cortisol, both in rodents (42,43) and in human beings (132), but also disrupt normal sleep patterns. At a practical level it is also expensive to use such specialist facilities and studies are dependent on space availability. Salivary cortisol offers an advantage in this regard and its use as such has increased exponentially in recent years. However, there are limitations to its use for various reasons, of which the two most important reasons are that they cannot be collected during sleep (nadir phase of cortisol) and that it is impractical to collect them continuously. In addition, the impact of variable daily routines on the day-to-day profiles of cortisol is not yet known.

### 6.1 METHODS

Male volunteers aged 18 to 24 years were recruited to this study conducted in Zurich (Switzerland), which was subject to local ethical committee regulations. They were considered healthy if they had no known medical conditions and were on no regular treatment. They had psychiatric assessment (Zurich health questionnaire and Coping strategies inventory) to exclude undiagnosed mental health disorders.

They arrived at the medical facility at their individual convenience, and the microdialysis system was set up as described in Chapters 2 and 5. The flow rate was set at 1  $\mu$ l/min and sampling frequency was every 20 minutes. Blood sampling was not carried out in this study. They were seen 24 and 48 hours later when collection device battery and perfusion fluid were replaced (Figure 6.1). 72 hours after the start of the study time the apparatus was disconnected. Cortisol samples from each 24 hour

period were decanted from the spool in to polypropylene vials immediately after it was retrieved. Samples were stored at -20°C until analysis at a later date.



**Figure 6.1.** SCHEMATIC OF 72 HOUR STUDY.

The microdialysis system was set up after midday on day 1. Participants were seen (arrows) approximately 24 hours apart on days 2 and 3 for fresh batteries and perfusion fluid replacement. The final visit was scheduled approximately 72 hours from the first meeting to conclude the study. Shaded areas represent approximate night periods.

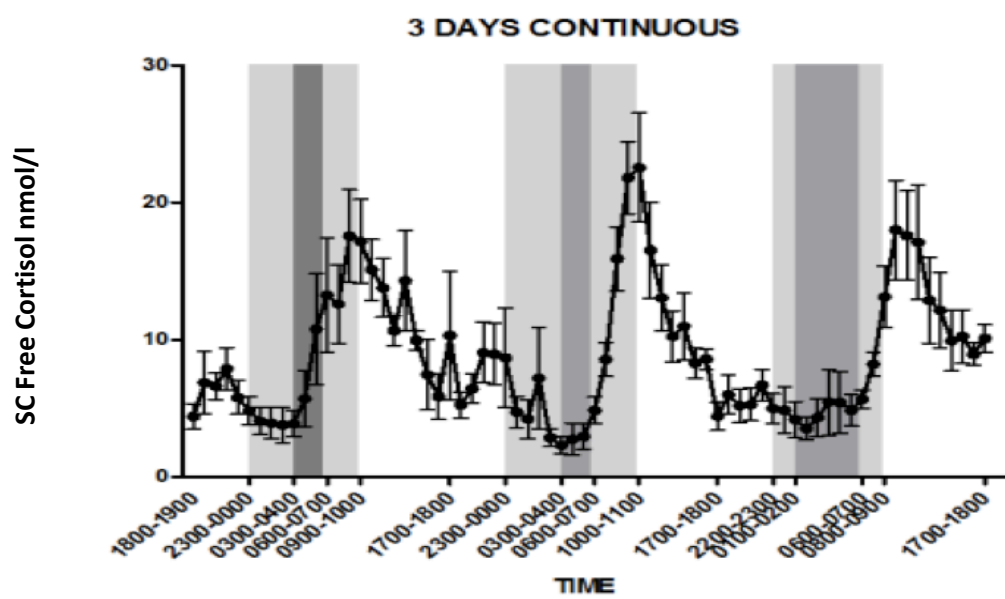
Participants were allowed to carry out all scheduled activities during the three days. The only restrictions related to contact sport, running and swimming which were fortuitously not planned by any of the participants. There were no impositions on sleeping or meal times. The participants in this study had already been recruited to another study (OPTIMI project), for which they recorded a sleep diary that we were able to incorporate into the sampling time. Other activities were generally reported by participants during their follow-up visits but were not logged sufficiently accurately for subsequent evaluation.

## 6.2 RESULTS

We compared the within subject variation in cortisol over the three days of this study. According to the sleep diaries completed by the participants, sleep period was divided into three: first to include the beginning of sleep for any individual (22:00-03:00); second when all individuals were asleep (03:00-07:00) and third when some were still asleep (07:00-11:00). The sleep period terminated when all of the participants were awake. As no other activity record was logged, the rest of the time was considered as the awake time period.

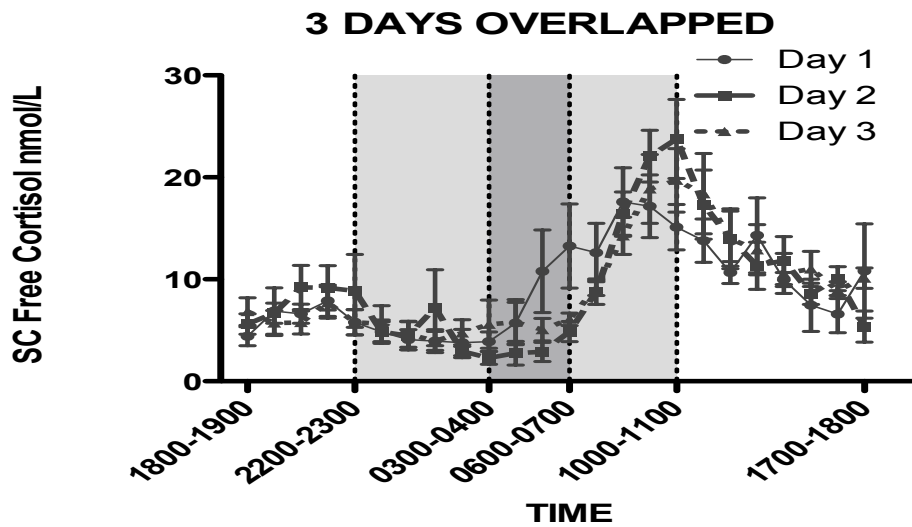
Figure 6.2 shows the mean free cortisol values for all 8 participants over the duration of the trial. It is evident from this figure that the circadian rhythm is present

on each of the three days. This circadian rhythm is characterized by a nocturnal nadir in early hours around sleep onset. The levels then begin to rise during the later part of sleep and reach a peak at or soon after waking. The participants as a group, were asleep for the longest duration on the third night. The mean peak level is achieved at a similar time i.e. 10:00-11:00, regardless of different waking times on the three mornings. The mean values for the three days are superimposed in Figure 6.3.



**Figure 6.2.** MEAN FREE CORTISOL VALUES FOR ALL PARTICIPANTS (n=8) OVER THREE DAYS.

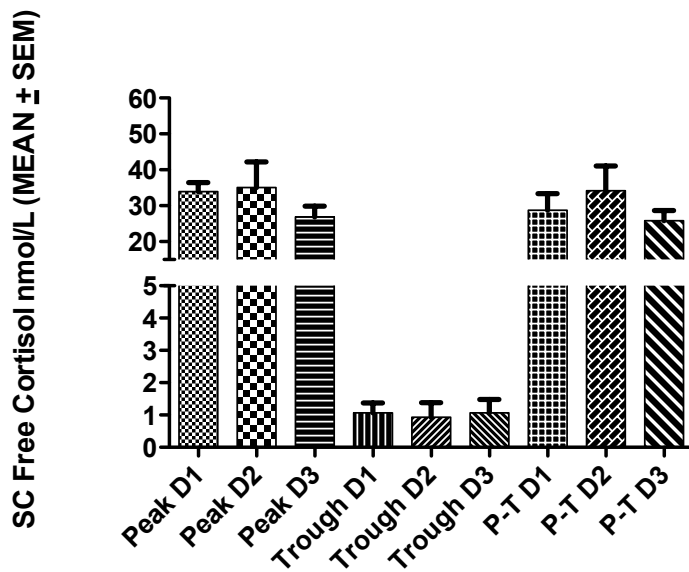
Light grey areas represent the time when some, but not all (dark grey), participants were asleep. Clock time is along the X-axis and SC free cortisol (nmol/L) along the Y axis.



**Figure 6.3.** MEAN FREE CORTISOL VALUES FOR ALL PARTICIPANTS (n=8) OVER THREE DAYS SUPERIMPOSED.

Light grey areas represent the time when some, but not all (dark grey), participants were asleep. Clock time is along the X-axis and SC free cortisol (nmol/L) along the Y axis.

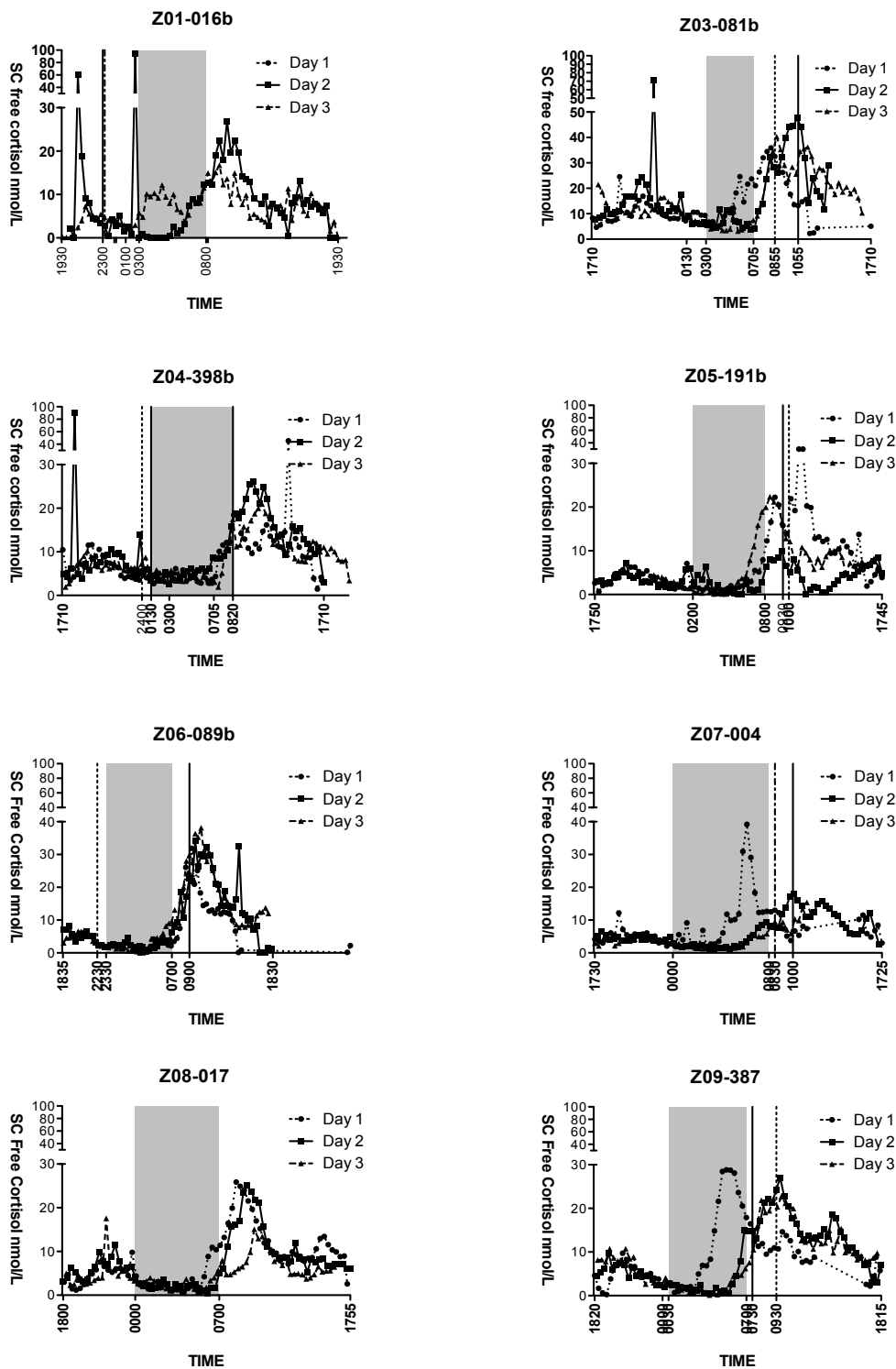
A good indicator of an individual's diurnal rhythm is the difference between the peak and trough values during a given 24-hour period. We therefore calculated mean peak and trough values, by averaging the peak and trough values in the daily profiles for each individual (n=7, as values for one out of 3 days was not available for one participant). We then calculated the average difference between the peaks and troughs (Figure 6.4). One-way ANOVA of peak, trough and the difference between the two showed no significant differences between the daily profiles (peak:  $p=0.2887$   $F=1.380$ ; trough:  $p=0.9907$   $F=0.009400$ , difference:  $p=0.2840$ ,  $F=1.401$ ).



**Figure 6.4.** AVERAGE PEAK, TROUGH AND PEAK MINUS TROUGH VALUES FOR 3 DAYS (n=7).

SC free cortisol values are along the Y-axis and the parameters calculated are along the X-axis as labeled. Days are denoted as D1, D2, D3.

Next, each individual's profile for the three 24-hour periods was superimposed upon each other to examine the day-day-variability. Only one participant of 8 had recorded identical bedtime and waking-up times for the three nights (Figure 6.5 Participant identifier Z08). Only one individual (participant Z01) recorded the same time of waking up on 2 out of 3 days but he could not continue sampling on day 3 and hence his data is incomplete.



**Figure 6.5.** INDIVIDUAL SC FREE CORTISOL VALUES FROM DAYS 1 TO 3 OVERLAPPED.

SC free cortisol is along the Y-axis and clock time along the X-axis. Shaded area represents the time an individual was asleep on all three nights. Vertical lines represent recorded bedtime and wake-up times, if outside of the shaded area.



4 out of 8 individuals went to bed at the same time on all three study nights. 6 out of 8 individuals woke up at different times everyday, ranging from 1 to 4 hours later/earlier than the previous morning. Participant Z04 got up progressively later having spent longer in bed over the three days. His peak levels and times did not show much variation, but there was only a difference of 90 minutes between the recorded waking-up times on days 1 and 3. Participant Z09 got up earlier each day (150 minutes earlier on day 3 compared to day 1) with shorter time spent in bed. His peak levels were achieved approximately 3 hours prior to waking on day 1, whereas although the peak occurred at similar times on days 2 and 3 when he was out of bed, but later compared to day 1.

Overall, despite their different activities reported informally, there was a remarkable consistency in each individual's own profile, with the lowest levels later part of the evening and early sleep hours, and acrophase around awakening time. Two individuals (Figure 6.5 Z05 and Z06) appeared to have high levels of free hormone in the early part of the night, soon after the recorded time of going to bed. As the information on time of going to bed was voluntarily entered by participants retrospectively after waking the following morning, it was dependent upon their memory of the events of the previous night and the level of pre-occupation about the day's activities (some experiment days coincided with examinations, others during preparation for a major social gathering). For both these individuals, the levels of SC free cortisol on the remaining two nights at the same time were significantly low, in keeping with the expected levels for early hours of the night.

### **6.3 DISCUSSION**

In rodents, SC free corticosterone on two consecutive days has shown remarkable consistency of both circadian and ultradian rhythm (43). Cortisol rhythm has previously been measured on more than one day in the same individual. Selmaoui et al repeated 24-hour serum total cortisol profiles over 3 separate days in 31 healthy individuals 2 to 4 weeks apart (159). The group of medical students was chosen for regular sleep/wake pattern, and was following a regular pattern on experiment days.

Their sampling regime was hourly during sleep hours (2300 to 0800) and 3 hourly during the awake period. Apart from one individual, they found no differences in the circadian profiles over 3 disparate days over 6 weeks. Our study imposed no activity structure on participants other than to avoid contact sport. They did not refrain from alcohol or other dietary ingredients, and were encouraged to lead as 'normal' a life as possible. A variety of activities including potentially stressful ones e.g. attending examinations, chairing a student body annual meeting, meeting with a tutor to discuss possibility of failing a term, chairing a meeting to allocate tasks towards organizing the high profile University annual ball, as well as leisure activities like playing poker, watching television/movies, and indulging in sexual activity were reported informally during the sampling period. It is striking how similar the day-to-day SC free cortisol profiles are despite such variations in their activities in the awakened state as well as a variable duration and pattern of sleep. The relationship of sleep duration (160) and quality of sleep (161) has been of much interest for decades, with no clear resolution. Our data therefore supports finding by Selmaoui et. al. of almost identical cortisol profiles across three days, the difference being that our sampling period was for 3 continuous days and the additional information from our study suggests that it may not be absolutely necessary to entrain behavior upon individuals when there is a desire to carry out sampling on more than one circadian cycle.

In another study, the effect of exercise at various times of the day on serum cortisol was observed where the individual underwent control (without exercise) sampling during the same time periods on another day (162). So, although cortisol was measured in a paired fashion in healthy men, it was done for a few hours on disparate days to evaluate the impact of an artificial intervention at a research facility. The assumption in this study was that on the two days, without the intended intervention, the profiles would show little or no variation.

Saliva sampling has in recent years offered the opportunity to record peoples' cortisol levels in their home environment. Many of such studies measure the cortisol awakening response (**CAR**-first sample within 10mintues of waking, and second 30min later) only with no further sampling in the day. One such study by Kunz-Ebrecht and

colleagues found higher CAR on a workday as opposed to that on a weekend day i.e. on a day off work (163). All of our sampling was done on weekdays with no intervening weekend day. Significant intra-individual variation in CAR across days has been reported elsewhere (164). Other studies measuring cortisol at multiple times during the day in individuals on several days have reported averaged values which prevents insight into the robustness of the rhythm across those days (165). The fundamental difficulty in relying on CAR values, and in making meaningful conclusions from the results on multiple days is that 'a different value' may simply be a product of the timing of saliva collection at a different time point along an endogenous pulse. Susceptibility to disease based on calculation of the nature of a circadian profile from few timed samples, even when collected on multiple days, must be drawn with caution (166).

To our knowledge, this is the first evidence of continuous measurement of free cortisol for 3 consecutive days in healthy people outside of a research facility setting, free to carry out their routine activities without major limitations. We have been able to demonstrate that free cortisol in the SC tissue shows remarkable consistency despite varied daily routines and activities of individuals. Cortisol has always been known to have a robust circadian rhythm, this being the first evidence of it in individuals who were performing all intended activities over three days. This provides considerable scope to plan future studies to investigate specifically the effect of an intervention on the circadian rhythm of free cortisol (or other hormones) on different days with or without the intervention secure in the knowledge that there would have been relatively little variation in the absence of the intervention even in individuals whose sampling days contain dissimilar activities/routines.

## CHAPTER 7      SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

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One of the key problems in research into the regulation of stress hormones and for the study of stress related disorders has been our inability to study ambulatory hormone dynamics in people within their home environments. I have attempted to overcome this limitation by the studies described in this thesis, and have:

- I. Validated the technique of microdialysis for measurement of free cortisol in the subcutaneous tissue (Chapters 4 and 5) and of intravenous free cortisol levels (Chapter 4)
- II. Coupled this technique together with the use of a novel automated ambulatory sampling system (Chapter 3) to compare intravenous serum total cortisol levels with subcutaneous free cortisol levels (Chapter 5)
- III. Used the portable collection device to collect subcutaneous free cortisol samples from ambulatory healthy male individuals to look at the day-to-day variation in their circadian profiles, when they are conducting their everyday activities.

First, in vitro testing of the catheters was carried out through a series of experiments in the laboratory to confirm good recovery rates. One of the main challenges in these as well as human experiments was the small individual sample size of no more than 20  $\mu$ l. For practical purposes, allowing flow variations, a volume of 15  $\mu$ l was expected per sample for the assay. A number of assays were tested until IBL Saliva Cortisol ELISA (IBL, Hamburg, Germany) was selected. The assay was then optimised to allow the expected sample volume, instead of the volume per sample recommended by the kit manufacturer, without losing the sensitivity and specificity. This was achieved with success, however it was not possible to have duplicates of each sample, as is standard practice with all enzyme-based assays. Attempts at using smaller volumes so as to allow duplicates failed and so, this compromise was accepted.

The catheters were subsequently employed in a series of clinical studies, the first of which involved administration of pharmacological agents in routine clinical use to healthy male volunteers. There was good correlation between serum total cortisol and free cortisol in the intravenous compartment and in the subcutaneous tissue (of the upper arm and abdomen) during both stimulation by 250 µg Synacthen and suppression by 1 mg of dexamethasone. In the placebo group as a whole, the correlation values were lower than in the other two groups, as a result of inter-individual variation in the timing of ultradian pulses - which would not have been apparent when the phase of cortisol release had been reset by administration of either ACTH or dexamethasone.

Following validation of subcutaneous microdialysis, this technique was coupled with the novel automated portable sampling device to measure circadian profiles with ten minute sampling of serum total and subcutaneous free cortisol in healthy individuals at a research facility with a pre-determined sleep-wake cycle. Standard meals were served to all participants and lights switched on and off at fixed times during the experiment. Circadian profiles of serum total cortisol were clearly present in the subcutaneous free cortisol measurements. However, ultradian pulses were not clearly demonstrated in the latter compartment. This may be an endogenous phenomenon related to the access to and clearance from this tissue as a result of one or more of the following reasons - relatively lower vascularity; change in serum levels being transmitted to the subcutaneous tissue only upon exceeding the capacity of corticosteroid-binding-globulin; tissue acting as a depot due to its high adiposity acting as a slow-releasing store of free cortisol; rapid conversion of free cortisol to cortisone by 11 β-HSD 2. Alternatively it may be methodological related to frequency of sampling from this compartment, carry over of cortisol from one sample to the next, or due to the fact that all samples were assays in singlicate. This final factor could have resulted in excess variance in the assay that was already in the lower third/quarter of its range of detection with associated increase in its intra-assay coefficient of variation and thus poor power to detect potentially small amplitude changes in free cortisol levels.

Finally, subcutaneous microdialysis coupled with the novel collection device was used in a group of male participants over three consecutive days to look at the day-to-day variation in the circadian profiles of free cortisol in people ambulating in their home and work environments. This endeavour proved to be safe and effective in achieving uninterrupted profiles, including during three nights and day-time periods. The participants found it no hindrance to carry this device in a travel bag secured around the waist with a trouser belt. One participant discontinued after 48 hours as he acquired a febrile illness towards the end of the second sampling day. The remaining participants adapted to the device well and informally reported no limitations to their activities by virtue of the device being attached to them for the duration. Every participant attended all the planned activities throughout sampling. They, as a group, and individually had varied sleep-wake routines on the days they were recruited to the study and no day was similar to the other two in terms of their activities, despite which there was remarkable consistency of circadian free cortisol profiles within individuals. There was no significant difference between peak and nadir levels of the group over the three days.

There are limitations to the application of this work. It was not possible to detect ultradian pulses within the circadian rhythm of the profiles collected. The possible explanations for this phenomenon are mentioned earlier in this section. Although the frequency of sampling can be altered within the novel collection device, the minimum interval is determined primarily by the amount per sample for the assay but also by the stability of the spool containing the samples (and separating air) within the collection device. The device's servo mechanism does not allow the pressure within the spool to rise above 10 cm of water *in vitro*, however this has not been rigorously tested in clinical protocols. Minimum sampling frequency of every 10 minutes can be recommended based on the experience during this study. Finally, most of the data presented in my thesis applies to free cortisol values obtained from the subcutaneous tissue of the abdomen and upper arm of healthy male volunteers. We do not know much about the local cortisol metabolic pathways in the skin-especially 11 $\beta$ -HSD2. Indeed future studies looking at both cortisol and cortisone in this

compartment is clearly a priority. This will undoubtedly open up a whole new area using microdialysis to look at the ratio of these hormones in different tissues undergoing our dynamic sampling technique.

## **CONCLUSION AND FUTURE DIRECTIONS**

Automated continuous free cortisol sampling from the subcutaneous tissue can be performed safely and reliably for up to 72 hours in individuals conducting their day-to-day activities within their natural environments. This novel system can be used to study disease processes and their effects on the circadian rhythm of free cortisol in the subcutaneous tissue. This includes diseases primarily affecting the HPA axis e.g. Cushing's syndrome, as well as other diseases believed to involve the axis e.g. affective disorders. In addition to helping understand disease pathology, it also has potential as a diagnostic modality. For example, the underlying abnormality in Cushing's syndrome has been reported to be flattening of the circadian rhythm of cortisol (167), or upward shift with maintenance of a rhythm (168). This has suggested that it might be diagnostically useful to sample at midnight i.e. nadir phase (121). However, midnight blood sampling has proved challenging for practical reasons as well as for obtaining a consensus cut-off for diagnosis (169), leading to the proposition of midnight salivary cortisol as a preferred measure (121), although one single sample may not be representative if the subject is anxious or stressed for any reason. It is also important to note however that it is cortisone and not cortisol in saliva, which is a better correlate of serum free cortisol (142). Our novel system presents the opportunity to study the uninterrupted dynamic rhythm of bioavailable free cortisol over an entire 24-hour (or longer) period, which may provide a much better diagnostic picture of the subject's cortisol status. This may of course be repeated after definitive treatment of Cushing's to assess whether there has been a return of a normal pattern of cortisol secretion.

Before it can be concluded that the ultradian rhythm in subcutaneous tissue of man is significantly less dynamic than that in serum, further studies are required. Future work should include measurement of both free cortisol and cortisone in the

subcutaneous (and other) tissue (s) to understand the role of 11  $\beta$ -HSD 2 in modulating the dynamics of the endogenous pulsatile pattern of subcutaneous glucocorticoids. A superior assay technique such as mass spectrometry would be ideal for this purpose, as it is able to quantify multiple analytes in a single sample.

My thesis has concentrated on the measurement of cortisol, but the technique along with the novel device may be utilised to measure multiple hormones and metabolites and related diseases namely, growth hormone in acromegaly, parathyroid hormone in hyperparathyroidism and aldosterone in primary and secondary hypertension. I have demonstrated the use of microdialysis in the subcutaneous tissue of man, but the technique has been used in other tissues like the brain (111), myocardium (170) and liver (171). Within the subject of this thesis alone however it is clear that there is great potential for using this technique to understand glucocorticoid physiology and metabolism in health and disease.



## APPENDIX I –

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### RIA AND NETRIA ELISA ASSAYS

During assay development, RIA and Netria saliva cortisol ELISA assays were used for a number of in vitro experiments.

#### **TESTING TWO FLOW RATES (2 $\mu$ L/min AND 1 $\mu$ L/min) USING SCC.**

Cortisol solution (50ml) of concentration 6.9 nmol/l was prepared. This concentration lies at the centre of the sigmoid shaped standard curve for the Netria ELISA assay. Simultaneous samples were taken from the solution as the dialysate on two occasions thirty minutes apart with the pump flow rate setting of 1 $\mu$ l/min.

The flow rate was changed to 2 $\mu$ l/min and after 30 minutes of stabilisation, two sets of samples were collected as above.

Tables I.I and I.II contain the results obtained during two separate experiments using the above protocol.

The results showed a wide variation in samples taken from the same cortisol solution when analysed with RIA, although the dialysate values were consistent with each other (Table I.I). These values lay in the plateau portion of the standard curve for RIA which in itself had a good configuration. RIA was not pursued due to lack of sensitivity in the range expected during our studies.

<b>TABLE I.I TESTING RECOVERY OF SC WITH TWO FLOW RATES OF CMA 107 PUMP – RIA</b>				
<b>FLOW RATE</b>	<b>TIME</b>	<b>SOLUTION nmol/l</b>	<b>DIALYSATE nmol/l</b>	<b>Recovery %</b>
1 µl/min	First 30 min	15.03	4.46	30
	Second 30 min	2.46	4.41	179
2 µl/min	First 30 min	3.96	4.21	106
	Second 30 min	2.99	3.33	111

TABLE I.I. RECOVERY OF SC WITH TWO FLOW RATES.

30 min dialysate and solution (expected 6.9 nmol/l) samples were analysed by RIA for two flow rates (1 and 2 µl/min) set for an hour each.

When Netria ELISA was used to analyse samples from another identical experiment, the variation in measured concentration of the prepared solution was less variable between the four samples than in the previous assay (Table I.II). The results were the reverse of what was expected as the dialysate values were higher and had a wider variation, giving recovery values of well over 150%.

<b>TABLE I.II TESTING RECOVERY OF SC WITH TWO FLOW RATES OF CMA 107 PUMP – NETRIA ELISA</b>				
<b>FLOW RATE</b>	<b>TIME</b>	<b>SOLUTION nmol/L</b>	<b>DIALYSATE nmol/L</b>	<b>Recovery %</b>
1 µl/min	First 30 min	6.54	10.49	160
	Second 30 min	4.08	7.20	176
2 µl/min	First 30 min	4.97	9.11	183
	Second 30 min	5.05	8.39	166

TABLE I.II. RECOVERY OF SC WITH TWO FLOW RATES.

30 min dialysate and solution (expected 6.9 nmol/l) samples were analysed by NETRIA ELISA for two flow rates (1 and 2 µl/min) set for an hour each.

## TESTING THREE SCs WITH ONE FLOW RATE IN ONE CONCENTRATION OF CORTISOL

Three cortisol solutions (6.9 nmol/l) were prepared for three catheters to be tested at the flow rate of 2  $\mu$ l/min. Three 30 minutely samples were collected concurrently, from the catheters and their respective solutions, and analysed with Netria ELISA.

TABLE I.III TESTING RECOVERY OF 3 SCs AT FLOW RATE OF 2 $\mu$ l/min – NETRIA ELISA				
CATHETER	TIME	SOLUTION nmol/l	DIALYSATE nmol/l	Recovery %
1	First 30min	5.93	6.10	103
	Second 30min	6.76	4.91	73
	Third 30min	5.02	6.71	134
	Average recovery for catheter 1			103
2	First 30min	8.89	9.22	104
	Second 30min	8.20	6.98	85
	Third 30min	9.66	7.34	76
	Average recovery for catheter 2			88
3	First 30min	8.64	6.13	71
	Second 30min	9.36	4.55	49
	Third 30min	8.11	7.07	87
	Average recovery for catheter 3			69

TABLE I.III. TESTING RECOVERY OF 3 SCs AT FLOW RATE OF 2  $\mu$ l/min.

Three simultaneous experiments with 30 min dialysate (at flow rates 2  $\mu$ l/min) and solution samples collected for 90 min. and analysed by NETRIA ELISA.

Samples taken from each individually prepared solution show a fair consistency across the three values (Table I.III). One dialysate concentration in each of the three samples obtained per catheter is either significantly lower or higher compared to the other two, which are consistent with each other suggesting a similar recovery as expected for a catheter. Average recovery for catheter 3 is less than expected, whereas that for the other two suggests good performance.

## **TESTING RESPONSE OF SC TO A CHANGE IN CONCENTRATION**

One SC was set up as in previous experiments and two cortisol solutions of concentration 6.9 nmol/l and 34.5 nmol/l were prepared. After 30 minutes of stabilisation, SC was immersed in the first solution (6.9 nmol/l) for 90 minutes followed immediately by transferring the membrane to the other solution. Three half hourly dialysates per 90minutes with simultaneous samples from the respective solutions were collected and later analysed with Netria ELISA. Three individual experiments were conducted with this protocol.

TABLE I.IV TESTING RESPONSE OF SC TO CHANGE IN CORTISOL CONCENTRATION (FLOW RATE 2µl/min)					
	CORTISOL CONC. nmol/l	TIME	SOLUTION nmol/l	DIALYSATE nmol/l	Recovery %
Experiment 1	6.9	First 30min	3.67	2.84	77
		Second 30min	3.95	5.16	131
		Third 30min	5.49	3.73	68
	34.5	First 30min		18.44	
		Second 30min	12.64	11.51	91
		Third 30min	17.36	25.06	144
		Average recovery for experiment 1			102
Experiment 2	6.9	First 30min	5.71	5.60	98
		Second 30min	7.84	6.32	81
		Third 30min	7.26	6.43	89
	34.5	First 30min	51.83	21.80	42
		Second 30min	40.85	31.90	78
		Third 30min	41.37	41.84	101
		Average recovery for experiment 2			81
Experiment 3	6.9	First 30min	8.67	5.55	64
		Second 30min	8.41	4.83	57
		Third 30min	6.73	6.76	100
	34.5	First 30min	31.35	26.80	85
		Second 30min	34.09	29.56	87
		Third 30min	37.78	42.20	112
		Average recovery for experiment 3			84

TABLE I.IV. RESPONSE OF SC TO CHANGE IN CORTISOL CONCENTRATION.

Three simultaneous experiments with 30 min dialysate (at flow rates 2 µl/min) and solution samples were collected for 90 min sequentially in solutions of two different concentrations. Samples were analysed by NETRIA ELISA.

Two important aspects were examined in this set of experiments namely; stability of the recovery during immersion in the same solution and the amount of recovery during the first thirty minutes in the second solution i.e. the rapidity of the response to change in concentration. Recovery varied from 68% to 144% in the first experiment with no clear pattern, and the first sample taken from the second solution was below detection limit suggesting analytical error making it impossible to calculate recovery for that duration

(Table I.IV). The average recovery for this experiment was 102%. The second experiment gave consistent values during the 90 min in solution one with a significant drop when moved to the second solution, suggesting a delay in reflecting the surrounding concentration, but improved during the next 60 min. Recovery was lower from solution one in experiment 3, but achieving 85% values for the first dialysate from solution two – an improvement from the previous experiment - which was maintained for the remaining time. The average recovery for both these experiments was above 80%.

### **TESTING RECOVERY OF SCS TO CHANGE IN CONCENTRATION OF CORTISOL**

For this experiment, the pump flow rate was set at 2  $\mu\text{l}/\text{min}$ . SC membrane was immersed for 90 min in each of three solutions of progressively higher concentrations (1.38, 6.9, and 34.5 nmol/l) of cortisol. Half hourly dialysates with simultaneous samples from the respective solution in which the membrane was immersed were collected and stored at  $-20^{\circ}\text{C}$  awaiting assay. Samples from two experiments were analysed by Netria ELISA.

For experiment 1, recovery could not be calculated for the first 90 minutes due to several missing values (Table I.V). Response to the first change in concentration was unacceptably low, but adequate for the second change. The values were more consistent for the second and third solutions. Average recovery for this experiment was 96%, similar to that of the next experiment of 97%. During experiment 2, the first sample when there is a change in concentration, the recovery is unexpectedly high. The recovery values for the later two samples in this experiment show significant variation, likely due to the difference in solution concentration albeit taken from the same solution. Nevertheless, there is a suggestion that when the surrounding concentration changes, the first samples may be affected by remaining dialysate within the outlet tube.

TABLE I.V TESTING RESPONSE OF SC TO A CHANGE IN CORTISOL CONCENTRATION (FLOW RATE 2µl/min)					
	CORTISOL CONC. nmol/l	TIME	SOLUTION nmol/l	DIALYSATE nmol/l	Recovery %
Experiment 1	1.38	First 30min	11.65		
		Second 30min	3.45		
		Third 30min			
	6.9	First 30min	7.07	3.45	49
		Second 30min	7.56	9.11	120
		Third 30min	6.71	5.77	86
	34.5	First 30min	27.99	30.19	108
		Second 30min	28.90	28.51	99
		Third 30min	23.87	26.77	112
		Average recovery for experiment 1			96
Experiment 2	1.38	First 30min	1.27	1.96	154
		Second 30min	1.49	1.74	117
		Third 30min	2.24	1.41	63
	6.9	First 30min	5.16	6.10	118
		Second 30min	6.27	5.77	92
		Third 30min	6.13	5.38	88
	34.5	First 30min	31.74	37.65	119
		Second 30min	54.51	27.13	50
		Third 30min	32.15	23.68	74
		Average recovery for experiment 2			97

TABLE I.V. RESPONSE OF SC TO CHANGE IN CORTISOL CONCENTRATION.

Two simultaneous experiments with 30 min dialysate (at flow rates 2 µl/min) and solution samples were collected for 90 min sequentially in solutions of three different concentrations. Samples were analysed by NETRIA ELISA.

As a strict protocol of labelling with documentation was followed sampling error was not thought to be the likely explanation. The solution in which SC is immersed was freshly prepared on the day, and its concentration varied not necessarily in one direction i.e. progressively lower or higher, which may have suggested membrane irregularities. There were several issues with reagents (leakages, precipitation and other such evidences of inadequate standard of product) when the Netria kit was being developed and tested and the results were thought be due to analytical errors.

Eventually, both these assays were abandoned.



## APPENDIX II

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### CHOICE OF A SEPARATION MEDIUM AND ITS INTERACTION

#### BACKGROUND:

The sampling spool in the automated collection device contains timed dialysate samples with successive samples being separated by an air bubble within the PTFE tubing. During most experiments, the collected spools had a clear definition between a dialysate column and the adjoining air bubble on either side. Occasionally however, this harmony was disturbed such that individual timed samples had disintegrated into even smaller droplets. This necessitated careful measurement of each droplet from the beginning of the collection to make an aggregate sample size corresponding to the sampling duration.

The factors influencing harmony could be external or internal (within the tubing). Rigorous testing of the device during its development had established that it was resilient to relatively moderate level of agitation, for example, those encountered during activities of cycling, walking, passenger journeys in a car/bus through speed-breakers. It was agreed that running or similar activities should be avoided, as those were almost certain to have adverse effect. Within the tubing, it was important to examine the interaction between the two phases – liquid (dialysate) and gas (air) – to find out if they played a role in disturbing the harmony.

#### METHODS AND RESULTS

First, an alternative to air as a separation medium was considered. The minimum requirements of such a medium were for there to be a clear definition between it and the sample, and in view of cortisol being hydrophobic in nature it should not adsorb or dissolve the hormone. In consultation with the department of Chemistry at the

University, several hydrophobic liquids of various viscosity levels were tested but none satisfied the first criterion. A suitable hydrophobic dye to colour the clear liquids proved unsuccessful. Moreover, movement of fluids with higher viscosity than water through the narrow bore collection tubing was not smooth and resulted in breakdown of dialysate columns. Hence, the search for an alternative to air was abandoned.

Later, in collaboration with the Nano-science department, the surface tension between the two phases i.e. air and liquid, also known as interfacial tension (172), was investigated. First, using volume tensiometry, the surface tension of a given volume (20 microlitres) of dialysate with a range of cortisol concentrations (0 to 138nmol/L) was measured (Table II.I). Ten consecutive measurements of a single drop showed minimal variation between the various concentration solutions (standard deviation 0.61 – Table II.I) and hence the strength of the solution itself was deemed unlikely to play a role.

TABLE II.I. INTERFACIAL TENSION (millinewtons/metre)

REPETITIONS	CONCENTRATION (nmol/L)									
	0	0.055	0.138	0.69	2.76	6.9	13.8	27.60	138	
1( mN/m)	71.45	70.95	71.57	71.28	70.01	71.19	71.88	72.08	71.16	
2( mN/m)	71.41	70.95	71.61	71.26	69.89	71.23	71.80	71.77	71.20	
3( mN/m)	71.41	71.10	71.64	71.29	69.86	71.23	71.80	71.51	71.14	
4( mN/m)	71.39	71.09	71.77	71.25	69.67	71.20	71.81	71.70	71.20	
5( mN/m)	71.39	71.03	71.66	71.27	69.81	71.20	71.84	71.65	71.12	
6( mN/m)	71.40	70.98	71.70	71.24	69.79	71.18	71.81	71.71	71.17	
7( mN/m)	71.44	70.94	71.64	71.26	69.70	71.20	71.86	71.61	71.17	
8( mN/m)	71.44	70.95	71.70	71.27	69.72	71.20	71.85	71.69	71.16	
9( mN/m)	71.51	71.01	71.72	71.26	69.72	71.21	71.85	71.53	71.15	
10( mN/m)	71.32	71.02	71.72	71.24	69.68	71.20	71.82	71.74	71.18	
Average( mN/m)	71.42	71.00	71.67	71.26	69.79	71.20	71.83	71.70	71.16	71.23
Standard Deviation										0.61

The interface between the dialysate sample and air, when visualised under an optical microscope, showed no evidence of instability.

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