The Measurement of 18-hydroxycorticosterone in Saliva

Chiew Yan Lee (B.Sc., M.Sc.)

A thesis submitted as part requirement for the degree of Doctor of Philopsophy of the University of London.

St. Mary's Hospital Medical School,

London W2 1 PG.

***

August 1987.

***

ABSTRACT

A radioimmunoassay system for measuring 18-hydroxycorticosterone (18OHB) using antibodies raised against 18OHB-3-(O-carboxymethyl) oximino-BSA hapten (18OHB-3-CMO-BSA) and 18OHB-3-CMO-2-[^{125}I] iodohistamine tracer is described. A novel procedure for preparing the 18OHB-3-CMO involved reacting stoichiometric proportions of 18OHB and carboxymethoxylamine-hemihydrochloride in pure methanol at room temperature (23^{o}C) for 15 minutes. Hydrochloride released during the reaction was neutralised with sodium hydroxide. 18OHB-3-CMO was simultaneously stabilised as its sodium salt.

The development, validation and application of the first, simple, specific, accurate and reliable method for measuring 18OHB in human saliva is described. A similar method was adapted for measuring plasma 18OHB concentration. The method involved a thin-layer chromatography with [1,2-^{3}H]18OHB incorporated as the procedural loss monitor. The changes of plasma 18OHB levels were reflected in the saliva concentrations under basal condition and after corticotrophin administration.

Diurnal fluctuations of saliva 18OHB were observed in normal subjects and in subjects with primary and secondary hyperaldosteronism. Frequent saliva sampling essential for such a study led to the observation that the secretion of 18OHB appeared to be largely, but not completely, synchronous with that of aldosterone.

Saliva 18OHB concentration in normal subjects declined significantly in response to 4 hours ambulation from overnight

recumbency but the saliva aldosterone concentration and the 18OHB:aldosterone ratio were unaffected. This finding contradicts previous reports of plasma 18OHB and aldosterone concentrations in response to ambulation.

In conclusion, the simple method for measuring 18OHB in saliva described in this thesis should facilitate investigation into the relationship between 18OHB and aldosterone secretions under physiological and pathological conditions.

Contents

Contents

Contents

Contents

List of Figures

List of Figures

List of Figures

List of Figures

List of Figures

List of Figures

List of Figures

List of Figures

List of Figures

List of Tables

List of Tables

List of Tables

List of Tables

List of Abbreviations

18OHB	18-hydroxycorticosterone
18OHB-3-CMO	18OHB-3-(O-carboxymethyl)oxime
ACTH	Adrenocorticotrophic hormone (Corticotrophin)
Aldo	Aldosterone
APA	Aldosterone producing adenoma
AII	Angiotensin II (an octapeptice)
$(CMO)_2$-HCl	Carboxymethoxylamine-hemihydrochloride salt
DCM	Dichloromethane
E	Cortisone
ETAC	Ethyl acetate
F	Cortisol
GC	Glucocorticoids (cortisol + cortisone)
IHA	Idiopathic hyperaldosteronism
n	Number of terms or subjects or samples etc.
PA	Plasma aldosterone
PB	Plasma 18OHB
PFB	Plasma free 18OHB
PF	Plasma glucocorticoids
^1R_E	Relative mobility of 18OHB to cortisone in which the chromatogram was developed once in a chromatographic solvent system
^2R_E	Relative mobility of 18OHB to cortisone in which the chromatogram was developed twice in

List of Abbreviations

	a chromatographic solvent system
SA	Salivary aldosterone
SB	Salivary 18OHB
SF	Salivary glucocorticoids
TLC	Thin-layer chromatography

Acknowledgements

I would like to thank Professor V.H.T. James and Mr. J. D. Few for their advice and encouragement throughout this project; their help is very much appreciated.

Similarly, I am thankful for the constant support and encouragement from my parents.

I am also extremely grateful to Professor D.N. Kirk for generous supplies of 18-hydroxycorticosterone and for the NMR spectrum on 18OHB-3-CMO and its interpretation, and to Dr. Angela Fairney for the immunisation of the rabbits.

*****

This thesis is dedicated to my parents.

August 1987.

*****

Chapter 1

Introduction

18-hydroxycorticosterone (18OHB, Figure 1a; Fraser and Lantos 1978, a review) is a steroid produced and secreted by the adrenal cortex (Knupper et al., 1965). The generally accepted pathway for aldosterone biosynthesis in the zona glomerulosa leads from acetate ➡ cholesterol ➡ pregnenolone ➡ progesterone ➡ deoxycorticosterone ➡ corticosterone ➡ 18OHB ➡ aldosterone (Figure 2). It has been demonstrated that 18OHB is an intermediate of aldosterone biosynthesis (Kojima et al., 1982 and 1984) by 'trapping' experiment. It was shown that the conversion of tritiated corticosterone to aldosterone was inhibited in a dose-dependent manner by non-radioactive 18OHB used as trapping agent. However, earlier studies failed to demonstrate that 18OHB is a precursor of aldosterone biosynthesis in vitro. In these studies it was found that the percentage conversion of corticosterone to aldosterone ranged from 26 to 81 times greater than using 18OHB as substrate (Ulick et al, 1964; Nicolis and Ulick, 1965) using frog and beef adrenal tissues. Therefore, it was proposed in 1973 by two groups of authors (Marusic et al. and Ulick) that the structure of 18OHB synthesised de novo has a steric configuration different from exogenous 18OHB. This is consistent with the existence of two tautomeric forms of exogenous 18OHB with equilibrium in favour of the cyclic hemiketal form (Figure 1b). The latter form was said to be resistant to oxidation. Hence, it was proposed that 18OHB synthesised de novo

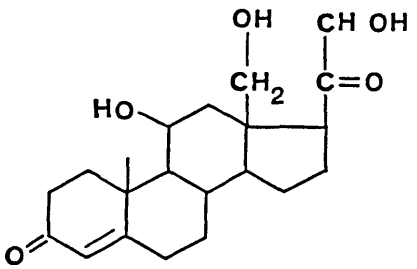


Figure 1a A Chemical representation of 18OHB with a mol. wt of 362

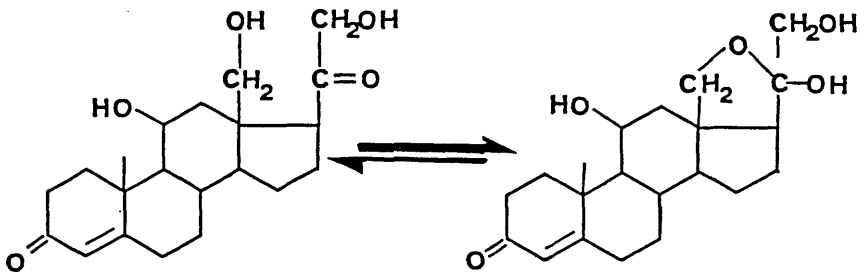


Figure 1b The tautomeric forms of 18OHB in solid and in solution.

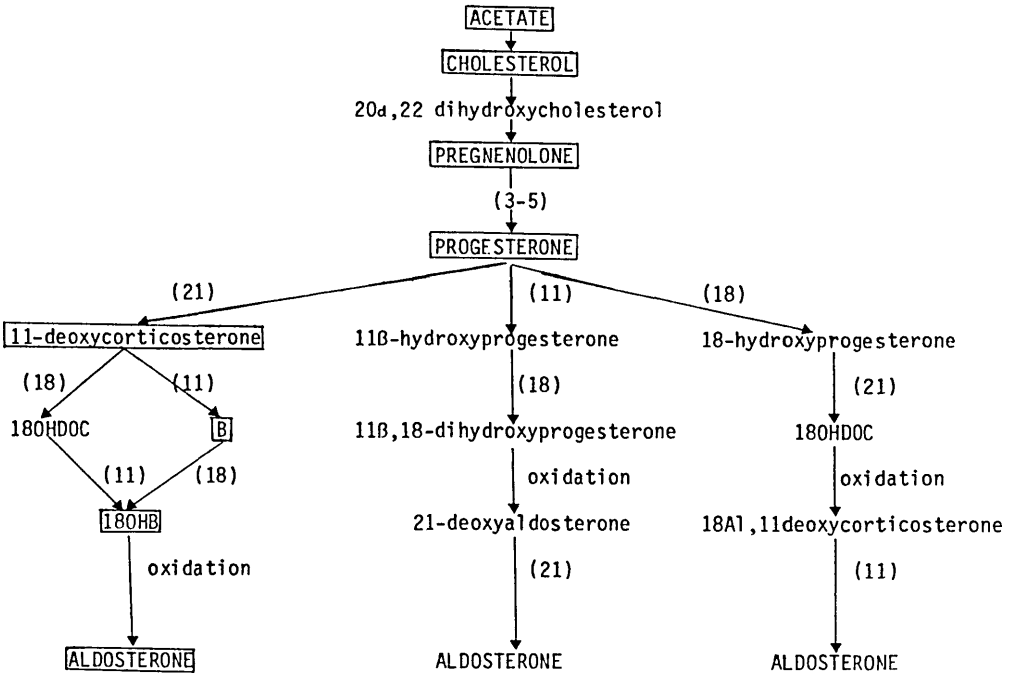


Figure 2

Possible biosynthetic pathways of aldosterone. The major pathway is highlighted by the rectangles.

(3-5) = 3β-hydroxysteroid dehydrogenase and Δ^5-3-keto-isomerase

(11) = 11β-hydroxylase

(18) = 18-hydroxylase

(21) = 21-hydroxylase

was associated with a metalloenzyme so that 18OHB was maintained in the readily oxidisable alpha-ketol structure. Alternate pathways of aldosterone biosynthesis have been suggested (Neher, 1979). There is some evidence that C-18 hydroxylation occurs before C-11 or C-21 hydroxylation after pregnenolone was synthesised. That is 18-hydroxylated derivatives of pregnenolone, progesterone and deoxycorticosterone are other possible precursors of aldosterone biosynthesis.

Conversion of corticosterone via 18OHB to aldosterone was indirectly implicated to occur via a two steps mixed function oxidation because of the requirement of cytochrome P_{450}, oxygen and NADPH (Watanuki et al., 1977; Yanagabashi et al., 1986). The generally accepted mechanism for the conversion of corticosterone to aldosterone is the oxidation of corticosterone to 18OHB which is further oxidised to 18,18' dihydroxycorticosterone. The latter undergoes spontaneous dehydration to give aldosterone (**Figure 3**; Ulick et al., 1976; Kojima et al., 1984).

Although 18OHB has little mineralocorticoid activity (Lanthier and Sandor, 1973; Ulick and Vetter 1962; Ulick et al., 1964; Huston et al., 1981) and no other proven physiological function (Damasco et al., 1979), it is a useful biochemical marker for the diagnosis and differentiation of primary hyperaldosteronism especially patients with aldosterone-producing adenoma (APA) from those with bilateral hyperplasia of idiopathic

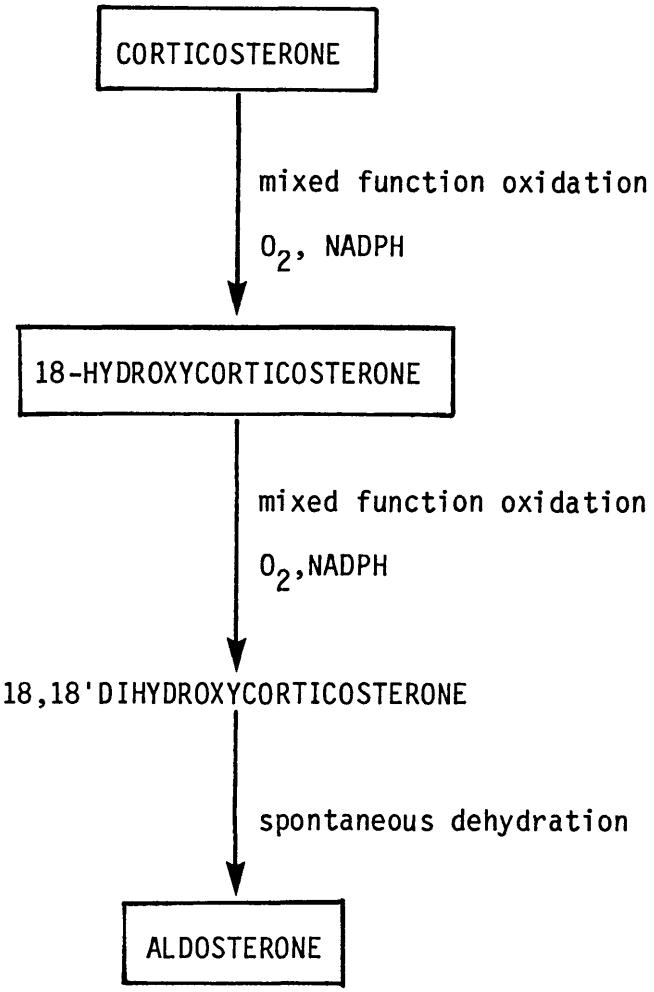


Figure 3

A general accepted mechanism for the conversion of corticosterone to aldosterone, via 18-hydroxycorticosterone being associated with a metalloenzyme as the intermediate, and a two step mixed function oxidation of corticosterone to aldosterone (proposed by Ulick et al., in 1976 and confirmed by Kojima et al., in 1982).

aetiology (IHA)(Biglieri et al., 1979; Bravo et al., 1983; Kem et al, 1985; Fraser et al., 1981). The distinction between these two groups of patients is important since the treatment of the two conditions differ, adrenalectomy or ablation being indicated in patients with APA whereas the raised blood pressure in patients with IHA could be controlled by anti-hypertensive drugs.

To date, several methods have been described for measuring 18OHB based upon the radioimmmunoassay of 18OHB directly (Connolly et al., 1978; Ojima & Kambegawa, 1979; Belkien et al., 1980; Witzgall et al., 1981) and of the 18,20 gamma lactone of 18OHB (Martin et al., 1975) or a method involving gas-liquid chromatography with electron capture detection , (GLC-ec), (Wilson et al., 1976). An enzyme immunoassay of 18OHB has also been described (Watanabe et al., 1984). All of these methods are either tedious or cumbersome to perform, since they involved solvent extraction and tedious chromatographic procedure (paper or column). Chromatography of the samples is necessary since most of the available antisera to 18OHB severely cross-react with 18-hydroxydeoxy-corticosterone (18OHDOC) and its gamma lactone and other steroids (cortisol, corticosterone, testosterone). The number of samples that can be assayed within a single batch is limited by these tedious procedures. Antibodies raised against 18OHB with negligible cross-reactivities with known substances have been achieved by Witzgall et al., 1981, but these authors

developed a chromatographic method so that other steroids in the same sample could be measured simultaneously. However, the titre of the antiserum using tritiated 18OHB with a specific activity of 31 Ci/mmol as the radioimmunoassay tracer was only 1:3,500. Therefore, the possibility of raising sufficiently specific antibodies against 18OHB using 18OHB-3-(O-carboxymethyl)oximino-BSA (18OHB-3-CMO-BSA) as the immunogen can be achieved. Antiserum with a higher titre coupled with the use of [^{125}I]radioligand as the tracer would be ideal for developing a method for measuring 18OHB simply and economically. Although the method using GLC-ec is simple and sensitive, it requires a high temperature to reduce the retention time and is limited by the deterioration of the material making up the electron capture detector with the high temperature used. The difficulty is overcome by reducing the number of samples to be measured.

This thesis describes the development and application of a radioimmunoassay for 18OHB present in saliva and plasma. The aims are to develop a method, sufficiently robust for routine use; a radioimmunoassay with sufficient sensitivity, reliability and precision; and an assay in which a large number of samples can be measured in a single batch. The development involved the raising of suitable antibodies against 18OHB, the preparation of the [^{125}I]radioligand namely 18OHB-3-(O-carboxymethyl)oximino-[^{125}I]iodohistamine (18OHB-3-CMO-[^{125}I]histamine), the validation

and optimisation of a radioimmunoassay and the establishment of a method for measuring 18OHB present in saliva and plasma specifically.

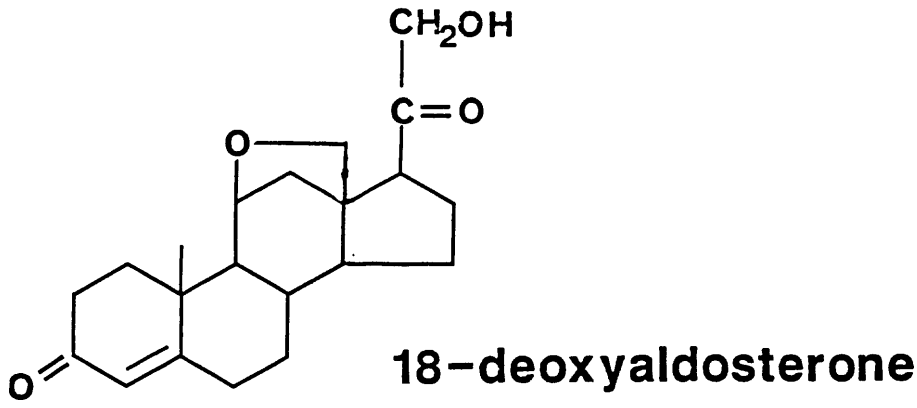
If the antibodies raised were insufficiently specific to measure 18OHB directly in plasma even after solvent extraction, it might be possible to measure 18OHB in saliva directly based on the development of a direct assay for measuring aldosterone in saliva (Few et al., 1984). Only a direct assay will facilitate the measurement of a large number of samples and reduce the cost of the assay.

The hapten of choice was to conjugate 18OHB via the C-3 carbonyl position to a protein. By doing so, the structure of the steroid molecule would be least distorted and the unique cyclic C18,20 hemiketal functional group would be exposed to the immune system of the rabbits in which the antibodies were raised. The derivative chosen was 18OHB-3-CMO, which was conjugated to bovine serum albumin and the antibodies were raised in rabbits. In addition, 18OHB-3-CMO was conjugated to a homologous protein namely rabbit serum albumin (Mckenzie and Clements, 1974; Schmied et al., 1975).

18OHB predominantly exists as the thermodynamically favoured cyclic hemiketal structure (**Figure 1b**) but this should not be a problem in the development of an assay for measuring 18OHB. The main problem is the formation of other artefacts in alcoholic

solution and in the presence of acid even at a concentration of 0.01M HCl (**Figure 4**, Aragones et al., 1978). Therefore, like 18-hydroxydeoxycorticosterone (18OHDOC, Roy et al., 1976) 18OHB dissolved in organic solvent could be stabilised by the addition of triethylamine at a concentration of 0.1% v/v (Usa et al., 1979). It is necessary to minimise the formation of 18OHB artefacts especially if a method developed for measuring 18OHB is based upon immunoreactivity. It was found that the two main forms of 18OHDOC have different immunological properties (Usa et al., 1979) using antibodies raised against 18OHDOC. This may also apply to 18OHB.

Relatively little is known of the physiological role of 18OHB and interest has largely focussed upon its presumed role as an intermediate in the biosynthesis of aldosterone where its measurement is used for the diagnosis and differentiation of primary hyperaldosteronism. The diagnosis of patients with primary hyperaldosteronism is frequently based upon high basal plasma aldosterone level, low plasma renin activity, hypokalaemia with metabolic alkalosis and elevated blood pressure (Ferriss et al., 1981 , 1983). The anomalous fall of plasma aldosterone level in patients with APA but not in those with IHA due to a change of posture from recumbent to upright has been shown to be useful (Ganguly et al., 1973). However, a rise in aldosterone level did not rule out patients with APA. Moreover, there was also an



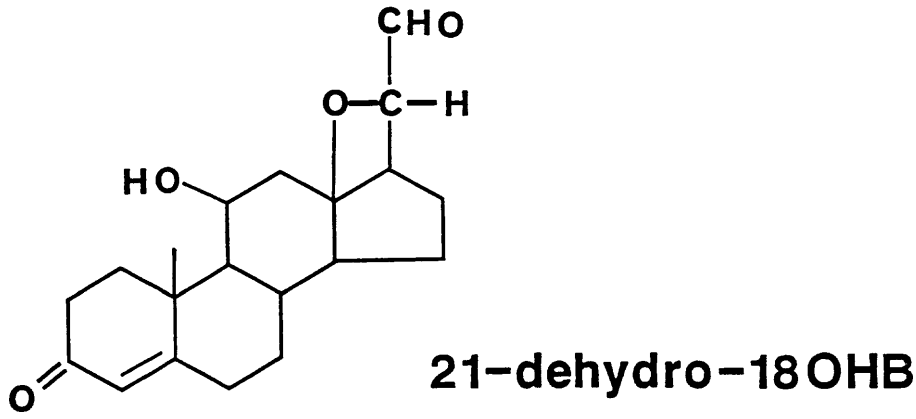


Figure 4

Two dehydration products of 18OHB in acidic medium. 21-dehydro-18OHB is the compound speculated by Aragones et al. (1978) whereas 18DAL is that speculated by various authors (Kirk and Rajagopalan, 1976; Kondo et al., 1965; Schmidlin & Wettstein, 1960; Tori et al., 1963.)

overlap of basal aldosterone but not 18OHB levels in patients with APA or IHA (Biglieri et al., 1979; Bravo et al., 1983; Chu and Ulick 1982; Kem et al., 1985). Other than having a high overnight recumbent 18OHB levels, patients with APA also exhibited the anomalous fall of 18OHB levels due to a change of posture from overnight recumbent to upright position. Perhaps measuring overnight 18OHB levels is a sufficiently sensitive marker of primary hyperaldosteronism.

The physiological variations of 18OHB with respect to aldosterone, cortisol and actual clock time have not been studied. Therefore, the diurnal variations of 18OHB in healthy subjects, patients with primary hyperaldosteronism and a patient with essential hypertension were studied. Simultaneously, the effect of postural change can be observed since the first saliva sample was collected while the subjects remained supine after overnight recumbency. Saliva samples were used to facilitate sampling since multiple samples were required for such studies and because collecting saliva is stressless, does not require supervision and hence can be done on an out-patient basis. In a couple of subjects, frequent (every 30 minutes) samples were collected in order to study the temporal pattern of the steroids secretion. Because collecting saliva is also non-invasive, saliva samples were collected from a group of healthy school children whose daily activities were similar and saliva samples were collected by these

children at the same time.

The secretion of 18OHB may be controlled mainly by the renin-angiotensin system and partly modulated by corticotrophin. The influence of these two factors was studied directly and indirectly. The direct method involved stimulation of normal subjects with corticotrophin. The indirect method was to measure salivary aldosterone and cortisol concentrations in addition to 18OHB levels under physiological conditions. Although measuring these steroid levels after dexamethasone and AII infusion are highly desirable to complete the studies, the investigation was not pursued.

It has been demonstrated, by measuring the specific activity of the major metabolites of the steroid after intravenous injection of the two steroids, that the secretion rate of 18OHB is twice that of aldosterone (Ulick et al., 1964, 1965). The plasma concentration of the two steroids in healthy subjects is approximately equal according to some authors (Wilson et al., 1976; Mason et al., 1977; Biglieri et al., 1979). Others found that the ratio of plasma 18OHB:aldosterone was between 2 and 3 (Martin et al., 1975; Witzgall et al., 1981). Yet others found that the ratio ranged from 2.6 to 103.7 with a mean $\pm$ SD of 18.3 $\pm$ 32.8 in healthy subjects (Kem et al., 1985). Therefore, the relationship between the two steroids was also studied.

Chapter 2

Materials and Methods

2.1. MATERIALS

2.1.1. For studying the methods of preparing 18OHB-3-CMO derivatives and its conjugation to proteins

	Chemicals	Suppliers
(a)	Carboxymethoxylamine hemihydrochloride	Aldrich Chemical co.
(b)	Bovine serum albumin (RIA grade)	Sigma
(c)	Rabbit serum albumin	Sigma
(d)	For mixed-anydride reaction:	
	1,4 dioxane A.R. grade	FSA
	tri-n-butylamine	BDH
	Iso-butylcholoroformate	BDH
(e)	Dialysis tubing (VT-26, 20 mm diameter)	FSA

2.1.2. **For the preparation of 18OHB-3-CMO[^{125}I] iodohistamine radioligand**

(a) For mixed anhydride reaction (see section 2.1.1. (d))

(b) Histamine (free base) Sigma

(c) Carrier-free sodium I-125 Amersham International

(d) Chloramine T BDH

(e) Sodium metabisulphite May & Baker Ltd., Dagenham

2.1.3. **For immunisation**

(a) Animals:

Sandy half lop rabbits (male, 3 months old) were purchased from National Institue of Medical Research (MRC Mill Hill, London)

(b) Complete and incomplete Freund's adjuvants Sigma

(c) Dried, heat killed tubercle bacillus, gift from Dr. I. Brown, Dept. of Bacteriology, St. Mary's Hospital Medical School, London W2 1PG.

2.1.4. **For radioimmunoassay**

(a)	Phosphate buffer (pH 7.4)	
	Di-sodium hydrogen orthophosphate dihydrate (Sorensons salt) (Mol. wt = 177.99)	FSA
	Potassium dihydrogen orthophosphate (Mol. wt = 136.09)	FSA
(b)	Bovine serum albumin (RIA grade)	Sigma
(c)	Dextran grade C (Mol. wt = 60,000 to 90,000)	BDH
(d)	Gelatin, from swine skin Type 1, approx. 300 blooms.	Sigma
(e)	Charcoal, activated, untreated powder, 250-350 mesh	Sigma

2.1.5. **For extracting plasma and saliva:**

(a)	Dichloromethane, A.R. grade, stabilised with 0.005% amylene.	FSA
(b)	Sodium sulphate (anhydrous)	FSA
(c)	[1,2-^{3}H] 18-hydroxycorticosterone	Amersham International

2.1.6. **For thin-layer chromatography**

(a)	Ethanol A.R. grade, redistilled before use	FSA
(b)	Ethyl aceate, A.R. grade	FSA
(c)	Chloroform, A.R. grade	FSA
(d)	Methanol, A.R. grade, redistilled before use	FSA
(e)	Aluminium backed thin layer silica gel plate with fluorescent dye (Kieselgel 60 F_{254}), 20 x 20 cm square, 0.2 mm thick, E. Merck, Darmstadl	BDH

2.1.7. **For paper chromatography**

	Whatman chromatography paper No. 1	Whatman
	23.0 cm x 100 cm roll	Ltd.

2.1.8 **For cross-reactivity studies**

(a) Steroids:

All 18-hydroxylated steroids were gifts from Professor D.N. Kirk, curator of the Steriod Reference Unit with present address at : Department of Chemistry, Queen Mary College, University of London, London E1.

18-hydroxycorticosterone (Mol. wt = 362)

18-hydroxydeoxycorticosterone (Mol. wt = 346)

18-hydroxyprogesterone (Mol. wt = 330)

18-hydroxycortisol (Mol. wt = 378)

Materials and Methods

All the following steroids were purchased from Steraloid Ltd.

Aldosterone (Mol. wt = 360)

Cortisol, compound F (Mol. wt = 362)

6ß-hydroxycortisone (Mol. wt = 366)

Deoxycortisol, Compound S (Mol. wt = 346.5)

Tetrahydrocortisol (Mol. wt = 366)

Cortisol-21-acetate (Mol. wt = 403)

Prednisolone (Mol. wt = 362)

Cortisone, compound E (Mol. wt = 361)

Corticosterone, compound B (Mol. wt = 346)

11-dehydrocorticosterone, compound A (Mol. wt = 344.4)

Deoxycorticosterone (Mol. wt = 330.5)

Testosterone (Mol. wt = 288.4)

Oestrone (Mol. wt = 270.4)

17ß-oestradiol (Mol. wt = 272.4)

Oestriol (Mol. wt = 288.4)

Progesterone (Mol. wt = 314.5)

17 alpha-hydroxyprogesterone (Mol. wt = 330)

5 alpha-androstan-3-ol-17one, Androsterone

(Mol. wt = 290.5)

Other substances used for cross-reactivity studies:

Glycyrrhetinic acid (3ß-hydroxy-11-oxo-olean-12-ene-30-oic acid, $C_{30}H_{46}O_4$ (Mol. wt = 470.67)

Cholesterol (Mol. wt = 386.64) Boehringer

Dexamethasone (Mol. wt = 392.45)

Synacthen, Tetracosactrin acetate (Mol. wt = 2,933.6) Ciba

2.1.9. **For adrenocorticotrophic stimulation:**

Synacthen Ciba

2.2. **APPARATUS:**

2.2.1. Glassware for solvent extraction

Quickfit extraction tubes and stoppers: FSA

MF 24/2/6 (capacity 31 ml)

MF 24/3/8 (capacity 75 ml)

MF 24/5 (capacity 195 ml)

2.2.2. Glassware for evaporating dichloromethane:

Pyrex conical tubes (capacity 14 ml) FSA

2.2.3. For dispensing dextran coated charcoal:

Peristaltic pump Watson & Marlow

2.2.4. Gamma counter LKB

2.2.5. ß-counter: Packard Tri-carb liquid scintillation spectrophotometer, Model 3380 Canberra Packard

2.2.6. Ultra-violet lamp Scientific supplies Co

2.2.7.	Flame photometer (Corning 435)	Corning
2.2.8.	"Dianorm machine, 1000	MSE, Crawley

2.3. Saliva and blood collection tubes:

7 ml Bijou	Sterilin
Lithium heparinised tubes	

2.4. METHODS

2.4.1. Preparation of 18OHB-3-CMO

Introduction

The most commonly used condition and reagent for preparing a steroid-3-oxime is the alkaline condition described by Erlanger et al. (1957 and 1959) and carboxymethoxylamine.hemihydrochloride ($(CMO)_2HCl$) respectively. Recently, Belkien et al. (1980) described a short reaction time (15 minutes) procedure for preparing 18OHB-3-CMO under acidic condition (pH1.6). However, a novel reaction procedure for preparing 18OHB-3-CMO derivative and the stabilisation of this derivative is described. In addition the conjugation of 18OHB-3-CMO to bovine serum albumin (BSA) or rabbit serum albumin and [^{125}I]iodohistamine ligand is described.

Procedure

(a) Kinetics of the formation of oxime

18OHB in methanol (5 µmole in 50 µl) with tritiated steroid (0.5 µCi) added as recovery marker and $(CMO)_2HCl$ in methanol (5 µmol in 50 µl) were reacted at room temperature (23^{o}C). At various time intervals 5 µl of the reacting mixture was spotted onto a TLC plate, which was kept cool. The TLC plate was then developed in a system of ethanol and ethyl acetate (60:40 v/v). The spots corresponding to the oxime were eluted with methanol. The amount of

oxime formed and steroid left unreacted were measured by radioactive counting and ultra-violet spectrophotometry. The time course of the oximation of aldosterone and cortisol was studied in a similar manner.

Results

The kinetics of the oximation of the three steroids are shown in Figure 5. It was found that these three steroids have very similar kinetics and the time required for maximum formation of the oxime was 15 minutes, which was similar to that reported by Belkien et al. (1980). Hence, an identical reaction condition was adopted for a macro-scale synthesis of 18OHB-3-CMO derivative described in the following section.

(b) Preparation of 18OHB-3-CMO for conjugation to BSA

Procedure

18OHB in methanol (35 mg, 96.7 µmol, in 4 ml) with added tritiated 18OHB (2 µci) was reacted with $(CMO)_2HCl$ in methanol (10.6 mg, 48.3 µmol, in 0.9 ml) for 15 minutes with stirring at room temperature (23^oC). At the end of the reaction, 5 µl of the mixture was spotted onto a TLC plate to check the purity of the oxime produced. Two spots were shown, one of which was the monoxime and the other spot was the unreacted 18OHB. No 'dioxime' was formed.

The remaining mixture was made alkaline (pH=11.0) with 0.1M

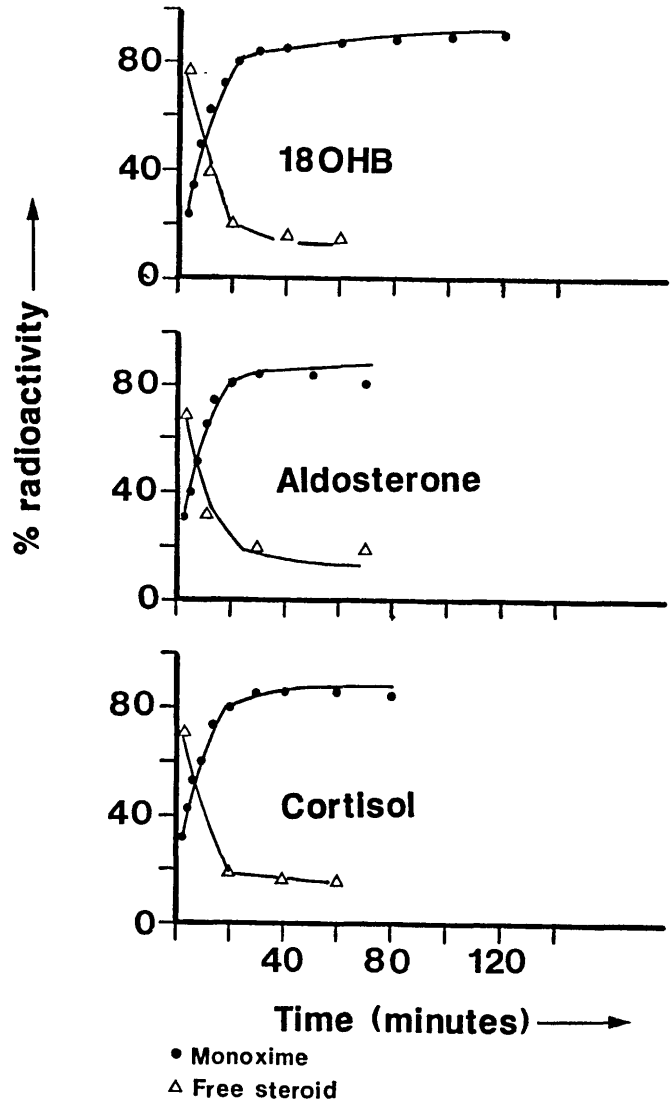


Figure 5

Time course of the oximation of steroids with two carbonyl functional groups at C3 and C21 using a mild reaction, reacting equimolar proportions of each steroid and carboxymethoxylamine.hemichloride in methanol at room temperature.

Na_2CO_3 (15 ml). Distilled water (20 ml) was added to increase the aqueous phase and the non-reacted 18OHB was extracted into dichloromethane (100 ml x 2). The aqueous layer was then acidified to pH 2.0 with 1.0 M HCl (12 ml) before extracting with freshly redistilled ethyl acetate (200 ml x 2). The extract was washed with water (20 ml x 2) and then dried with Na_2SO_4 before reducing it to dryness and then crystallisation was attempted.

Results

It was found that a white amorphous material was formed which could not be crystallised. It has a melting point of 225^{o}C (decomp.). The product was then found to contain both the monoxime and an 'artefact' which was absent prior to the crystallisation. The material could not be purified without the formation of an artefact and hence impure 18OHB-3-CMO was conjugated to BSA. This conjugate was used for immunising 6 rabbits. It was then postulated that 18OHB-3-CMO derivative could possibly be stabilised by the formation of its sodium salt, which simultaneously has an advantage of being a better substrate for the mixed anhydride reaction, which was the reaction procedure used for conjugating the 18OHB-3-CMO derivative to [^{125}I]iodohistamine or protein. Hence, the experiment was pursued with one mg of 18OHB as described below.

(c) Preparation of 18OHB-3-CMO stabilised as its sodium salt

Procedure

18OHB (1 mg, 2.762 µmoles, in 120 µl methanol) with added tritiated 18OHB (1 µci) was reacted with $(CMO)_2$-HCl (1.381 µmoles in 83.8 µl) for 15 minutes at room temperature. 5 µl of the reaction mixture was spotted onto a TLC plate. Then NaOH in methanol (4.1 µmoles, 41 µl of 0.1M NaOH in methanol obtained by diluting 2.0 M NaOH with methanol) was added. The mixture was evaporated to dryness and reconstituted with methanol containing 0.1 % triethylamine. 5 µl of the reconstituent was again spotted onto a TLC plate to check the purity of the oxime produced.

Results

Only 18OHB-3-CMO derivative was formed with trace amounts of 18OHB left unreacted. The percentage yield of 18OHB-3-CMO, determined by radioactivity, was 90%. This 18OHB-3-CMO derivative was conjugated to rabbit serum albumin (RSA) for raising antibodies in 2 rabbits.

Subsequently, another 3 mg of 18OHB was reacted in a similar manner for identification by Nuclear Magnetic Resonance (NMR) spectroscopy. The NMR spectrum is shown in **Figure 6** and shows that Z and E stereoisomers of 18OHB-3-CMO in the proportion of 4:5 were formed, retaining the cyclic C18-20 cyclic hemiketal structure of 18OHB. No other compound was found.

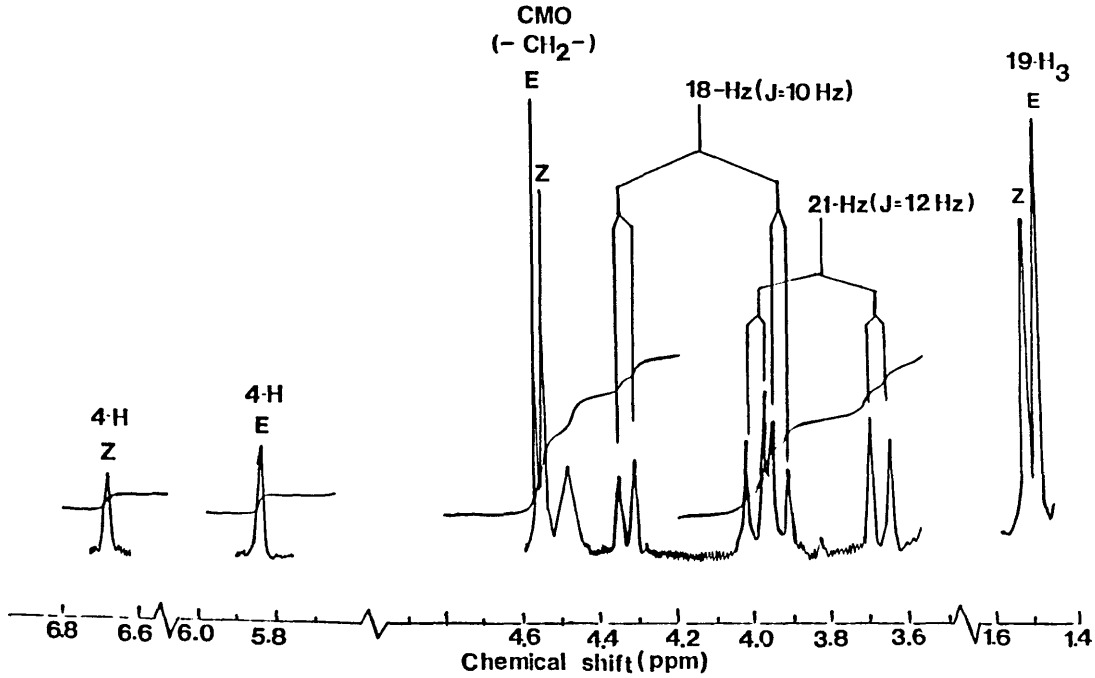


Figure 6

Nuclear magnetic resonance spectrum of 18OHB-3-CMO-Na (Reproduced with the kind permission of Professor D.N. Kirk). The spectrum was recorded at 100MHz in $CDCl_3$ and compared with that of 18OHB (Kirk and Slade, 1981). It was found that 18OHB-3-CMO-Na in this preparation consisted of two isomers Z and E, based on the nomenclature of Cahn, Ingold and Prelog (1985) and in the ratio of 4:5 (Z:E). The cyclic hemiketalic structure of 18OHB remained unaltered indicating the absence of 18OHB-dioxime.

(d) Conjugation of 18OHB-3-CMO to protein by the formation of peptide linkage

The peptide linkage between carboxylic group of 18OHB-3-CMO derivative and the proteins (either BSA or RSA) was formed using the mixed anhydride procedure of Vaughan et al. (1953) and Erlanger et al. (1957,1959). The isolation of the conjugate and the determination of the number of steroid molecules incorporated into the protein residue was however different from that of Erlanger's. Latter method involved the precipitation of the conjugate with ethanol prior to lyophilisation; and the spectroscopic determination of the molar incorporation of steroid molecules into the protein residues assuming that the extinction coefficients of the steroid-oxime and its protein conjugate were similar.

Procedures

The 18OHB-3-CMO (7.0 mg, 16 µmoles) prepared as decribed in section (b) was dissolved in tri-n-butylamine (16 µmoles, 38 µl of a 1:10 dilution with anhydrous dioxane, which was prepared by standing dioxane over KOH pellets for one week and freshly redistilled with reflux for 20 minutes). Some 18OHB-3-CMO was dissolved in anhydrous dioxane and spotted onto TLC plate to check the purity of the compound. The solution was cooled in an ice bath ($10^{o}C$). Isobutylchloroformate (16 µmoles, 21 µl of a 1:10 dilution with anhydrous dioxane) was added. The reaction mixture was stirred for

30 minutes and then added as a portion to a solution of BSA dissolved in a mixture of dioxane and water in the ratio of 1:1 v/v. The reacting molar ratio of BSA and 18OHB-3-CMO was 1:59. 1.0M NaOH (37 µl) was added to the mixture. After one hour, another volume of NaOH (18 µl of a 1.0M solution) was added and the mixture was stirred for another 3 h in the ice bath. The conjugate was then dialysed against running water for 18 hours. The product was lyophilised without first precipitating the protein-conjugate, and weighed. The number of molecules of 18OHB-3-CMO incorporated was determined by tritium counting. The initial BSA concentration was determined gravimetrically and by Lowry's method. It was compared to the gravimetric measurement of the lyophilised conjugate after sub tracting the number of 18OHB-3-CMO residues being incorporated. The mixed anhydride procedure described here was also used to conjugate 18OHB-3-CMO-Na salt to rabbit serum albumin. A carbodiimide method was tested for conjugating cortisol-3-CMO to BSA but the molar incorporation of the cortisol-3-CMO molecules into the BSA molecules was very low (10:1). Hence, this method was not used for conjugating 18OHB-3-CMO molecules into BSA residues.

Results

(i) Conjugation of 18OHB-3-CMO to BSA

The concentration of BSA measured gravimetrically and by Lowry's method of estimation agreed with 90% accuracy. The calculation of the incorporation of 18OHB-3-CMO into BSA was as follows:

Weight of 18OHB-3-CMO conjugate	= 36.8 mg
Weight of 18OHB-3-CMO from radioactivity measurement	= 6.8 mg
Therefore, weight of BSA in the conjugate	= 30.0 mg
Hence, the molar ratio of 18OHB-3-CMO : BSA conjugate (Note: Mol. wt of BSA was taken to be 66,000)	= 34:1

(ii) Conjugation of 18OHB-3-CMO-Na to RSA

The molar ratio of 18OHB-3-CMO : RSA was found to be 45:1 (Note: Mol. wt of RSA was taken to be 70,000)

Discussion

Pilot studies showed that pure 18OHB-3-CMO derivative could not be prepared using the methods which were found to be feasible for preparing pure cortisol-3-CMO derivative. These methods included the short reaction acidic condition (Belkien et al., 1980), the mild basic condi tion (Arnold and James 1971) or the selective procedure for preparing C-3-oxime in compounds containing C-3,20 dicarbonyl functional groups via the formation of enamine using pyrrolidine (Janoski et al. 1974).

Even though the novel procedure described in this thesis is suitable for preparing pure cortisol-3-CMO crystal (Appendix I) in high yield, it has to be slightly modified in order to prepare stabilised 18OHB-3-CMO derivative. This involves the preparation of 18OHB-3-CMO derivative as its sodium salt.

Initially, the difficulty of obtaining pure 18OHB-3-CMO derivative lies with the formation of an artefact, which has the same thin-layer chromatographic mobility as the dioxime. This artefact was not a dioxime as would have obtained with using excess $(CMO)_2HCl$ to 18OHB molecules for the reaction. This artefact was formed when chromatographic^{ally} pure monoxime was concentrated and rechromatographed. If a dioxime is formed from two molecules of the monoxime, a molecule of 18OHB would be formed. This was not found. The artefact could possibly be a 20,21';20'21- 'anhydro dimer'.

2.4.2. PREPARATION OF 18OHB-3-(O-carboxymethyl)oximino [^{125}I] iodohistamine and ITS ASSESSMENT

Introduction

A modified method of Al-Dujaili et al.(1978) was used. In their procedure, 1.45 mg of the oxime was used in the mixed anhydride reaction but only 50 µg of the activated steroid was used for the coupling. 50 µg of the oxime was activated in our procedure and directly used for coupling. The amount of all the reagents used was scaled down in proportion. Secondly, the 1 % acetic acid present in the solvent mixture used for the thin-layer chromatographic purification of the radioligand was omitted. One of the modifications made by Al-Dujaili and their colleagues to the method described by Nars and Hunter (1973) was the use of a lower proportion of oxime:iodohistamine. Instead of a 100:1 proportion, a 5:1 was used.

Results

A typical autoradiogram of the radioligand after chromatographic separation is shown in **Figure** 7. The percentage yield of the most polar product (P) having a relative mobility to the solvent front of 0.29 has improved from 4% to 20% on average. Simultaneously, the yield of the less polar band (LP) has decreased from 38% to 15% on average. The difference was attributed to the stabilised

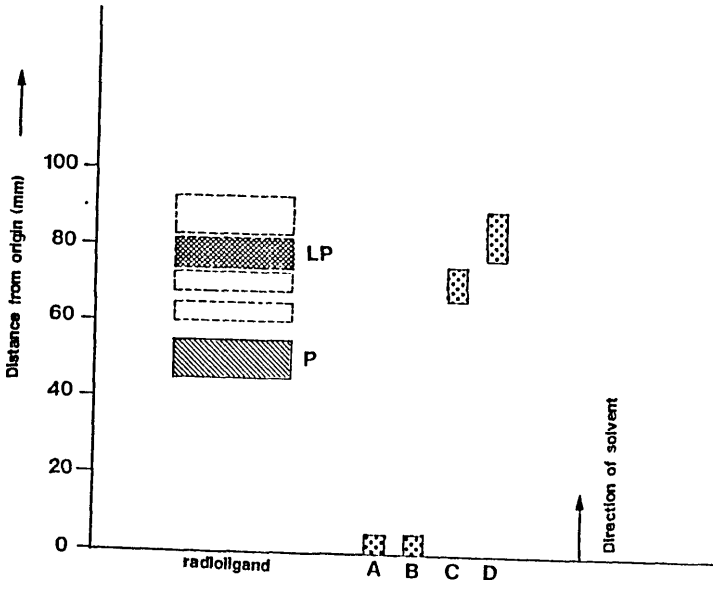


Figure 7

A typical autoradiochromatogram of the iodinated products of 180HB-3-CMO derivative. A modified solvent system of that of Nars and Hunter (1973) was used. It consisted of toluene:ethanol in the ratio of 70:30. The room temperature was 17°C on this occasion. The relative mobility varied with ambient temperature. The only immunoreactive label was P. LP bound strongly and was not displaceable by 180HB standard even at a dose of 500 pg/tube. The relative mobilities of various substances to the solvent front (R_f) were :-

Code	Content	R_f
P	Polar label	0.29
LP	Less polar label	0.46
A	[^{125}I]iodohistamine	0.01
B	180HB-3-CMO-Na	0.01
C	180HB	0.42
D	Cortisone	0.51

18OHB-3-CMO-Na salt being used.

Only band P was immunoreactive to the antibodies raised against 18OHB-3-CMO. Band LP had a strong binding to the antibodies but could not be displaced by 18OHB even in concentration as high as 1000pg/tube (5525 pmol/l). However, it could be displaced by the 'dioxime'. Both bands were equally immunoreactive to the antibodies given to us by Dr. Belkien.

Displacement curves due to varying mass of [^{125}I]radioligand are shown in **Figure 8**. At an optimum antibody dilution of 1:30,000, the mass of the label which could be used to achieve reasonable sensitivity and statistically acceptable count rate was between 4000 cpm and 14,000 cpm. However, the amount of label used for all subsequent assays was between 4000 cpm and 7000 cpm. By using label in this range of count rates, the sensitivity of the standard curve was maintained throughout a 6 months period. The values of the QC samples were also maintained. Early preparations of the radioligand deteriorated rapidly. At that time the original Nars & Hunter (1973) solvent system was used for TLC which contained 1 % acetic acid. Because of the known instability of 18OHB in acid medium this was omitted from more recent preparations. This resulted in no loss of chromatographic resolution but in the radioligand being usuable for over 6 months.

The specific activity of the product was estimated by radioimmunoassay. Tubes containing varying amount of the label were

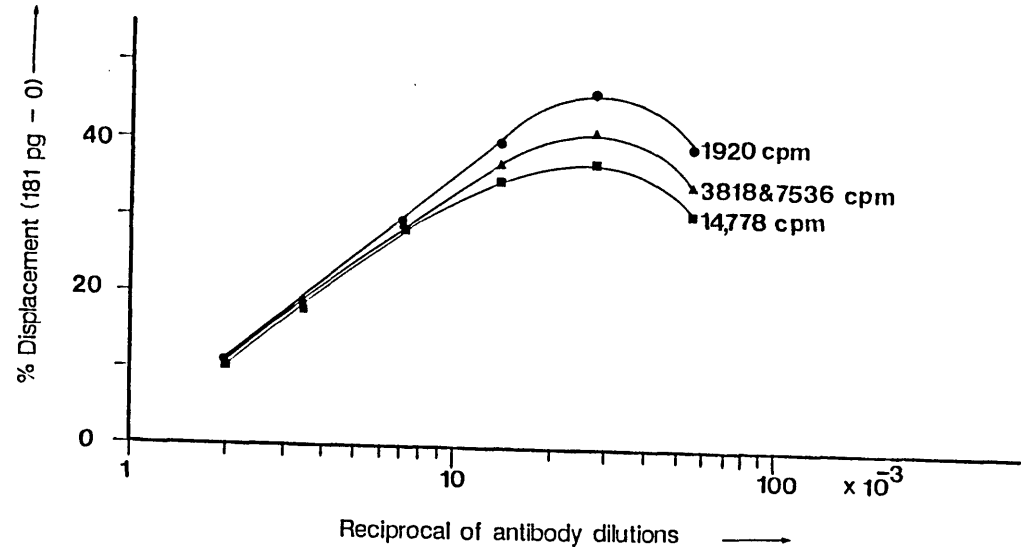


Figure 8

Displacement curves due to varying mass of [^{125}I]radioligand at varying antibody concentrations.

assayed as standard tubes. The standard curve and the curve containing varying amount of label was shown in **Figure 9a** and **9b** respectively. The specific activity of one batch of label was estimated to be 105.9 Ci/mmol. Standard curves using [1,2-^{3}H]18OHB (specific activity of 60 Ci/mmol) and [^{125}I]radioligand are shown in **Figure 10.** The sensitivity of the standard curve has improved using the [^{125}I]radioligand and the antibody dilution was also increased.

The % yield of the usable radioligand was consistently found to be 20%. Improvement could be achieved by using a higher molar ratio of oxime:iodohistamine.

Conclusion

The [^{125}I]radioligand prepared by the method described here is usable for more than 6 months and the sensitivity of the standard curve is improved compared to using tritiated 18OHB as the radioligand.

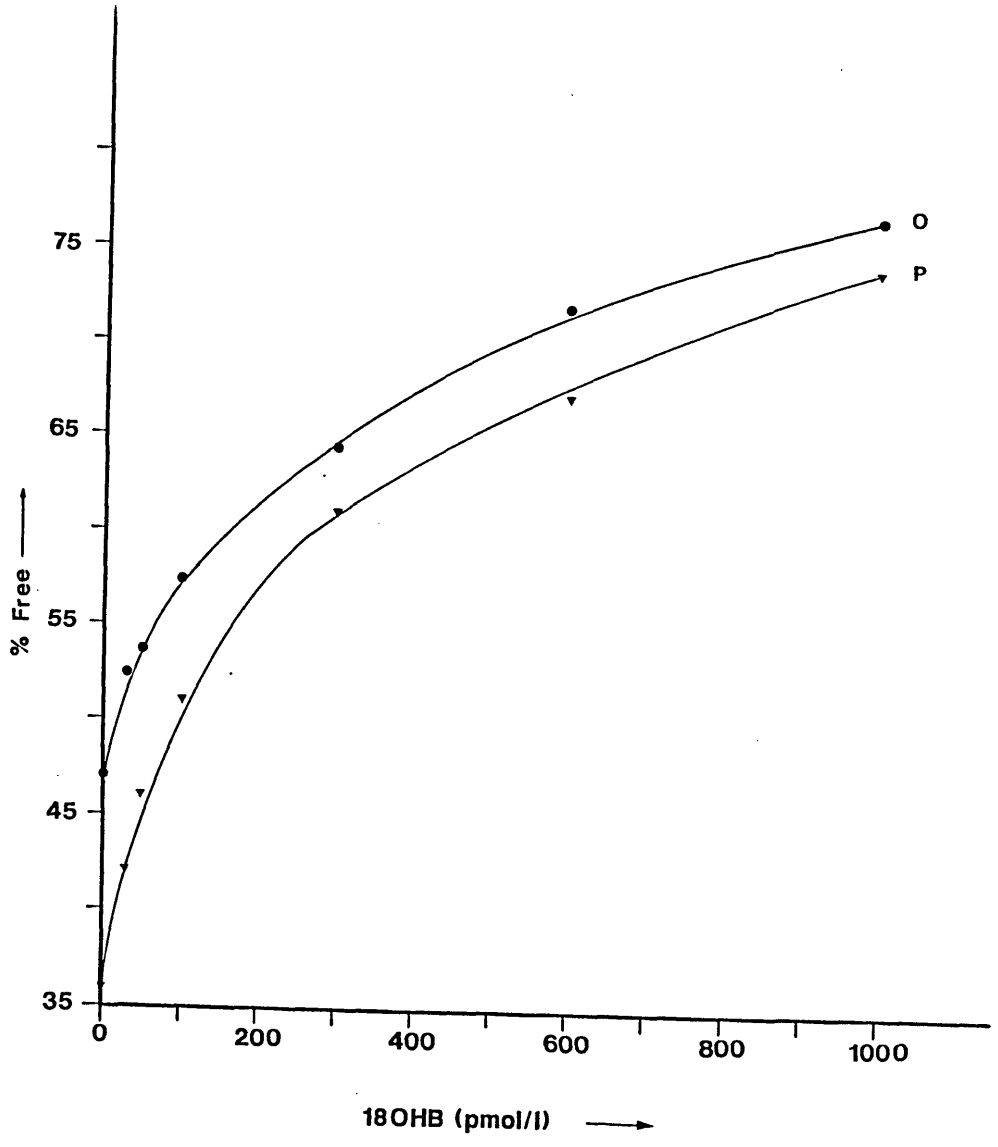


Figure 9a

Comparison of standard curves using a 7 months old (O) [^{125}I] radioligand and the same label after being purified (P). The total count used was 4000 counts/min. Although the standard curves were significantly different, the values of the quality control samples were not affected.

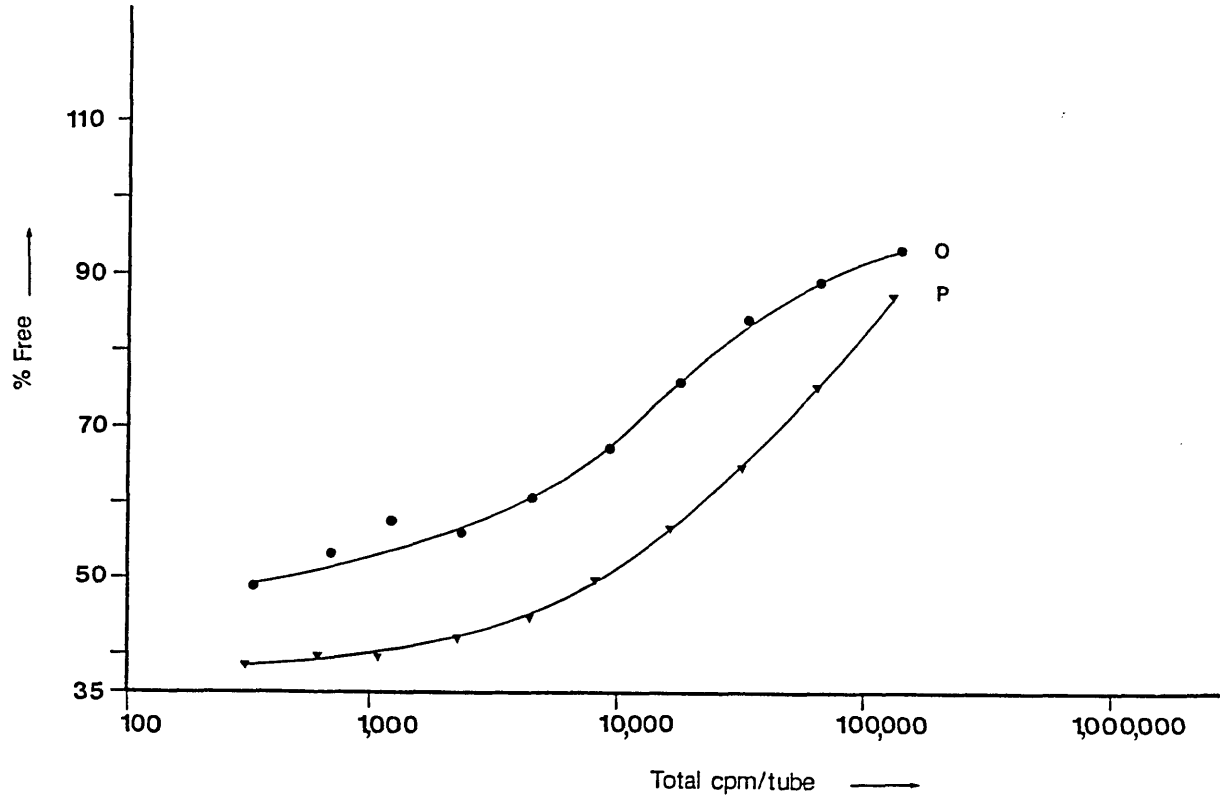


Figure 9b

Dose response curves using varying mass of the old (O) and purified (P) [^{125}I]radioligand mentioned in Figure 12a. The specific activities of both labels were calculated based on the respective standard curves shown in Figure 15a and were found to be 30.7 Ci/mmol and 109 Ci/mmol for the O and P labels respectively.

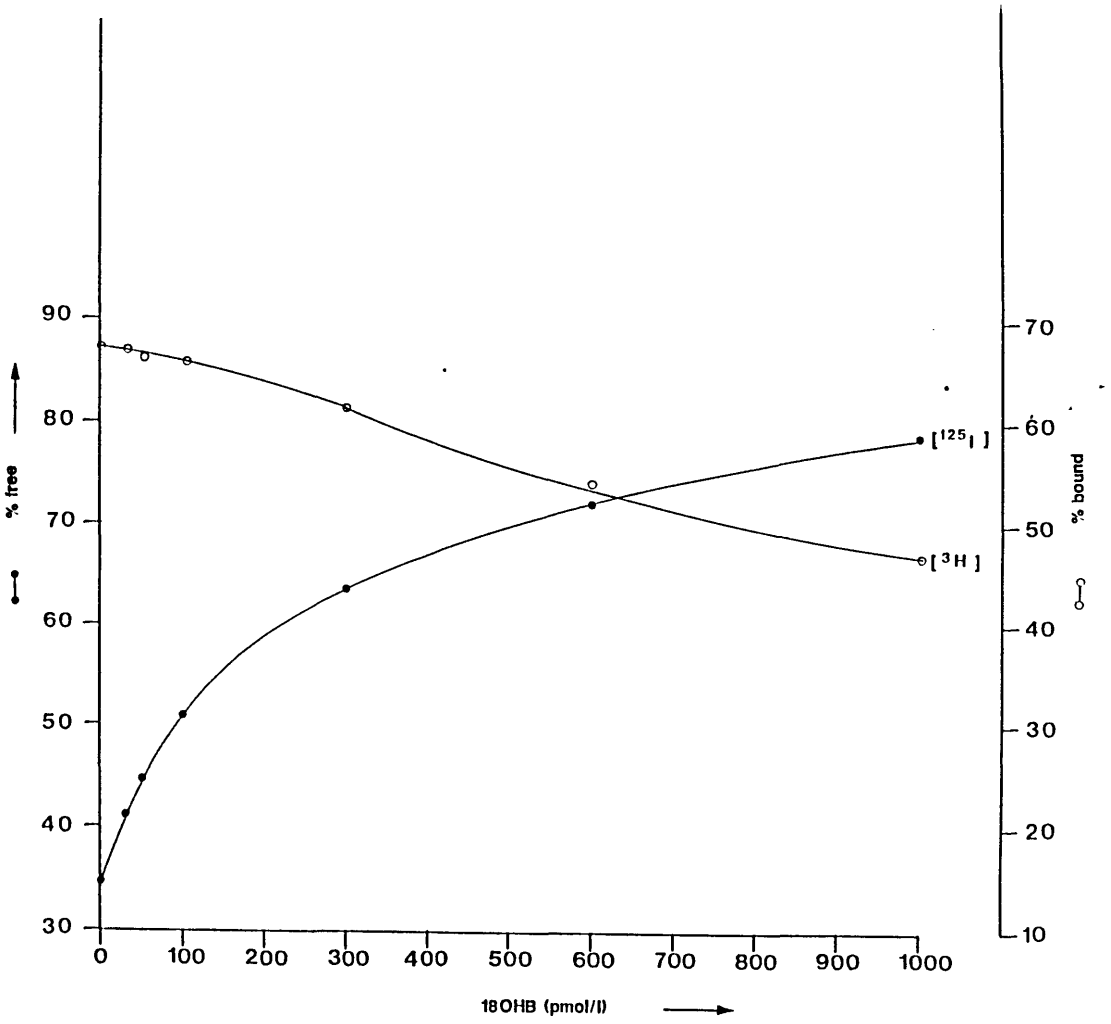


Figure 10

Comparison of the standard curves set up with 18OHB-3-O-(carboxymethyl)-oximino-[^{125}I]iodohistamine and [1,2-^{3}H]18OHB labels. The final optimum antibody dilution using the iodo-radioligand and the tritiated ligand was 1:30,000 and 1:2,000 respectively. The specific activity of the tritiated 18OHB specified by the supplier was 60 Ci/mmol. That of the iodo-radioligand was estimated to be 105 Ci/mmol. This might hence explain the sensitivity of the standard curve using the [^{125}I]radioligand.

2.4.3. (a) IMMUNISATION OF RABBITS

Introduction

The method used was based on the low dose (100µg antigen/rabbit) multiple sites intradermal immunisation technique described by Vaitukaitis (1971,1981). One modification made by them during the ten years was to omit the use of pertussis vaccine. This latter protocol was followed.

2.4.3. (b) CHARACTERISATION AND EVALUATION OF THE ANTISERA RAISED USING [^{125}I]IODORADIOLIGAND AS TRACER

(a) Titre

Titre is arbitrarily defined as the final antibody dilution of the antiserum which binds 50% of the immunoreactive label of a certain mass. It was determined by means of an antiserum dilution curve where no antigen was added. At very high antibody concentration (1:100 to 1:1000) the binding of the ligand was 98 $\pm$ 1.3 % (mean $\pm$ SD) using antiserum #5/12/6. This is sufficiently near to 100% that no correction to the theoretical 50% binding is necessary. Therefore, in the subsequent calculation of the titre, a 50% binding of the added label was used.

Results

The titres of the various bleeds from rabbit #5 are shown in Figure 11. The titre assessment using antiserum #5/12/6 ranged from 1:30,000 to 1:66,000 . This was attributed to the batch to batch variation of the label and the age of the label. The titre has now stabilised at 1:66,000 for antiserum #5/12/6 tested with a few batches of both freshly prepared and aged radioligand. However, only results obtained using the same label in the assay set up the same day are presented here. The titres, optimum % displacement and brief specificity assessment of the sera from the

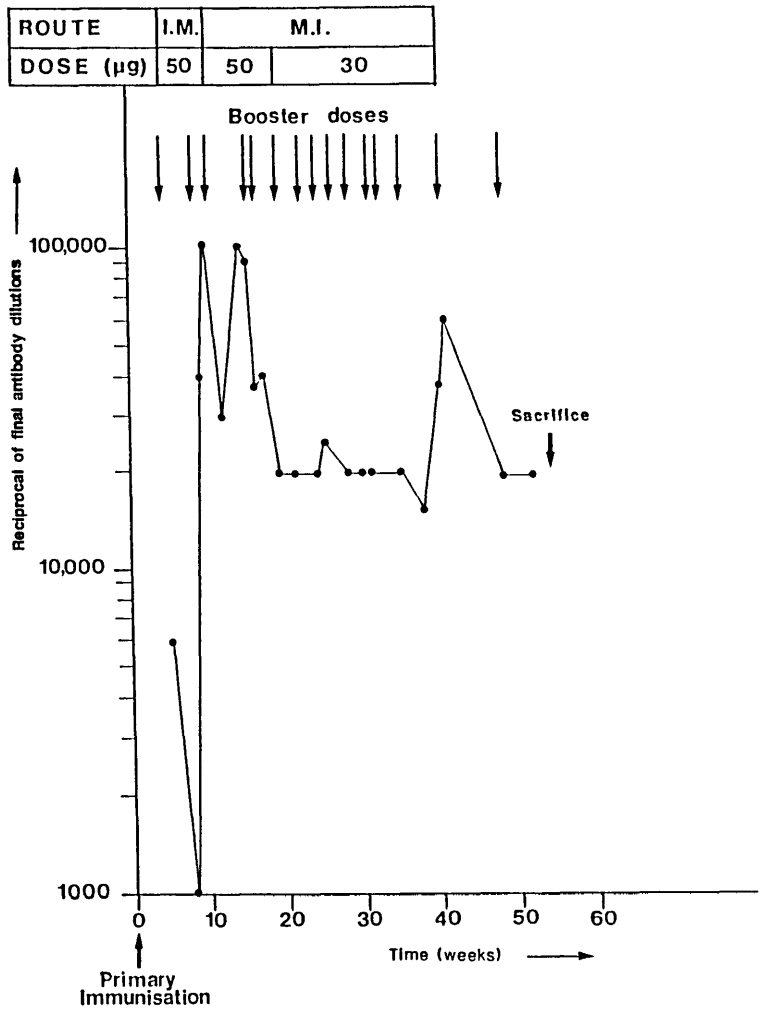


Figure 11

Titres of antiserum #5/12/6. The titres were assessed using the [^{125}I]radioligand as tracer and dextran coated charcoal to separate the free and the bound fractions. The titres shown above were assessed at the same time in two batches. The immunogen used for the boosting was initially done by intramuscular (I.M.) injections (50 µg immunogen in 1.0 ml saline). Later, it was reverted to multiple intradermal (M.I) sites injection (10 sites, 50 µg in 1.0 ml incomplete Freund's adjuvant, IFJ). The amount of immunogen was reduced to 30 µg in 1.0 ml IFJ from 21 weeks post primary immunisation.

other 5 rabbits immunised against 18OHB-3-CMO-BSA conjugate have been assessed and are not going to be presented here but will be discussed.

(b) Optimum antibody dilution

A displacement curve was done in a similar fashion for determining the titre except with the addition of 18OHB standard of 181pg/tube (1000 pmol/l), which was used as the top standard of the standard curve.

Results

A typical displacement curve with 18OHB standard using antiserum #5/12/6 was shown in **Figure 12.** The optimum antibody dilution using the same antiserum ranged from 1:16,000 to 1:30,000. This again was attributed to the initial batch to batch variation of the radioligand. The optimum antibody dilution has now stabilised at 1:30,000 for antiserum #5/12/6 tested with the last batches of radioligand. It varied slightly with age of the label but it did not affect the results of the samples used as quality control. A typical standard curve is shown in **Figure 13.**

Antiserum #1/16/9, whose titre was 1:330,000, bound very strongly to the label. To obtain a reasonable degree of displacement, a high concentration of 18OHB standard was required. It was not possible to improve the sensitivity of the standard

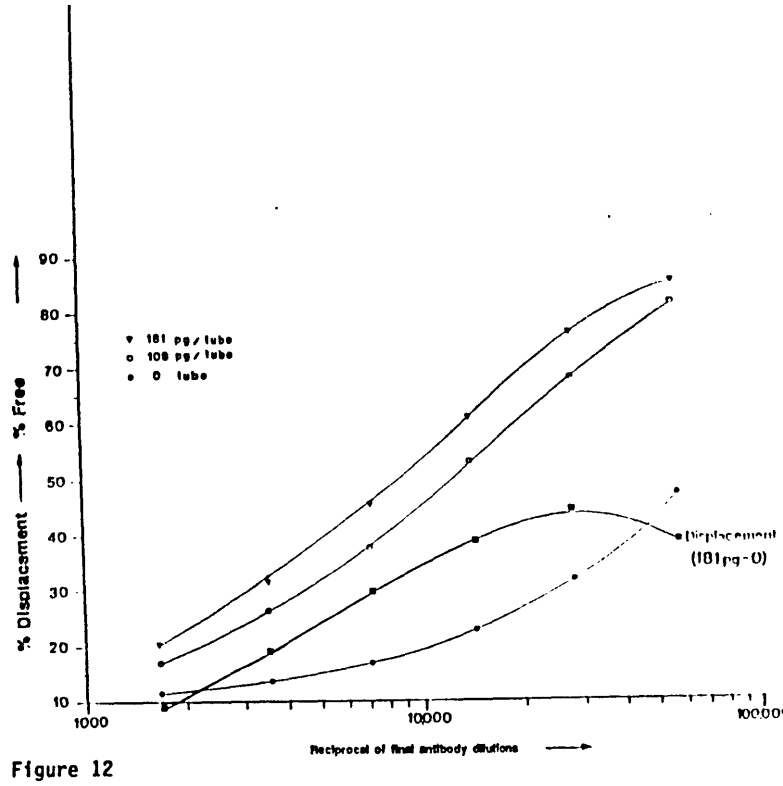


Figure 12

A typical antibody dilution and displacement curves using antiserum #5/12/6 and [^{125}I]radioligand.

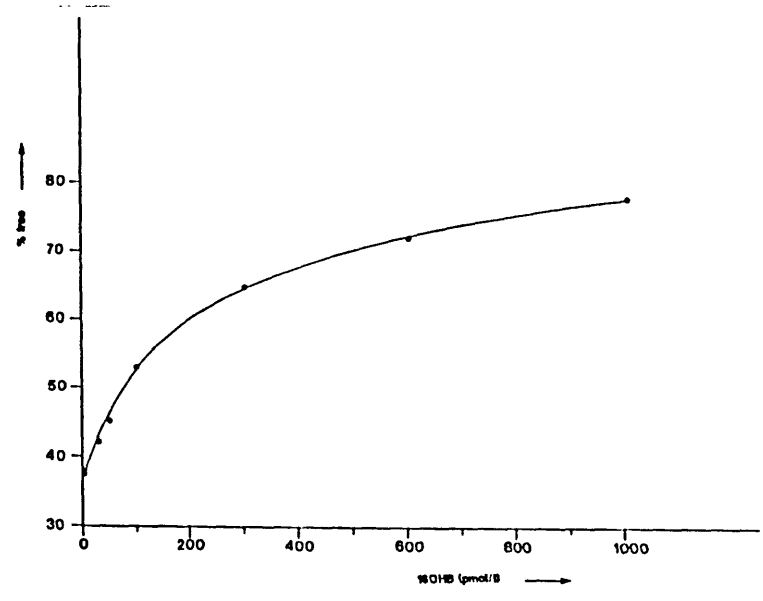


Figure 13

A typical standard curve of 18OHB using antiserum #5/12/6 at a final antibody dilution of 1:30,000 using [^{125}I]radioligand (ca. 4000 cpm/tube).

curve using this antiserum by increasing the antibody dilution (**Figure 14**). This phenomenon is hence an exception to the general observation that the higher the antibody dilutions used within a reasonable limit, the more sensitive is the standard curve.

(c) Specificity

Procedure

This was studied in two stages. The first stage was done in order to select the best bleed of antiserum to use for developing a radioimmunoassay. A direct assay of a pool of saliva, a pool of plasma diluted 1:5, cortisol and aldosterone standards were studied. Cortisol was used because cortisol concentrations in the saliva and plasma were higher than any other hormonal steroids. Aldosterone was studied because of its related chemical structure to 18OHB.

The second stage involved the use of known steroids and substances used for experimentation by setting up a series of standard curves for 18OHB and the cross-reactants in question. Also a technique was developed, to assess cross-reactivity with unknown substances present within biological fluids. This technique has been termed as 'radioimmunochromatography'. It involved the extraction of substances from saliva and plasma into dichloromethane, chromatographic separation and testing of

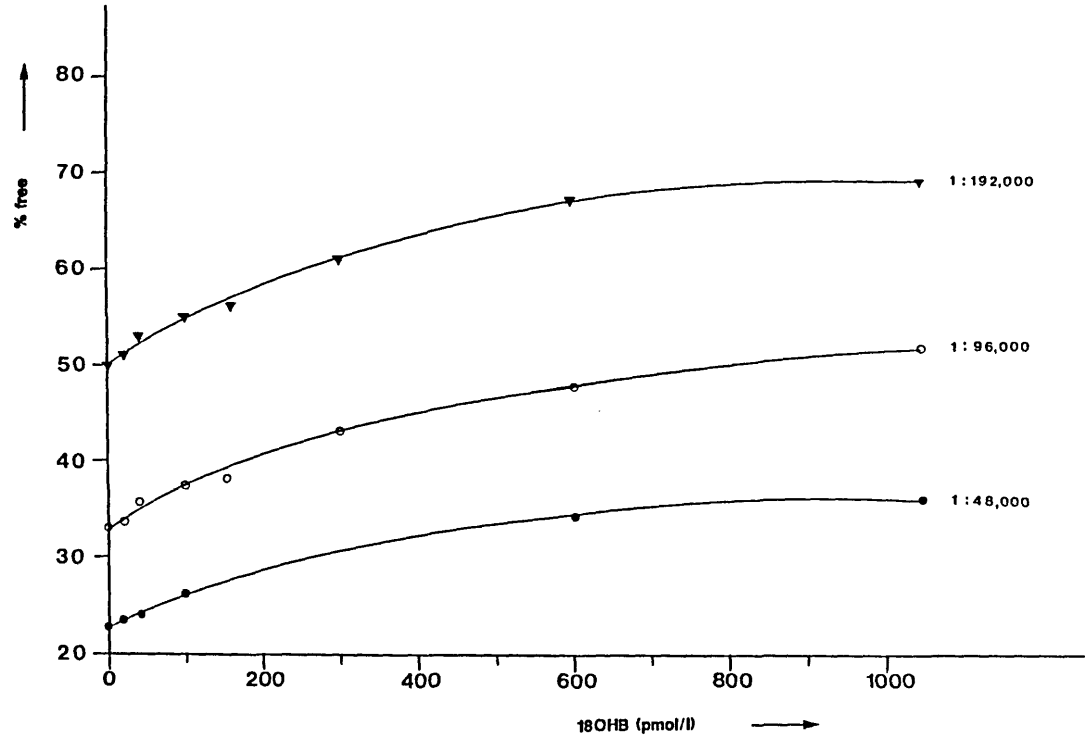


Figure 14

Standard curves of 18OHB at three antibody dilutions using antiserum #1/16/9 and [^{125}I]radioligand. Increasing the antibody dilution did not improve the sensitivity of the standard curve with this antiserum.

immunoreactivity throughout the whole length of the TLC plate using the antiserum chosen from the first stage. In addition, saliva samples were assayed directly and after solvent extraction (including the measurement on the aqueous residue after solvent extraction), and the results were compared with the results obtained by radioimmunochromatogram. Similarly, plasma extract and the residue after extraction were measured by radioimmunoassay and the results were compared with that obtained by the radioimmunochromatographic method. This method was developed because not all interferring substances are known or are available in pure form. The saliva and plasma used were from normal subjects, pregnant women, subjects during salt restriction and salt loading tests, patients with renal disorders, primary hyperaldosteronism, essential hypertension and ileostomy.

Results and discussion

From the first stage of analysis, it was found that all the antisera raised against 18OHB-3-CMO-BSA conjugate cross-reacted with cortisol. The best antiserum to use was from rabbit #5 out of the 6 rabbits immunised with 18OHB-3-CMO-BSA conjugate, between bleed 5 (at 10 weeks) onwards till the animal was sacrificed at 52 weeks after primary immunisation. The brief cross-reactivity study in some of the antisera is shown in **Table 1.**

It was found that the cross-reactivity data obtained using

Rabbit #/wks/ bleed #	Reciprocal Optimum antibody dilutions	Amount of immunoreactive 18OHB (pg/tube) 'Saliva'	'Diluted Plasma'	% cross reactivity F	Aldo	E
#5/10/5	20,000	12	46	0.15	-	-
#5/12/6	20,000	12	27	0.20	-	-
#5/14/7	20,000	8	65	0.15	-	-
#5/15/8	20,000	15	75	0.25	-	-

Table 1.

Brief antibody assessment of the specificity of antisera from rabbit #5. Iodinated radioligand was used and the assessment was done as a single batch. Data using the antisera from other rabbits showed that the amount of immunoreactive material using the same saliva and plasma pool was at least 3 fold higher than the results shown here. - indicates that crossreactivity is less than 0.0001%.

known steroids (**Table 2**) were similar to that obtained by 'radioimmunochromatogram' with respect to the steroid the antibodies cross-reacted. Interestingly, however, one other spot of cross-reactivity of unknown identity was found in 2 of the three patients with primary hyperaldosteronism studied. This spot was more polar than 18OHB and remained at the origin of the thin-layer chromatographic system used. The amount of this spot was approximately 10% of the 18OHB spot. The identity of this spot is uncertain because no authentic compound studied has the chromatographic mobility in the solvent system used. It was also deduced from the data of the 'radioimmunochromatogram' that patients with primary hyperaldosteronism and patients with renal diseases have elevated concentration of corticosterone. Therefore, a method involving the removal of corticosterone and these less polar substances by solvent extraction may simplify the method used in this thesis, which serves as a reference method. This simplified method will be useful for screening samples. Although it may be a screening method, this simplified method could enable meaningful physiological data to be obtained. This method will involve a solvent extraction using ether or a solvent mixture such as hexane and ether in varying proportions to remove, in particular corticosterone, and other less polar substances.

It was also found that the antisera tested did not have any immunoreactivity with the aqueous saliva or plasma residue

Steroids and other substances	% cross-reactivity	Mobility relative to cortisone	
		A	B
1. 18OHB	100.00	0.47	0.43
2. 18-hydroxydeoxycorticosterone	3.57	0.84	1.01
3. 18-hydroxyprogesterone	45.40	1.07	1.48
4. 18-hydroxycortisol	-	0.51	0.38
5. Progesterone	0.74	1.22	1.71
6. 17a-hydroxyprogesterone	0.33	1.21	NC
7. Androsterone	-	NC	NC
8. Testosterone	0.18	1.09	NC
9. 17β-oestradiol	-	1.21	NC
10. Oestrone	-	1.00	NC
12. Oestriol	-	0.68	NC
13. Aldosterone	-	0.69	0.89
14. Deoxycorticosterone	0.25	1.07	1.58
15. Corticosterone	2.70	0.93	1.10
16. Compound A	-	0.91	NC
17. Compound S	-	1.07	0.91
18. 6β-cortisone	-	0.83	NC
19. Cortisone	-	1.00	1.00
20. Prednisolone	0.02	0.88	NC
21. Tetrahydrocortisol	-	NC	NC
21. Cortisol-21-acetate	-	1.25	NC
22. Cortisol	0.15	0.94	0.65
23. Glycerol	-	NC	NC
24. Cholesterol	-	NC	NC
25. Synacthen *	-	(origin)	NC
26. Dexamethasone	-	1.08	NC
27. Glycyrrhetinic acid	-	NC	NC

Table 2

Cross-reactivity data of antiserum #5/12/6 and chromatographic mobilities of these substances in two solvent systems as follows:-.

system A: 5 % ethanol in ethyl acetate (developed twice)

system B: 10% methanol in chloroform

- indicates cross-reactivity of less than 0.0001 %; * located with ninhydrin.

NC not chromatographed

remaining after dichloromethane extraction.

Antiserum #5/12/6 cross reacted with authentic 18-hydroxyprogesterone (18OHProg) by 45.4%. This will allow the investigation of the presence of 18OHProg in biological fluids. This will be investigated. 18-hydroxyprogesterone has been shown to be a poor substrate of aldosterone biosynthesis using frog and ox adrenals (Ulick et al., 1964; Kahnt et al., 1965). However, it has been shown that the patients with 21-hydroxylase defect have elevated concentrations of 18-hydroxyprogesterone in their urines in comparison to controls, patients with primary hyperaldosteronism and essential hypertension (Lewicka et al., 1986).

The cross-reactivity data obtained using authentic steroids are interpreted with the following considerations in order to determine the severity of the cross-reactivity in biological fluid. The severity of the cross-reactivity depends on the concentrations of the analytes in the biological fluids. For example, by considering the presence of a maximum amount of progesterone present in the plasma of 3.0 nmol/l at a cross-reactivity of 0.736%, the equivalent apparent amount of 18OHB would be 22 pmol/l (3.9pg/tube). Therefore, the cross-reactivity would be minimal in comparison with a cross-reactivity of 0.2% with cortisol. At an average concentration of 30 nmol/l of cortisol in saliva, an equivalent

apparent amount of 60 pmol/l (10.8pg/tube) of 18OHB would be measured.

In addition to the cross-reactivity with cortisol (**Figure 15a**), antiserum #5/12/6 cross-reacted with corticosterone (2.7%). But corticosterone did not affect the specificity of the measurement because it was well-separated from 18OHB in the thin-layer chromatographic system chosen for routine assay.

The conventional method for calculating percentage cross-reactivity was to determine the ratio of the weight of the specific antigen required to reduce the binding of the label at zero by 50% to the weight of the cross reactant which would reduce the binding by the same amount, multiplied by 100%. The percentage cross-reactivity at various degree of binding ranging from 65% to 35% at three antibody dilutions are shown in **Figure 15b**. The percentage cross-reactivity was higher at higher degree of binding of the label to the antibody than at lower degree of binding. Therefore, the percentage cross-reactivity calculated at 50% binding was an estimate of cross-reactivity. The percentage cross-reactivity was also lower when the optimum antibody concentration was used for the assay than when higher or lower antibody concentration than the optimum was used. This therefore implies that the higher the binding of the antibody to 18OHB (i.e. at excess antibody concentration) the higher will be the binding of the antibody to the cross-reactant as well resulting in a

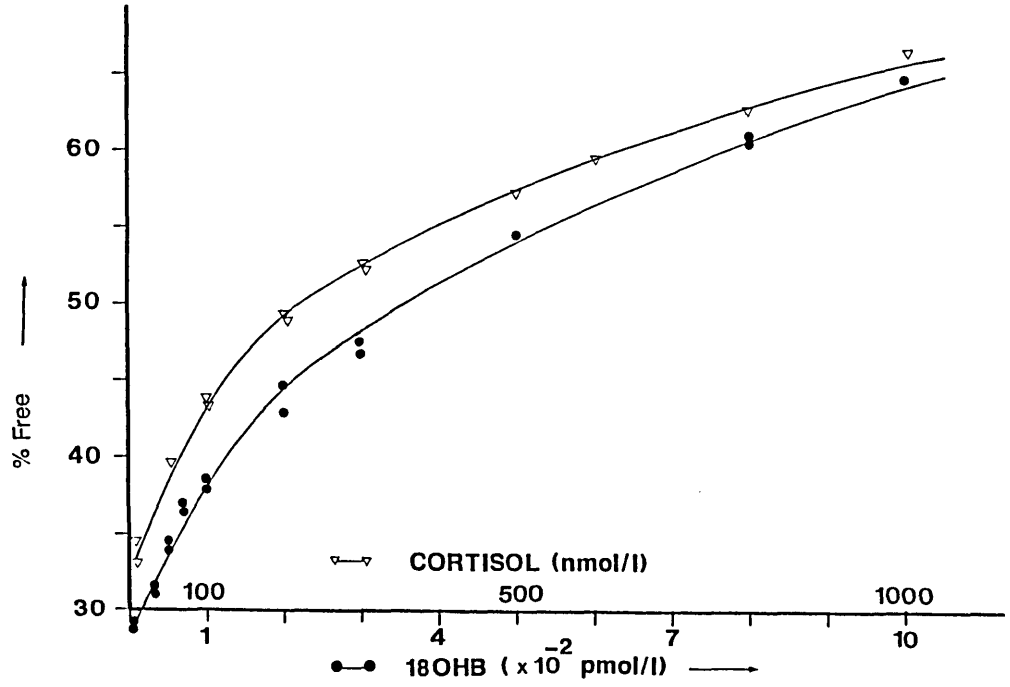


Figure 15a

Standard curves of 18OHB and cortisol for the evaluation of the cross-reactivity of cortisol with antibodies against 18OHB using 18OHB-3-CMO-L[^{125}I]iodohistamine radioligand as tracer.

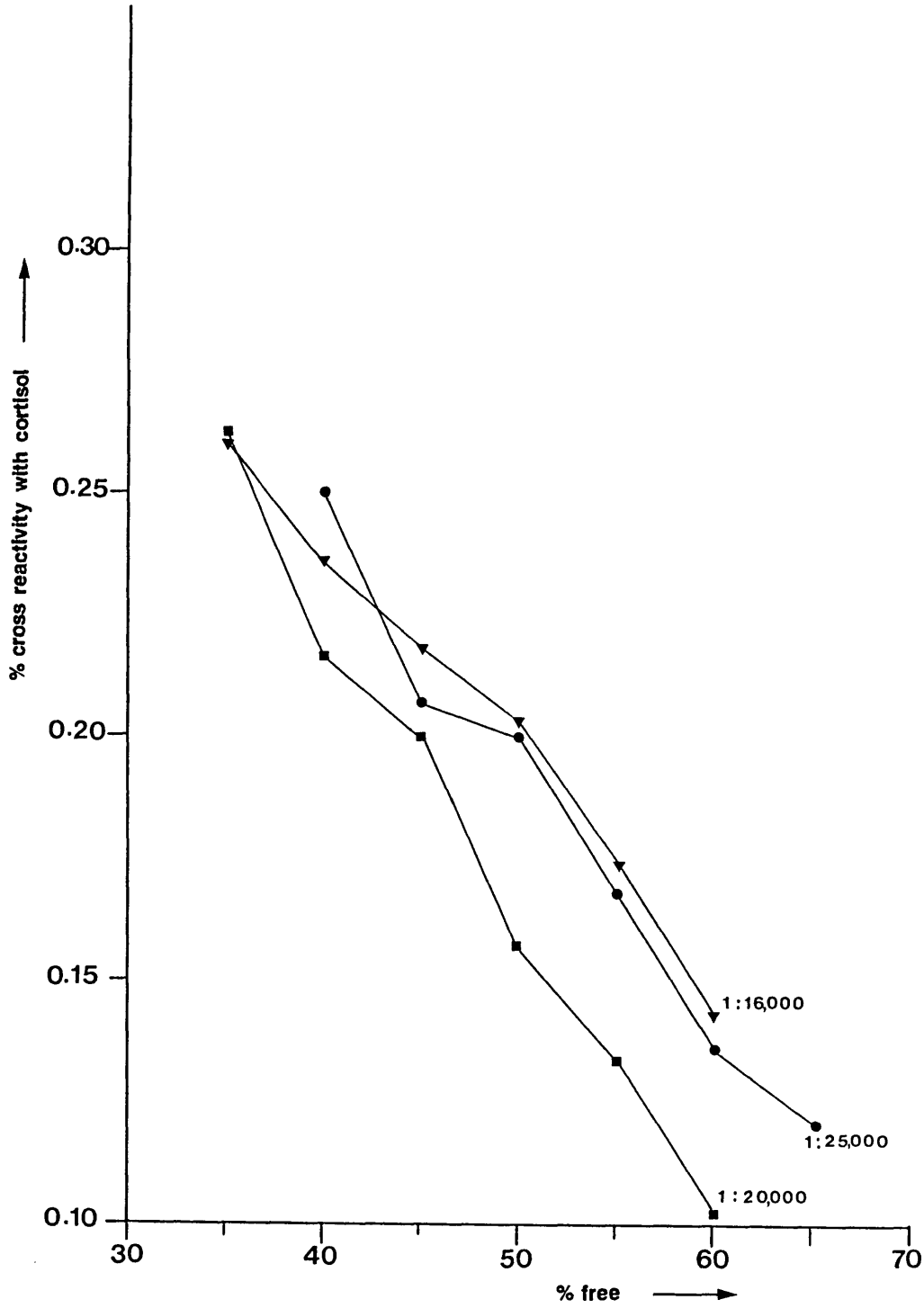


Figure 15b

Percentage cross-reactivities with cortisol calculated at varying levels of displacement of the [^{125}I]radioligand from the antibody at three antibody dilutions. The antiserum used was #5/12/6 and the optimum antibody dilution assessed at that time was 1:20,000.

higher percentage of cross-reactivity. On the other hand, improving the sensitivity of the standard curve by using higher antibody dilution will similarly increase the magnitude of the cross-reactivity. It is thus of no advantage to use an antibody dilution other than the optimum.

Neither was the cross-reactivity of the antiserum #5/12/6 to cortisol reduced by using a second antibody precipitation technique for separating the free from the bound fractions at the end of the antigen-antibody reaction.

Although the rabbit antiserum obtained with 18OHB-3-CMO coupled to RSA was specific, it was limited by the problem of 'drift' where the label bound to the antibody was stripped during the separation of free from the bound fractions during dextran charcoal separation. Other separation procedures were not pursued, since it was felt that this antibody did not have a suitable titre, despite it's greater specificity.

(d) Avidity

Introduction

This has sometimes been used interchangeably with the term 'affinity' though avidity usually refers to the properties of the antibody whereas affinity refers to the antigen. In kinetic language, avidity refers to the binding energy of the antibody for

its antigen and is denoted as K, termed as affinity constant of the antiserum. K can be calculated by Scatchard's plot (Feldman et al., 1971) or by the Michaelis-Menten's hyperbola (Abraham et al., 1975). The latter technique was used for calculating the affinity constants of the antisera described in this thesis. The affinity constant is defined as the reciprocal of free steroid molar concentration at half saturation of the antibody binding sites.

RESULTS

The affinity constants for a few batches of antisera from rabbit number 5 after a certain period of primary immunisation are shown in **Table 3.** Since all the antisera produced cross-reacted with cortisol and corticosterone to a large extent, the choice of the antiserum was based on the one which produced a sensitive curve. In addition, the antiserum chosen was one which would enable a large number of samples to be set up in a single batch using dextran charcoal separation without the problem of 'drift' and available in sufficient quantity.

Table 3 Affinity constants, K, of five bleeds of antisera from one rabbit. The calculation of K is based on that of Michaelis-Menten's hyperbola (Abraham et al., 1975):

Antisera rabbit #/weeks/bleed no.	Affinity constant, K, (x 10^5 l/mole)	
#5/10/5	3.2	
#5/12/6	72.0*2	*1, *2, one and two
#5/14/7	31.0	weeks after boosting
#5/15/8	38.0	with the immunogen
#5/16/9	79.0*1	respectively

2.4.4. (a) RADIOIMMUNOASSAY

The method used was based on that developed in this department for measuring aldosterone (Few et al.,1984). However, optimisation of the assay conditions for 18OHB assay was studied. In addition, the cross-reactivity with cortisol under these conditions was studied simultaneously.

General Reagents:

(a) Phosphate buffer(0.15M, pH 7.4)

(b) Albumin solution (0.1%)

(c) Dextran-gelatin solution (0.1%) with 0.1% sodium azide as preservative

(d) Charcoal in dextran-gelatin solution (1% wt./v)

(e) 18OHB standards

A stock solution of 18OHB dissolved in ethanol containing 0.1% triethylamine (1mg/ml, 2.762×10^9 pmol/l) was prepared and kept at $-20^{o}C$. It was further diluted twice (1:27.6 then 1:50) to give a solution (2×10^6 pmol/l), which was stored at $-20^{o}C$ and used for further dilution in phosphate buffer (pH7.4) to give a top standard

of 1000 pmol/l. This top standard contained 0.05% ethanol and this was shown to have no effect on the radioimmunoassay. The top standard was further diluted to give a series of concentrations 30, 50, 100, 300, 600 and 800 pmol/l).

(f) Tracer and antibody solution

[^{125}I]18OHB-3-CMO solution in ethanol containing 0.1% triethylamine was added to the albumin solution (0.1%) to give a total count of 4000 cpm in 0.2 ml. The percentage of ethanol in the buffer was kept to less than 0.2%. This was pre-calculated during the preparation of the gamma labelled ligand. Otherwise, the ethanol had to be evaporated prior to the addition of the albumin solution. The antibody (#5/12/6, 1:250 in 0.1% albumin containing 0.1% sodium azide) solution was added to the solution containing the tracer to give an antibody dilution which was determined from antibody dilution and displacement curves with each new batch of tracer.

General Methods

(a) 18OHB radioimmunoassay

An aliquot of the standards and other samples such as TLC gel eluates and QC samples (0.5 ml per tube) was pipetted into disposable LP3 polystyrene tubes in duplicate. Then the tracer containing the antibody (0.2 ml) was added. The assay tubes were

left in the cold room($4^{o}C$) overnight and then taken out of the cold room in an ice bath ($4^{o}C$).

Pre-chilled well-stirred dextran coated charcoal suspension was added using a pulsatile dispenser delivering 0.5 ml of charcoal suspension per pulse. The charcoal was dispensed into the tubes in a 'mirror image' manner (1 - n, n - 1) so that the mean time of exposure to charcoal for each pair of duplicates was similar. The tubes were left in the ice bath for a standardised time of 8 minutes prior to centrifugation at $4^{o}C$ and 1500g for 15 minutes (the carriers for holding the LP3 tubes were pre-chilled in the centrifuge). At the end of the centrifugation, the supernatant were decanted and the pellet was left to drain upside down for a few minutes in a rack containing tissue paper. Traces of the supernatant were lightly tapped off onto the tissue paper.

The pellets were counted for 120 seconds in a 12 wells gamma counter. The curve was fitted using the spline fitting program provided by the manufacturer of the counter. It is based on knotted polynomial functions. This was checked by manually plotting the curve and comparing the values of the QC samples for the assay using the two methods. The standards were recounted so that the values of the standards could be intrapolated using the splined fitted curve. This was a method used for ensuring the values obtained from the spline fitted curve were accurate. In addition, only values which fell on the linear part of the standard curve

were used.

(b) Aldosterone radioimmunoassay

The method has been previously described (Few et al., 1984).

(c) Glucocorticoids radioimmunoassay

The method has been previously described including specificity for measuring cortisol (Few et al., 1986a; Few et al., 1986b)

2.4.4. (b) Optimisation

(a) Incubation time

Standards, NSB and saliva QC samples were incubated with the [^{125}I]iodohistamine radioligand and antibody at 4^{o}C for 4h, 8h, 12h, 24h and 48h and were then treated with charcoal. Saliva is chosen as the immunological material for evaluating the effect of incubation time on the specificity of the antibody as it is known that the antibody used crossreacted with cortisol and corticosterone. It is hoped that a direct measurement of 18OHB in saliva samples might be possible. The results of the standard curves are shown in **Figure 16** and that of the saliva QC samples are shown in **Table 4.**

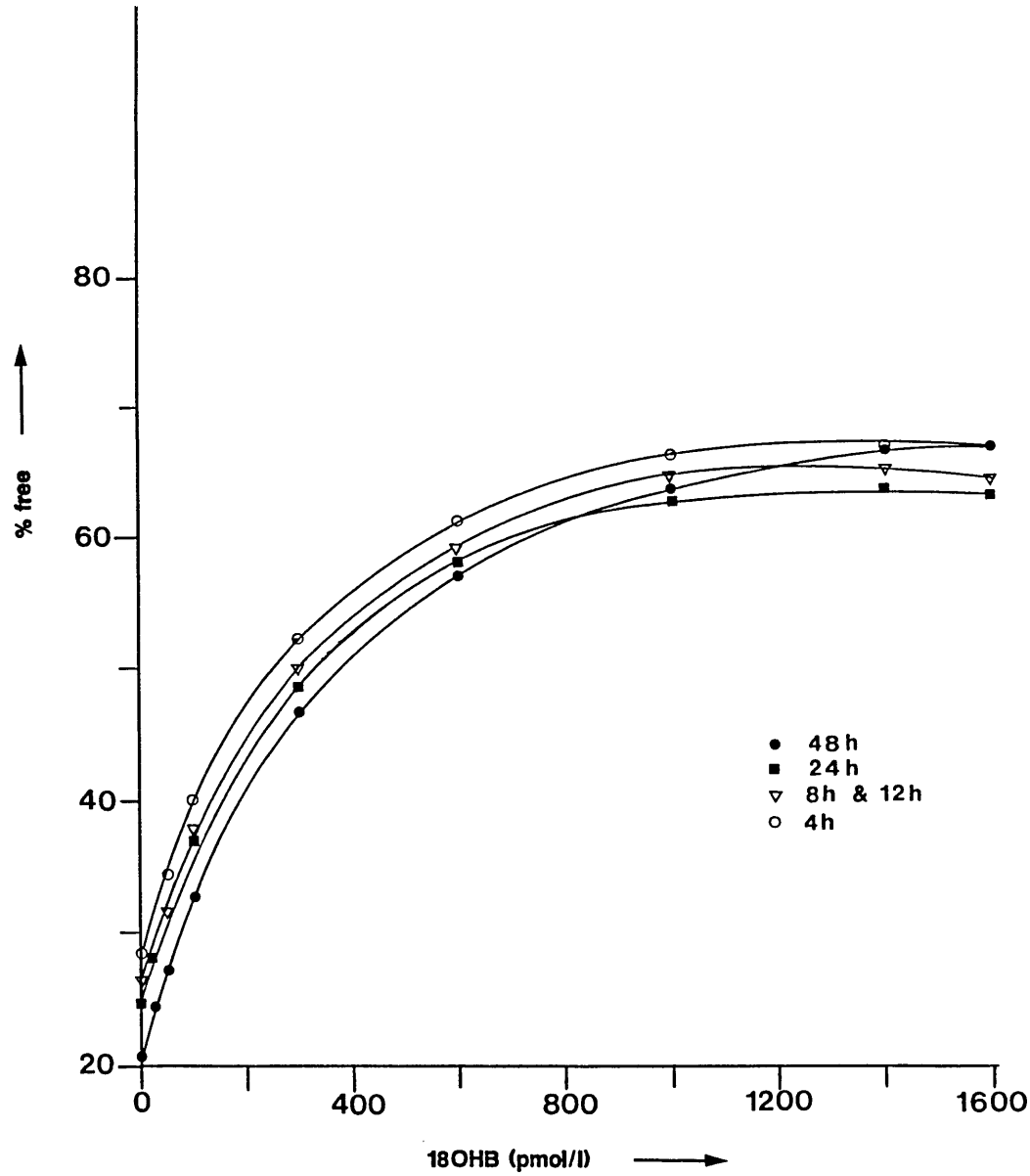


Figure 16

The effect of varying the incubation time at $4^{o}C$ on the 18OHB radioimmunoassay and on the cross-reactivity with cortisol. The antiserum used was #5/12/6 at a final antibody dilution of 1:16,000. The standard curves obtained after 8h and 12h were superimposable. Similarly, this applied to the 12h and 24h incubation. After 48h of incubation, the binding increased slightly. Under all these various incubation times, the percentage cross-reactivity with cortisol was not altered.

Table 4 Assessment of various periods of incubation on 18OHB radioimmunoassay using saliva QC samples of three different doses:

Time (h)	Concentration (pmol/l) mean ± SD (n=6)		
	Low	Medium	High
4	30.4 ± 5.0	60 ± 10.0	130 ± 10.4
8	31.5 ± 6.0	65 ± 6.0	140 ± 15.0
12	30.4 ± 7.0	68 ± 4.0	135 ± 6.0
24	35.0 ± 6.0	70 ± 5.0	138 ± 8.0
48	32.8 ± 6.7	65 ± 6.0	140 ± 9.0

The values of saliva QC samples obtained at 4h, 8h, 12h and 48h were compared with those at 24h by paired t-test and they were not significantly different. For convenience, the time of incubation was chosen to be an overnight incubation for all the subsequent assays.

(b) Varying the amount of dextran coated charcoal

Varying mass of charcoal (2.5mg/tube, 3.1mg/tube, 6.25 mg/tube and 12.5 mg/tube) were added to a series of 18OHB and cortisol standards, zero (for drift assessment) and NSB tubes in a 'mirror image' manner. The tubes were left in an ice bath for 8 minutes

before centrifugation. The results obtained are shown in Figure 17. Since the results obtained at the 5 mg/tube (i.e 1% solution used in general method) and 3.1mg (0.625%) of charcoal were superimposable, the former dose was used for subsequent assays, no 'drift' being detected. The standard curves obtained using the 1.25% and 2.5% dextran coated charcoal were statistically significantly different (paired t-test, $p < 0.001$) from the 1% solution. This could be due to the disturbance of the equilibrium between the antibody and the labelled antigen.

'Drift' was also assessed by leaving the charcoal in contact with zero tubes for varying lengths of time up to 30 minutes in the ice bath before centrifugation using the 1% charcoal suspension. At each period of time dextran coated charcoal solution was added to 10 pairs of zero tubes and NSB. The results showed that 'drifts' did not occur till 25 minutes after the addition of charcoal. Therefore, it was concluded that it was necessary to centrifuge the tubes in less than 25 minutes after the addition of charcoal. However, the time of contact was standardised for 8 minutes. This was independent of the number of tubes and the time was sufficient to charcoal 192 tubes using a pulsatile pump for dispensing the charcoal.

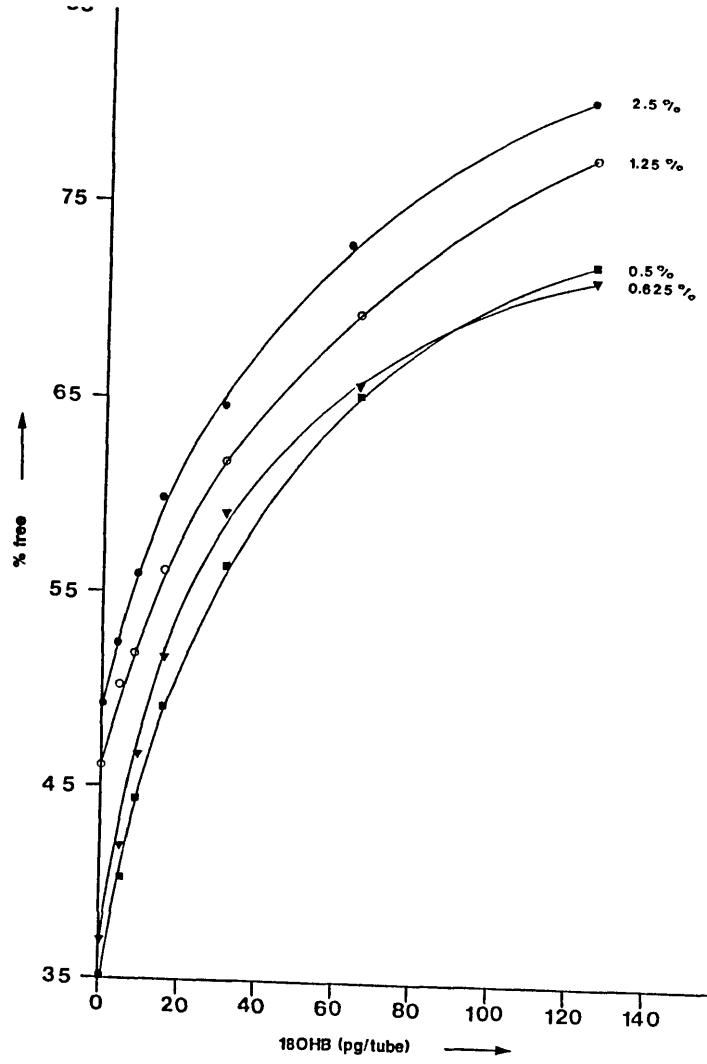


Figure 17

Effect of varying amount of dextran coated charcoal on the 18OHB radioimmunoassay and cross-reactivity with cortisol. The amount of dextran coated charcoal in 0.5 ml dispensed into each tube was 2.5 mg, 3.1 mg, 6.25 mg and 12.5 mg using a concentration of charcoal in dextran-gelatine solution of 0.625%, 0.5%, 1.25% and 2.5% respectively. Cross reactivity with cortisol at these various charcoal concentrations was not altered. 'Drift' was not detected.

(c) Assay buffers

Citrate phosphate buffers (pH2.2, 3.0, 4.0, 4.6 5.0, 6.0, 7.0 and 7.8) and phosphate buffer (pH7.4) were used for preparing the albumin and dextran coated charcoal. The 18OHB and cortisol standards were dissolved in distilled water for this experiment instead of phosphate buffer. The results are shown in **Figure 18.** It was found that the standard curves using citrate phosphate of 7.8 and phosphate pH7.4 were superimposable. In addition, the cross-reactivity with cortisol was not altered. Therefore, in all subsequent experiments, phosphate buffer (pH7.4) was used. Despite a 70% binding when the pH2.2 citrate-phosphate buffer was used, a standard curve was not obtainable due to lack of displacement of the label from the antibody with 18OHB standards.

(d) Assay buffer proteins

Bovine serum albumin (BSA) and bovine gamma globulin (BGG) at a dose of 0.1% and 0.5% were investigated. The standard curves were superimposable. Hence, a 0.1% BSA solution was used in all subsequent assays.

(e) Assay volume

Initially all assays were carried out using a total volume of 1.0 ml (0.5 ml standard and 0.5 ml binding reagent). It was tested against a total assay volume of 0.7 ml (0.5 ml standard and 0.2 ml

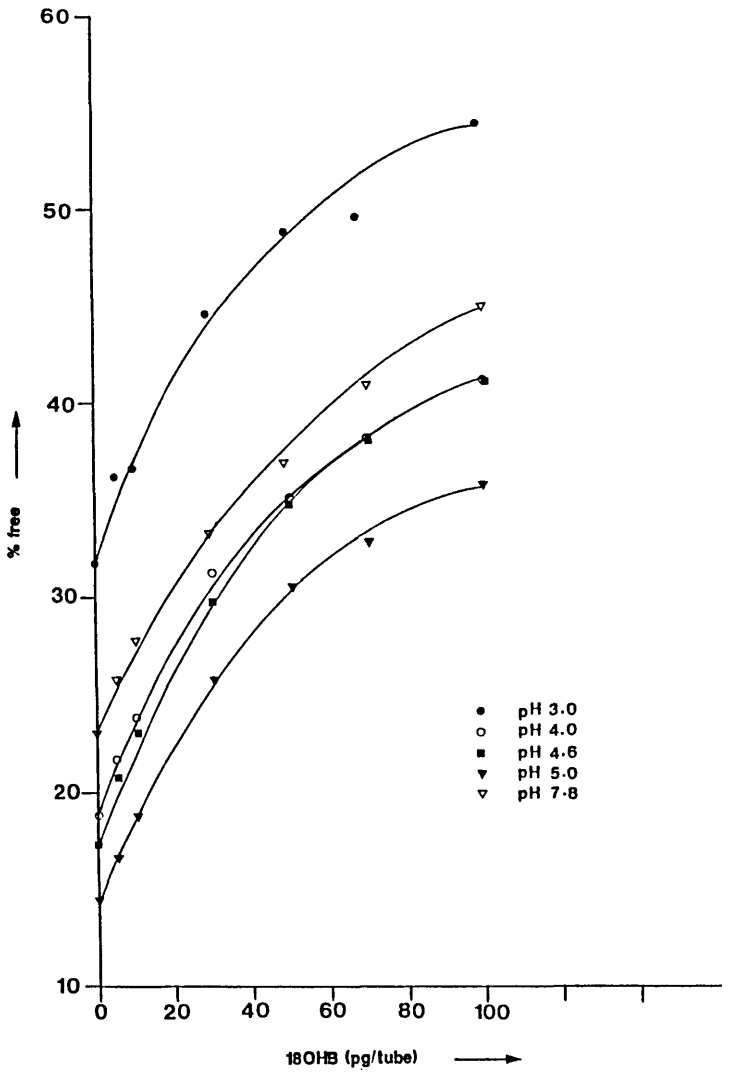


Figure 18

Comparison of the 18OHB standard curves using citrate-phosphate buffers of varying pH (2.2, 3.0, 4.0, 4.6, 5.0, 6.0, 7.0 and 7.8). Cross-reactivity with cortisol was not diminished at any of the pH studied. The antiserum (#5/12/6) was studied at an optimum final antibody dilution of 1:16,000 titrated at pH7.4. The standard curves at pH4.0, pH4.6, pH6.0 and pH7.0 were superimposable on each other. Using a buffer of pH2.2, the standard curve was flat but the slope of the standard curve was very low even though the zero binding was similar to that at pH 7.4.

binding reagent). It was found that there was no difference between the two assay volumes tested using QC samples. Therefore, the microassay was used for subsequent experiments.

(f) Stability of the standards

The stability of the 18OHB standards in phosphate buffer (pH7.4) was tested with temperature and time. A series of 18OHB standards kept at $4^{o}C$ were removed and left at room temperature (23^{o}) for one hour daily for one month. Another series were left at room temperature for varying periods of time (2, 4 and 6 months). Standards treated in these ways were compared with standards aliquoted into assay tubes and stored at $4^{o}C$ until use. Every month a new series of standards were prepared and compared with the old stock. Values of the QC samples and the standards from each treatment were compared using paired t-test. The conclusion was that the 18OHB standards were extremely stable even when being kept at room temperature for 6 months.

(g) Cross-reactivity with cortisol

It was found that varying the incubation time, pH of the assay or the amount of dextran-coated charchoal for separating the free and the bound fractions did not change the cross-reactivity of antiserum #5/12/6 to cortisol.

2.4.5. : Validation of a chromatographic method for measuring 18OHB in saliva and plasma

Initially, the saliva samples were concentrated by freeze-drying and the residue was taken up in methanol and chloroform mixture three times and centrifuged twice in between (Lee et al., 1985). The extract was then spotted onto the TLC plate sequentially. The method was found to be cumbersome and hence was abandoned. The present method utilises concentration of saliva by freeze-drying saliva before extraction with dichloromethane and then TLC. Similarly, plasma was extracted with DCM prior to TLC. Tritiated 18OHB was used to assess procedural loss in all the experiments.

General methods established :

(a) Saliva samples

Debris in saliva was removed by freezing them overnight, which was then thawed and centrifuged. Three ml of cleaned saliva was pipetted into the extraction tube containing tritiated 18OHB (2000 cpm in 10 µl ethanol containing 0.1 % triethylamine). The saliva was gently vortexed and equilibrated at 37^{o}C for 30 minutes. The tubes were then removed and left at room temperature for 15 to 20 minutes. The saliva samples were snap

frozen using a mixture of dry ice in methanol then freezed dried. Samples were reconstituted with one ml of phosphate buffer and extracted once with dichloromethane (10 ml) (**Figure 19**). The extraction was done by roller mixing the two phases for 30 minutes. The two phases were allowed to separate for 10 to 15 minutes. The aqueous phase (top layer) was removed leaving a trace of the aqueous phase behind. Anhydrous sulphate (1.5g) was added slowly and very gently to the extraction tube while rotating the tube so that the sodium sulphate made contact with the aqueous phase first. The tube was gently vortexed and left to stand for 1 minute. The dichloromethane (DCM) layer was decanted into a conical tube and a further volume of DCM (3 ml) was added to the tube containing the sodium sulphate. The DCM extracts were pooled and evaporated to dryness in a water bath ($43^{o}C$ to $46^{o}C$) with one crystalline anti-bumping granule added.

(b) Plasma sample

Plasma (1ml) was added to the extraction tube containing [^{3}H]18OHB (2000 cpm in 10 µl). It was proceeded as for the reconstituted saliva samples.

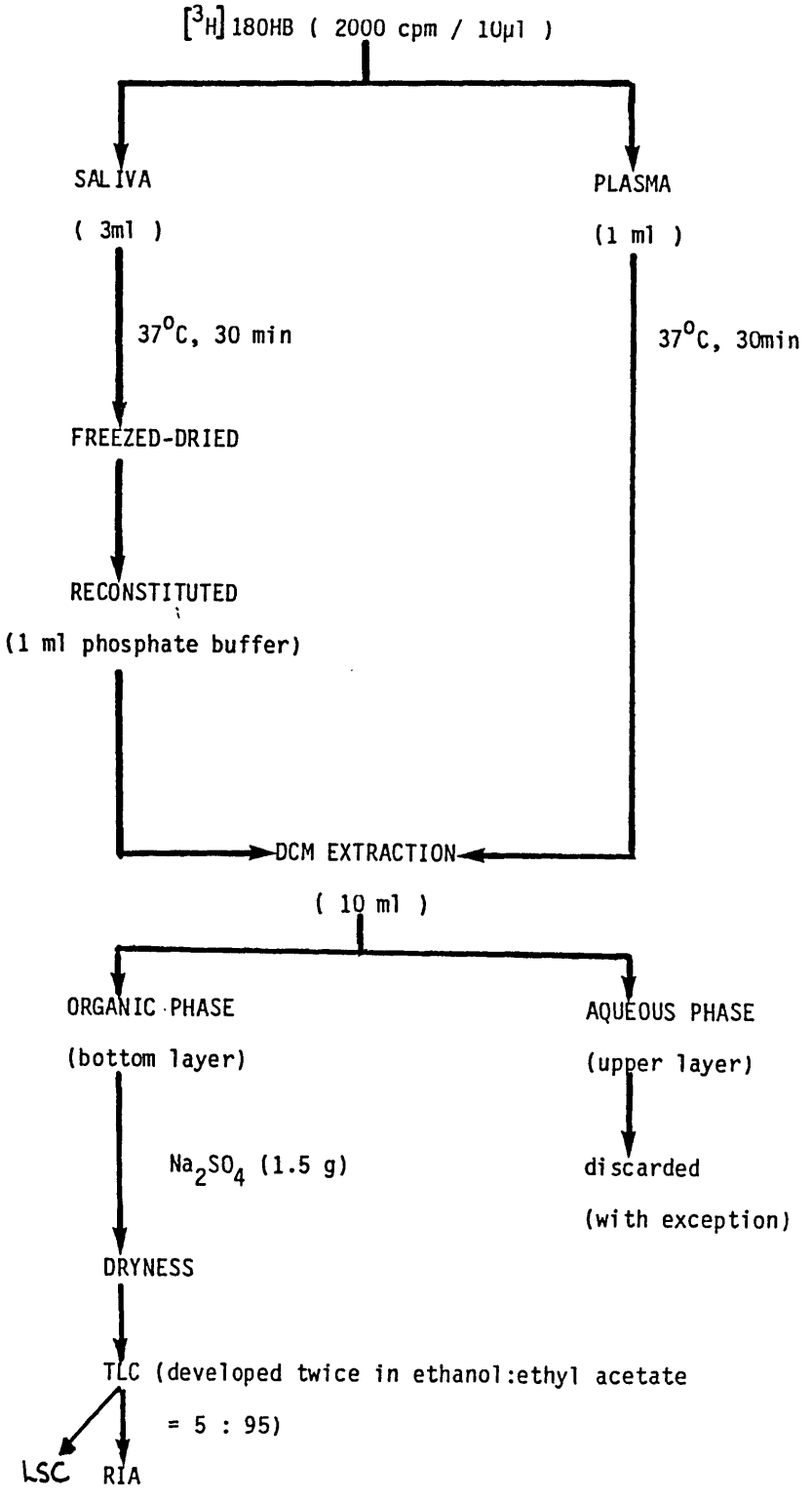


Figure 19

Established procedure for purifying saliva and plasma samples prior to radioimmunoassay (RIA). LSC, liquid scintillation counting of the tritium label for recovery assessment.

(c) Chromatography

The residues of the extracted biological samples were transferred onto a TLC plate using a mixture of methanol and chloroform (1:1) freshly prepared. The mixture (50 µl) was added into the residue by dispensing down the sides of the tube while the tube was rotated in a slanting position. The tube was stood upright for 5 minutes to allow the solvent to reach the bottom of the tube . The material was then transferred with a pasteur pipette onto a scored TLC plate 1.5 cm below the origin. A further 100 µl of the solvent was then added in the same fashion as the first. The mixture was gently mixed with for a few seconds. This was then followed by a further 200 µl of the mixture.

A reference marker (cortisone), which did not cross-react with the antiserum, was used. It could be spotted on two empty lanes at the edges of the TLC plate or lanes containing the samples. The samples were run up with methanol to the origin and left to dry. Then it was developed in a tank equilibrated overnight with solvents (ethanol:ethyl acetate = 5:95 v/v) twice. In between the two developments, the TLC plate was left to dry. The apparent 18OHB position inferred from the relative mobility of 18OHB to cortisone was 0.47. If time did not allow the elution to be done on the same day, the TLC plate was stored at -20^{o}C. The assay could be accomplished in a working day for 10 samples ran on one TLC plate.

The TLC strips were eluted with phosphate buffer (pH7.4, 2.5

ml) by roller mixing for 45 minutes or left to elute overnight at $4^{o}C$. The eluate was decanted into a LP3 polystyrene tube and centrifuged for 15 minutes. The supernatant was removed into another LP3 tube leaving 0.8 ml behind in the first LP3 tube. This was to minimise agitation of the gel pellet. A pasteur pipette was used and was not allowed to touch the sides of the LP3 tube. The clean eluate (2 x 0.5 ml) in the second LP3 tube was used for radioimmunoassay whereas the eluate in the first LP3 tube was used for liquid scintillation counting.

Samples expected to have high or low values were adjusted by freeze-drying the appropiate volume of saliva and then reconstituting in one ml of phosphate buffer before DCM extraction. This was to keep the volume of the gel eluate used for radioimmunoassay constant. It was not strictly necessary because the standard curves using standards diluted with TLC eluates and phosphate buffer were superimposable.

The rest of the eluate corresponding to the 18OHB position was pooled and freezed dried. The residue was reconstituted with methanol:chloroform mixture before being rechromatographed in another TLC solvent system (methanol:chloroform = 10:90). This was to verify the specificity of the 18OHB measured in the first TLC system.

(d) Glassware cleaning for the extraction :

The used glassware was washed with warm tap water and rinsed once with distilled water. Then they were soaked overnight in Decon washing detergent (3 %) and sonicated. They were rinsed with tap water followed by distilled water and then soaked in acetic acid (5%) containing EDTA (0.08%) overnight . They were again washed with warm tap water (10x) and distilled water (3x). The tubes were vortexed with redistilled methanol once before being dried in the oven ($58^{o}C$). However, if the sample extract were to be measured by RIA after solvent extraction, the tubes were not rinsed with methanol.

Extraction with dichloromethane (DCM)

(a) Purity of tritiated 18OHB

Commercially available tritiated 18OHB has a stated radiochemical purity ranging from 96.9% to 97.8% and its specific activity from 52 Ci/mmol to 60 Ci/mmol. The label was stable with time. For example, according to the manufacturer, a batch of label (H/6024/2) has a purity of 97.8% when analysed on 31/10/83 was found to have a purity of 96.9% when reanalysed on 3/5/84.

The purity of the label (H/6024/2) was tested by comparing the immunoreactivity of purified and unpurified tritiated 18OHB at varying mass. The purification was done by chromatographing the

label in a solvent system of 20% ethanol in dichloromethane. The radioactivity was located by ß-graphing and then the TLC plate was cut into 1 cm strips, eluted with phosphate buffer for liquid scintillation counting and radioimmunoassay.

Results

The radioactivity on the TLC plate mapped by ß-graphing ran in parallel with ultra-violet absorbing authentic 18OHB spot in both cases where the tritiated 18OHB was spotted onto the plate with and without authentic 18OHB as carrier. The results of the liquid scintillation counting is shown in **Figure 20a & 20b**. Approximately 94% of the total radioactivity has a chromatographic mobility of the authentic 18OHB. The rest of the radioactivity was spread all over the TLC plate and could be detected only by liquid scintillation counting.

The mass of the eluate of the main tritiated 18OHB spot (between 60 to 80 mm) was measured by radioimmunoassay. **Figure 21** shows that the purified and unpurified material have similar immunoreactive properties when [^{3}H]18OHB was assayed in the routine iodine ligand assay. The sensitivity of the iodine-ligand assay is 1 pg/tube (5 pmol/l) which is equivalent to 330 cpm [^{3}H]18OHB. It is therefore possible to calculate the maximum quantity of [^{3}H]18OHB which can be used as an internal standard. When 2,000 cpm

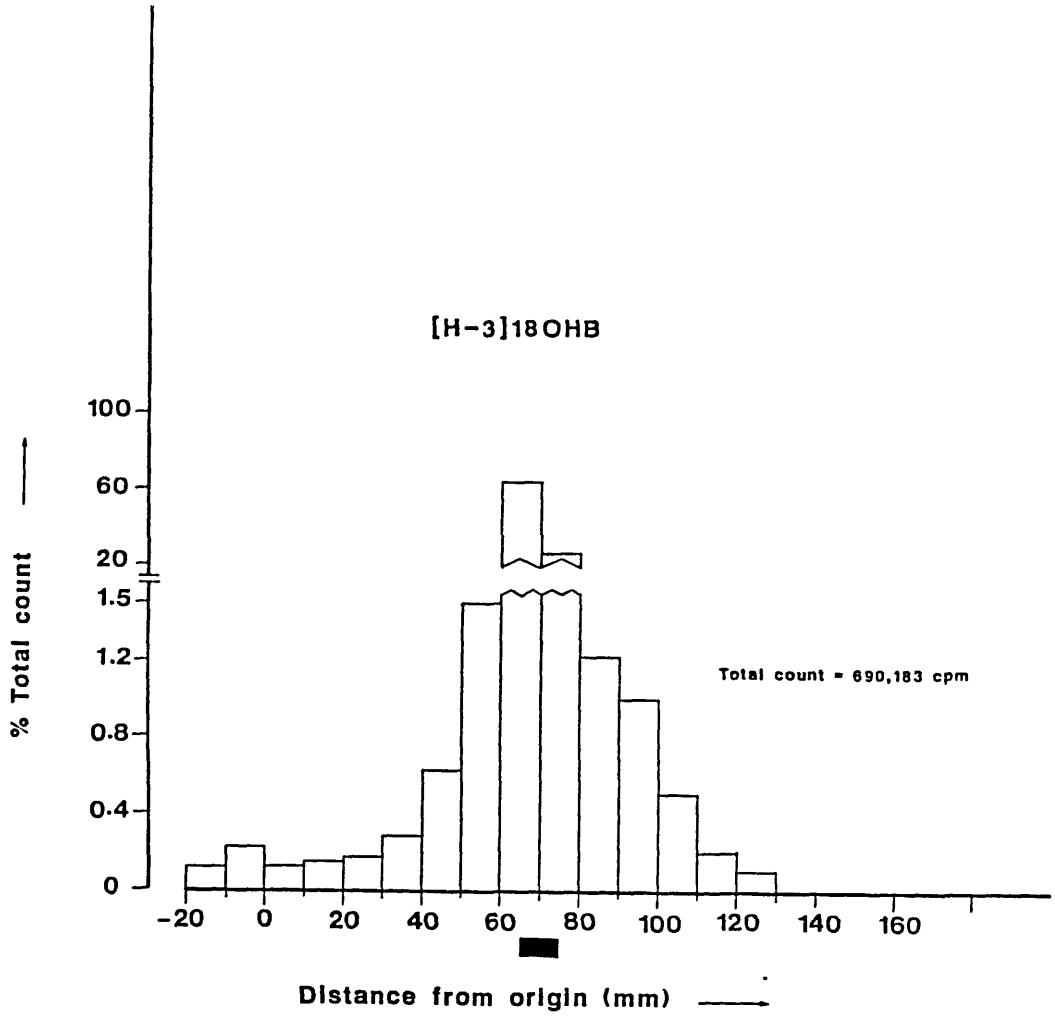


Figure 20a

Radiochromatogram of [1,2^{3}H]18OHB which was being chromatographed without authentic 18OHB as carrier. The TLC solvent used was 20% ethanol in dichloromethane and authentic 18OHB (■) has a relative mobility to the solvent front of 0.41.

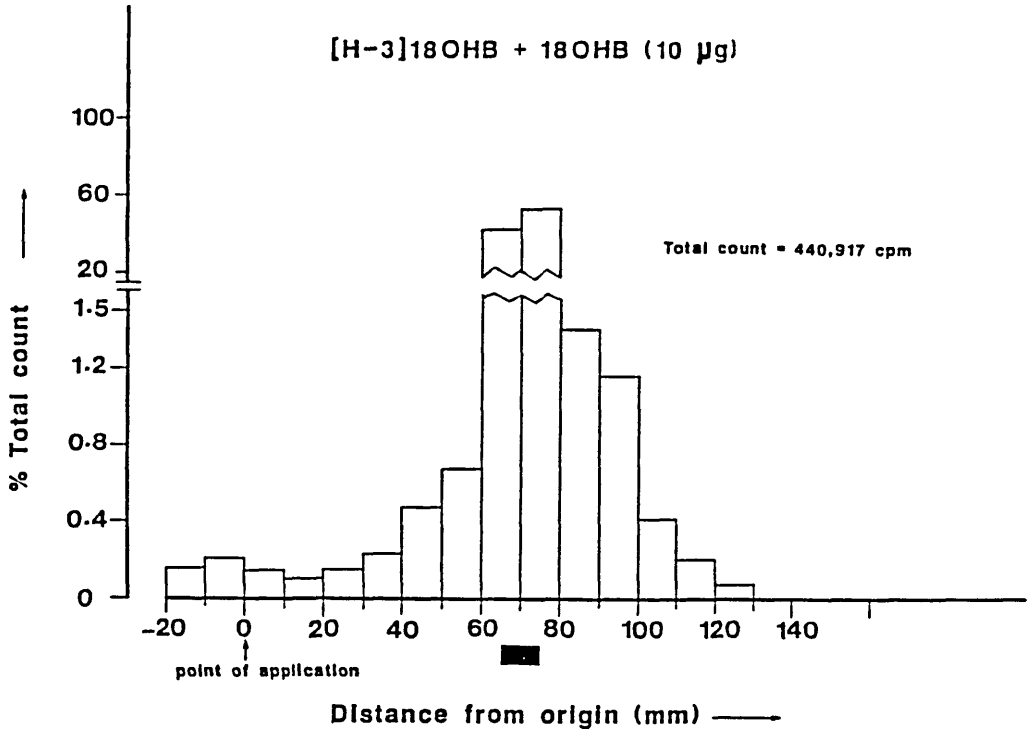


Figure 20b

Radiochromatogram of [1,2^{3}H]18OHB being chromatographed with authentic 18OHB (■) as carrier using the 20% ethanol in dichloromethane solvent system.

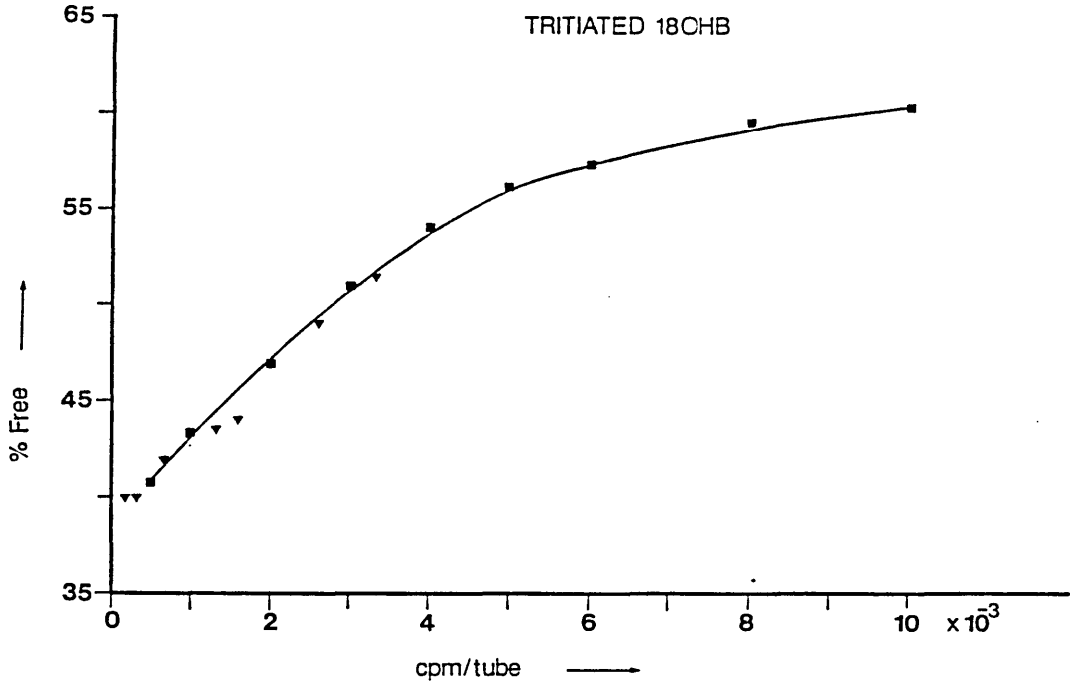


Figure 21

Immunoreactivity of purified (■) and unpurified (▼) [1,2^{3}H]18OHB.

is added to a sample and 20% of the TLC eluate is taken for radioimmunoassay, 50% recovery gives 200 cpm/assay tube which will therefore contribute negligible mass to the assay. The tritiated 18OHB when stored at -20^{o}C was usuable even after 6 months without any purification.

(b) Extractability of tritiated 18OHB with DCM

This was to determine if extractability of tritiated 18OHB is dependent on the weight of the tritated 18OHB used. This was done by equilibrating varying amounts of tritiated 18OHB (equivalent to 6 pg to 99 pg/extraction tube) in pH7.4 phosphate buffer (1.0 ml) for 30 minutes at 37^{o}C prior to extraction with DCM in a ratio of aqueous:DCM layer of 1:10. The percentage recovery ranged from 78.9% to 83% (**Table 5**). The extraction of tritiated 18OHB was found to be independent of the weight of tritiated 18OHB.

This experiment was repeated using saliva and plasma samples. Similar results were obtained when saliva or plasma was used instead of buffer.

II Thin layer chromatography

(a) Choice of solvent system

The purpose is to separate 18OHB from other known steroids which would potentially cross-react with the antiserum such as cortisol and corticosterone. From a few single and binary systems studied, it

Amount of radioactivity used for DCM extraction		Wt. of label (pg)	% recovery (mean of duplicates)
µCi	cpm		
0.0165	9788	99.5	82.8
0.0082	4775	49.8	83.1
0.0041	2382	24.9	78.9
0.0021	1211	12.4	82.5
0.0010	621	6.0	83.2

Table 5

Varying weight of tritiated 18OHB in pH7.4 phosphate buffer (1.0 ml) was extracted with dichloromethane (DCM, 10 ml). The % recovery of tritium was measured. The specific activity of [1,2^{3}H] quoted by the manufacturer was 60 curies/mmole. The weight of tritiated 18OHB given above was derived by calculation using the specific activity quoted by the manufacturer. The results showed that the extractability of DCM was not dependent on the weight of the radioactivity tested (ca. 6 pg to 99 pg).

was found that 18OHB was more polar than cortisol, corticosterone and aldosterone. The separation of 18OHB from cortisol was studied by using different solvent systems (**Table 6a to 6c**). Although the cross-reactivity with aldosterone using antiserum #5/12/6 was negligible, a solvent system was studied to separate them. It was found that methanol:chloroform (10:88) separated 18OHB from aldosterone but did not adequately separate from cortisol. On the other hand ethanol:ethyl acetate (5:95) separated 18OHB from cortisol but not from aldosterone. This latter separation was improved by developing the TLC plates twice in the same solvent system and this moved 18OHB well away from the origin. As shown in **Table 2** this system also separates 18OHB from other known steroids and was adopted for routine purposes while the methanol:chloroform system was utilised for further investigation of the specificity of the routine method.

(b) Linearity studies

Varying volumes saliva using three saliva pools were extracted with DCM and chromatographed. The results are shown in **Figure 22** and linearity over the volume of saliva assayed was shown.

In another experiment, double dilution of a pool of TLC eluate containing up to 1000 pmol/l of 18OHB was compared with the 18OHB standard curve. Parallelism between the curves was obtained. This implies that the TLC system separates 18OHB from other substances

Table 6a TLC 1, 10 % ethanol in ethyl acetate;
TLC 2, 15% t-butanol in t-butyl methyl ether
(spots had 'cometic' appearance);
*, detected by blue tetrazolium test.

	Ambient temperature = 22oC	
STEROIDS	Distance from origin (mm)	
	TLC1	TLC2
18OHB	42.5	38
Cortisol	74	72
Aldosterone		40
THE *		62
Cpd B		64
Cortisone		72
Prednisolone		72

Table 6b TLC at varying percentage of ethanol in ethyl acetate at 22°C:

	% of ethanol in ethyl acetate			
STEROIDS	5		7	
	Run 1	Run 2	RUN1	
	Distance from origin (mm)			
18OHB	25	46.5	34.5	
Aldo	35	60	44.8	
Cortisol	62	105	72	
Cortisone	65	110	76	

Table 6c TLC at varying percentage of ethanol in ethyl acetate at 25°C:

	% of ethanol in ethyl acetate			
STEROIDS	5		10	15
	RUN 1	RUN 2	RUN1	RUN1
	Distance from origin (mm)			
18OHB	25.5	46.5	27.0	55.0
Aldo	34.0	59.5	37.0	62.0
Cortisol	57.0	94.0		87.2
Cortisone	61.5	99.0	70.0	95.5

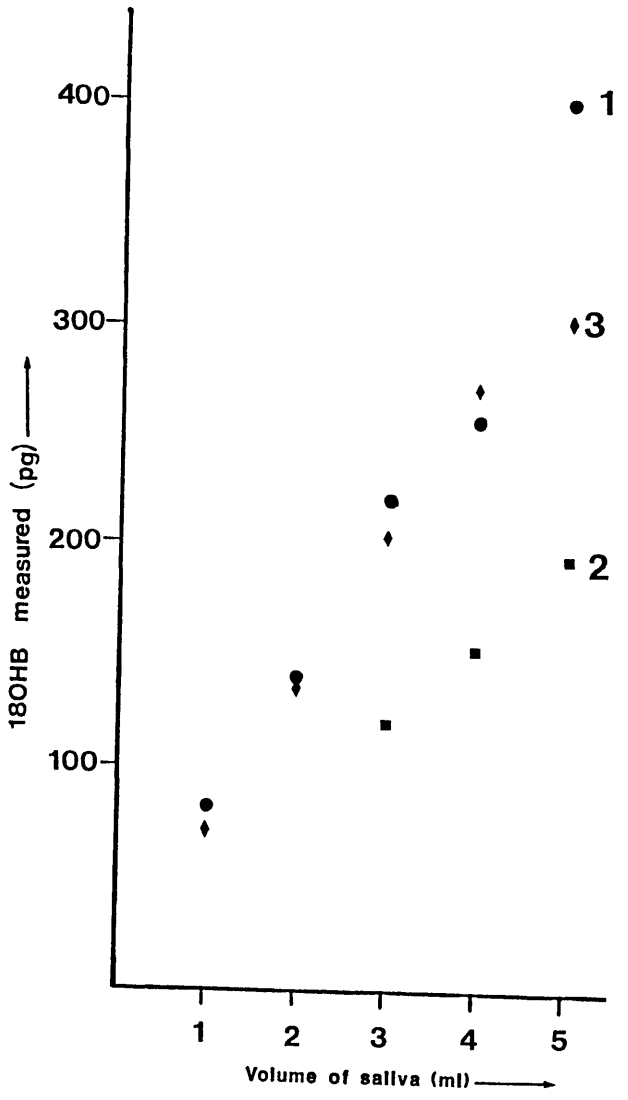


Figure 22

Linearity studies by measuring the amount of 180HB recovered from varying volumes of saliva pool. Three saliva pools (1,2 and 3) were evaluated and the lines of linear regression are shown below:

Pool	Line of linear regression (r)
1	Y = 82.8x -14.8 (0.99)
2	Y = 36.4 + 10.6 (0.99)
3	Y = 73.5x + 38.9 (0.97)

Y= Amount of 180HB measured

x= Volume of saliva processed

r= correlation coefficient

present in biological fluid adequately and is evidence of specificity.

(c) Specificity

The TLC eluates of 18OHB from the 5 % ethanol in ethyl acetate separation were pooled and freeze -dried. The residues were spotted on a TLC plate and developed in a solvent system of 10% methanol in chloroform. Radioimmunochromatographic technique was used and it was found that the radioactivity and immunoreactivity ran in parallel with authentic 18OHB. Therefore, it shows that the routine TLC system is reliable.

(d) Accuracy

Two saliva pools were spiked with three doses of 18OHB (100, 200 and 500 pmol/l). The saliva pools were made up of saliva from healthy subjects and patients respectively. The amount of 18OHB recovered after chromatography and the amount added was shown in **Table 7a**.

A plasma pool was similarly spiked with three doses of 18OHB (200, 300 and 800 pmol/l) and assayed for 18OHB after chromatography. The amount of 18OHB in the plasma was also quantitatively recovered as for the spiked saliva samples and the results are shown in **Table 7b**. Therefore, the assay developed for both the saliva and plasma samples are reliable over the range of

18OHB concentration investigated.

Table 7a Recoveries of authentic 18OHB from saliva:

Amount of 18OHB added (pmol/l)	0	100	200	500
	Amount of 18OHB measured (pmol/l)			
Saliva pool (healthy subjects)	30	120	220	600
Saliva pool (patients)	50	180	250	400
	% recovery			
Saliva pool (healthy subjects)		90	95	114
Saliva pool (patients)		130	100	70

Table 7b Recoveries of authentic 18OHB from plasma:

Amount of18OHB added	0	200	300	800
Plasma pool (healthy subjects)	300	550	690	1000
	% recovery			
		125	130	87.5

In routine assay, authentic 18OHB (543 pg) was included in the assay to check the recovery. It was found that the mean recovery was 96.5 $\pm$ 2.5 (SD) % (n=100). This again shows that the method developed is reliable.

(e) Sensitivity

The sensitivity of the method is defined as the lowest detectable dose measurable by RIA. This may be determined from the standard deviation of zero and 10pmol.l^{-1} standards, water blanks and saliva samples containing very low concentration of 18OHB. In Table 8 I have summarised data on the percentage free ligand for zero and 10pmol.l^{-1} (1.8 pg. $tube^{-1}$) standards, water blanks and samples of a pool of saliva low in 18OHB, obtained from 10 consecutive assay sets. If we consider the zero standard then the minimum value distinguishable from this with 95% confidence will be the mean + 2SD which is approximately 36.2%. If we assume a linear relationship between mass of 18OHB and % free ligand, 36.2% is equivalent to 1.5 pg.$tube^{-1}$. The mean % free ligand for the water blanks is essentially the same as the zero standard but the SD is rather higher (34.8 $\pm$ 1.28%). The sensitivity calculated from these data is 2.2 pg.$tube^{-1}$. I must emphasise that these calculations are approximate as they are based on the assumption of a linear relationship between % free ligand and mass of 18OHB. It is very difficult to determine the precise nature of the relationship over small increments of concentration, especially at this bottom end of the calibration curve. Neither of the computer systems to which I have had access make satisfactory predictions of mass from values for % free ligand close to that of the zero standard. Nevertheless, even if there was a substantial

error on this account, my data would still show that the assay was of adequate sensitivity for the purposes for which I have used it.

Table 8 Evaluation of sensitivity based on zero and 10 pmol/l standards, and a saliva pool low in 18OHB concentration:

	% free ligand		
	mean	SD	CV
zero	34.5	0.86	2.5
10 $pmol.l^{-1}$	36.6	1.6	4.3
Water blank	34.8	1.28	3.7
Low pool	36.8	0.65	1.8

In practice, an appropriate volume of saliva was used so that the amount of 18OHB measured in 0.5 ml of TLC gel eluate was brought into the optimal range (30 $pmol.l^{-1}$ to 500 $pmol.l^{-1}$) of the standard curve. This was made possible by measuring the salivary aldosterone concentration before 18OHB. By adjusting the volume of saliva to be used, the precision of the assay was maximised.

(f) Quality control (QC)

SALIVA

The inter-batch precision using QC samples was 12.6 % at a dose of 45.5 ± 5.6 (30) pmol/l (mean ± SD (n). The intra-batch precision was 8.9 % using the same QC samples.

PLASMA

The inter-batch precision for plasma samples had a cv of 12.0% using QC samples with a mean ± SD (n) value of 400 ± 48 (n=30) pmol/l. No intra-batch precision was done.

III Direct assay versus TLC assay

Saliva samples were assayed directly and after thin-layer chromatography (TLC). The results are shown in **Figure 23**. The 94 saliva samples were from normal women and men, pregnant women and from patients with primary and secondary hyperaldosteronism. The values for the direct assay (X) ranged from 62 - 4420 pmol/l (900 ± 969 pmol/l) and for the TLC assay (Y) 39 - 2892 (440 ± 518 pmol/l). The two values were related by the expression Y=0.463X + 1.3 and were highly correlated (r=0.92).

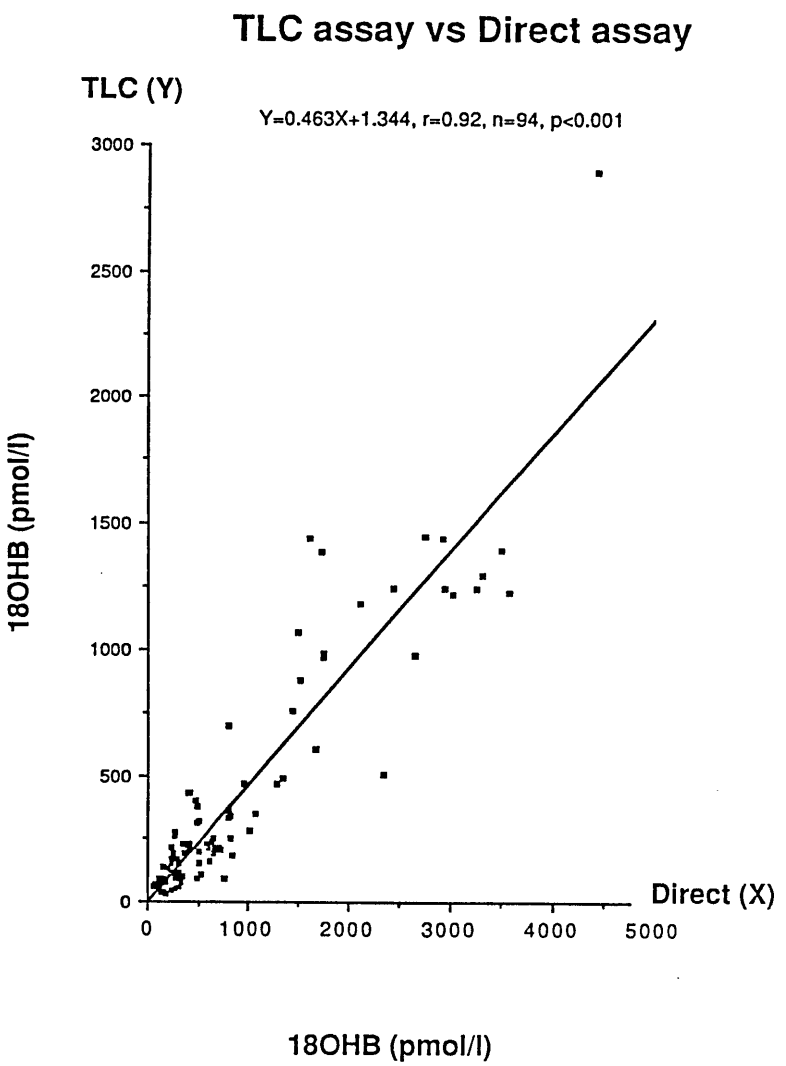


Figure 23

Comparison of a TLC method with a direct assay for measuring 18OHB in saliva. The saliva samples were from women and men, pregnant women and patients with primary and secondary hyperaldosteronism. and with renal diseases. The results were compared by paired t-test and found to be significantly different (t= 7.9; n= 94 $p < 0.001$).

2.4.6. Stability of 18OHB in saliva and plasma

(a) Saliva

A saliva pool (50ml) was collected over 45 minutes from one subject. An aliquot of the saliva was frozen immediately and various aliquots were left at ambient temperature ($23^{o}C$) for various durations of time (4h, 8h, 12h and 24h) before being frozen. The samples were thawed, centrifuged and assayed for 18OHB using the routine chromatographic method. The results of the assay were 150, 180, 140, 160 and 170 pmol/l for samples left at ambient temperature for 30 minutes, 4h, 8h, 12h and 24h respectively.

(b) Plasma

20 ml of blood was collected into lithium heparinised tubes. An aliquot was centrifuged immediately at $4^{o}C$, 1500g for 15 minutes and various aliquots of whole blood were left at ambient temperature ($23^{o}C$) for various periods of time (4h, 8h, 12h and 24h) prior to centrifugation. Plasma obtained were stored immediately at $-20^{o}C$ until use. The results were 480, 500, 450, 490 and 480 pmol/l for samples centrifuged at various time intervals (0, 4h, 8h, 12h and 24 h) respectively. The results implies that blood samples can be left up to 24h at ambient temperature prior to centrifugation and storage at $-20^{o}C$.

These two experiments show that saliva and blood samples can

be left at ambient temperature ($23^{o}C$) up to 24h prior processing before storage at $-20^{o}C$. This is important especially when the saliva samples are collected on an out-patient basis and the blood samples collected in the hospital wards cannot be centrifuged immediately.

(c) Freezing and thawing

The surplus saliva and plasma samples from the above experiments were pooled and thawed once, twice, thrice and four times prior to assay. The results are shown in Table 9. Hence, comparing these results and those in section (b) above, it was shown that freezing and thawing had no deleterious effect on immunoassayable 18OHB. This is important especially when the samples have to be repeated for some reasons.

Table 9 Stability of 18OHB in saliva and plasma after freezing thawing for various number of times:

No. of times of thawing	0	1	2	3	4	5
			pmol/l			
SALIVA		154	180	156	161	140
PLASMA	480	450	400	488	485	

Chapter 3

Applications of 18OHB Measurement

3.1. Demonstration of the presence of 18OHB in saliva

Introduction

Katz and Shannon (1969) found radiolabelled aldosterone, oestradiol and androstenedione in human saliva following the intravenous (i.v.) infusion of these labelled hormones. It was decided to use this technique to determine whether 18OHB could pass from plasma to saliva prior to developing a method for the measurement of 18OHB in saliva. Having taken the decision to administer tritiated 18OHB i.v., the opportunity was taken to make some observations on the kinetics of the disappearance of 18OHB from plasma as well as the rate of appearance in the saliva. Initially a bolus injection of tritiated 18OHB was employed but the plasma level of tritiated 18OHB declined so rapidly that insufficient saliva radioactivity was collected for characterisation of the chemical nature of the tritium labelled material. Consequently, a constant continuous infusion protocol was used in an attempt to solve this difficulty.

In man the liver is a major site for the catabolism of steroid hormones and it was assumed that this would also be true for 18OHB. It was therefore decided to include measurement of hepatic blood flow in the protocol by including indocyanine green

(ICG) in the bolus injection experiments and measuring the concentration of the dye in plasma samples by spectrophotometry.

The kinetics of steroids especially with respect to aldosterone and the mathematical treatment of the results have been comprehensively described (Tait et al., 1961; Tait, 1963; Baird et al., 1969). One of the most important assumptions made to enable the calculation to be done is that a steady state exists in an open, two compartmental system. In my study, the quantitative treatment of the data was restricted to the calculation of the double exponential equation which described the disappearance curve of the dichloromethane (DCM) extractable radioactivity. The main reason for not calculating the sizes of the compartment and the rates of flow of the tracer into and out of the compartment is that a steady state condition had not been established for 18OHB. This could be done by measuring the specific activity of the tracer during the course of its disappearance. At the time of the study, the method for measuring 18OHB was not available. Therefore, only qualitative description of the transport of 18OHB was presented.

Materials

(i) For intravenous injection and infusion:

Indocyanine green (ICG) used was purchased from Hynson, Westcott and Dunning, U.S.A. and [1,2-^{3}H]18-hydroxycorticosterone was from

Amersham International.

(ii) For liquid scintillation counting:

For non aqueous samples 0.5% PPO in toluene was used. For aqueous samples one third volume of Trition X-100 was added.

(iii) Paper chromatography

T_{50}, Toluene: Methanol: Water: = 2:1:1, V:V:V; E_2B, Iso-octane: t-butanol : water = 100:50:90, V:V:V.

(iv) Glucuronidase hydrolysis

ß-glucuronidase was purchased from General Diagnostics.

Methods

(i) Single intravenous injection of tritiated 18OHB and indocyanine green (ICG):

Two healthy male subjects (Subject JF and subject CN) weighed ca. 70 kg were given a single bolus injection of tritiated 18OHB (20 µCi with a specific activity of 52 Ci/mmol; 97.8% radiochemical purity) and ICG (25mg) in 10 ml of sterile saline injected in less than 20 seconds through an indwelling cannula. Blood was collected from the other arm via another indwelling cannula. Whole mixed saliva was collected. Initially, saliva and blood were collected every minute then 2 minutes before prolonging to 5,10,15 and 20 minutes intervals. Urine was collected at various times during and post injection.

(ii) Continuous infusion of tritiated 18OHB:

A healthy male subject (Subject MH) weighed 66.8 Kg and 21 years of age was infused with 18.45 µCi of tritiated 18OHB at a constant rate of 0.615 µCi in 1.16 ml/minute for 30 minutes. Saliva and blood were collected at 5 and 10 minutes intervals. Urine was collected at various times of the day for 24 hours.

Collection of samples

(i) Blood:

Blood (10 ml) was collected into lithium-heparin tubes and spun immediately at 1500g and $4^{o}C$ for 15 minutes.

(ii) Saliva:

Whole mixed saliva was collected into 7 ml sterile Bijou bottles and frozen overnight. The saliva was thawed and centrifuged for 15 minutes at 1500g and $4^{o}C$ to remove any debris.

Assay protocol

I Plasma:

Study 1: Single i.v. injection of tritiated 18OHB

ICG and radioactivity (DCM extractable and unextractable fractions)

ICG was estimated spectrophotometrically at 805nm using ICG standards made up in autologous plasma. This is a non-destructive

assay and the samples were then extracted using a protocol shown in **Figure 24** but omitting the addition of tritiated 18OHB. The procedure illustrated in Figure 28 has been validated using a pool of plasma spiked with tritiated 18OHB. This pool of plasma was subsequently used as quality control samples. The radioactivity in the DCM extractable and unextractable fractions were then measured and corrected for counting efficiency, which was done by spiking with a radioactive isotope with known counting efficiency.

Study 2:Continous infusion of tritiated 18OHB

The radioactivity in plasma was extracted with DCM. Both the DCM-extractable and DCM-unextractable radioactivity were measured. Metabolic clearance rate was calculated from the rate of infusion and plasma radioactivity at 'steady state' (see section on calculation presented below).

The DCM extractable and DCM-nonextractable radioactive isotope in the plasma was characterised by paper chromatography without and with ß-glucuronidase hydrolysis respectively (see III below, pp132).

Calculations :

Study 1:

(a) Fractional disappearance of ICG

When the concentration of ICG against time was plotted on a

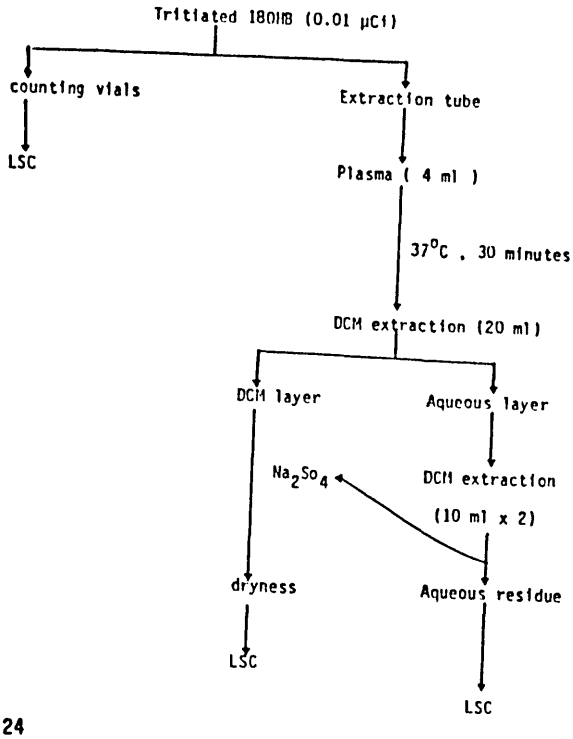


Figure 24

Protocol used for validating the extraction procedure for plasma. (LSC denotes measurement of β-emission by liquid scintillation counting). The recoveries of tritiated 18OHB added into the QC plasma samples during the extraction of samples obtained from the two single intravenous injection studies and the continous infusion study were as follows:-

Processing samples of subject:	% recovery of tritiated 18OHB mean + SD (CV%) (n=6)
JF	97.8 ± 6.1 % (6.3%)
CN	96.5 ± 5.0 % (5.2%)
MH	95.8 ± 6.5 % (6.8%)

semi-logarithm scale (i.e. log [ICG]) a linear relationship was observed. The slope (^{ICG}k) and intercept of the linear regression line were computed. The intercept is the notional zero time concentration of ICG and when divided into the dose of ICG administered gives an estimate of the volume of distribution of the dye in the plasma (VD). $^{ICG}t_{1/2}$ is determined by dividing ln 2 by ^{ICG}k. The estimated hepatic plasma flow (EHPF) was calculated from the formula : EHPF = (VD)(^{ICG}k)/E where E is the hepatic plasma extraction rate, which has been assumed to be 0.70 (Cherrick et al., 1960; Caesar et al., 1961). The calculated VD obtained was then compared to the expected plasma volume determined from the nomogram for obtaining plasma volume corrected for age and body mass. The clearance of ICG was determined by using the formula : (^{ICG}k)(VD) / body mass.

(b) Resolution of multi-exponential decay by the 'stripping method' in the single rapid intravenous injection study

The raw data in cpm/ml were converted into % dose in the total plasma volume (calculated from ICG data). A semi-logarithmic plot of % dose tritiated 18OHB in plasma against time (**Figure 25 & 26**) suggested that the clearance of 18OHB was multi-exponential. However, from about 30 minutes post injection a linear relationship between log [^{3}H]18OHB and time was seen. An attempt was made to resolve the data using a 'stripping' technique as

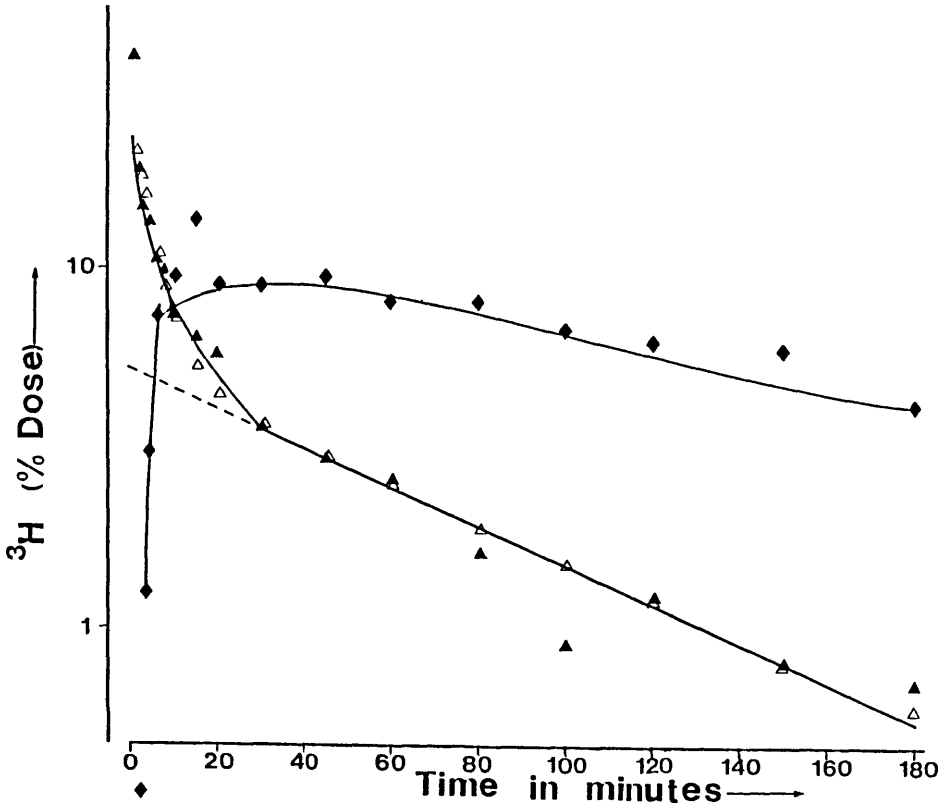
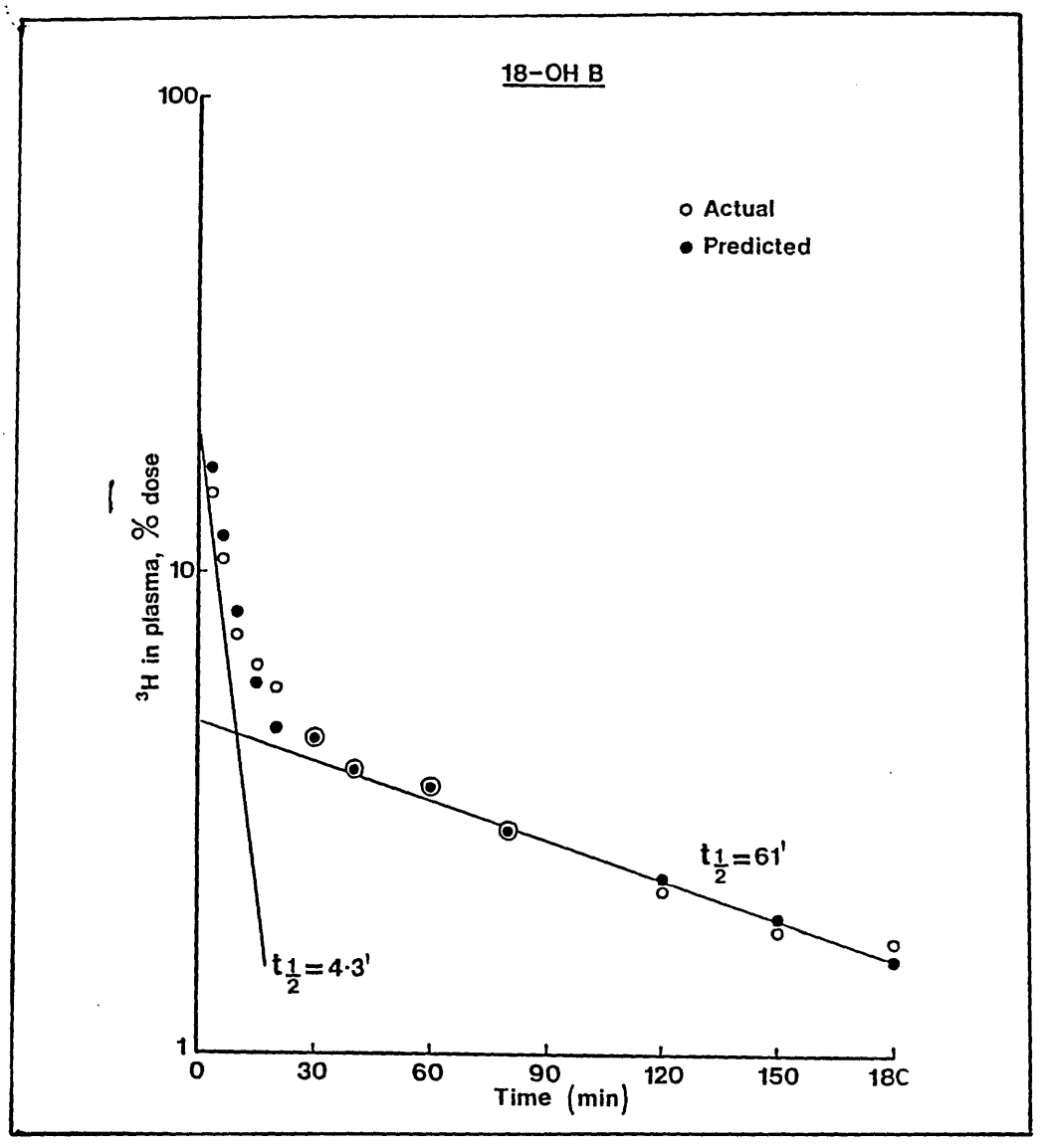


Figure 25

Kinetics of tritiated 18OHB (▲) and its conjugate (♦) in the plasma after a single rapid intravenous injection of the tritiated steroid in subject JF. The disappearance of the tritiated steroid is described by a bi-exponential equation :

% dose = $13.4e^{-0.119t} + 5.23e^{-0.0124t}$ where the half lives are 5.8 and 55.9 minutes. This equation can be used to predict values of y(% dose) for the sampling times. These predicted values (△) are highly correlated with the observed values (r=0.995) showing that the equation accurately describes the data.



Inset of Figure 25 to illustrate the rapid decline of the injected radioactivity on first sampling after 1 minute and the rapid appearance of the conjugated steroid.

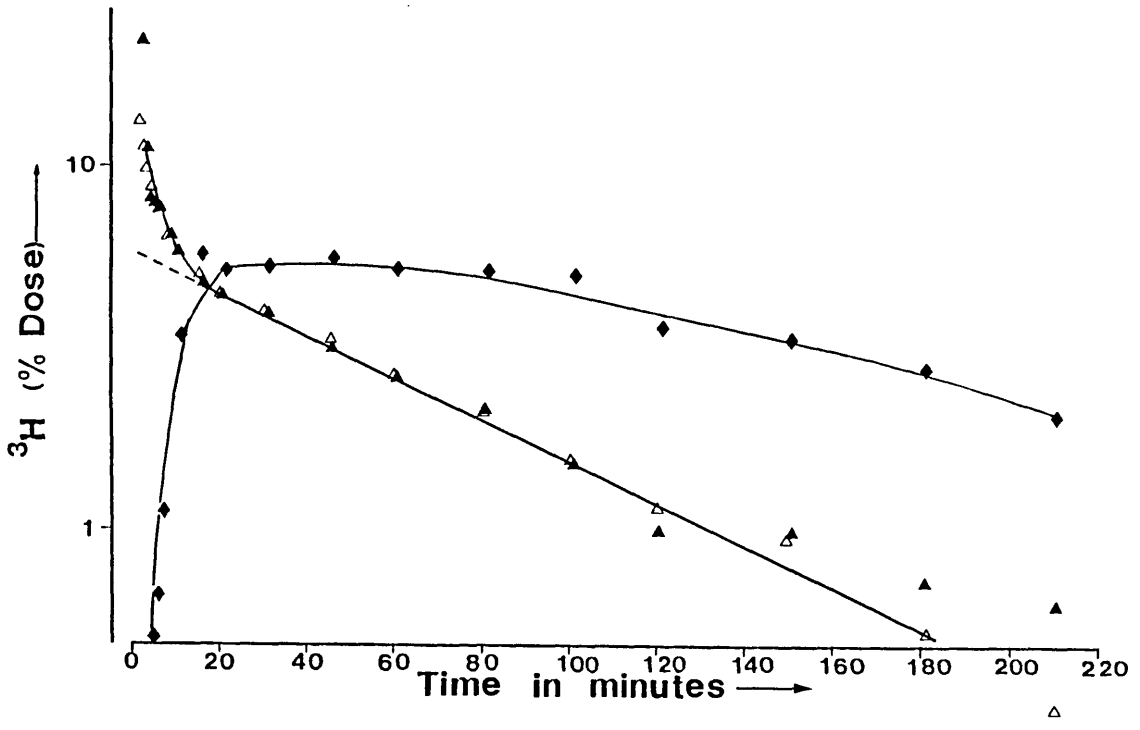
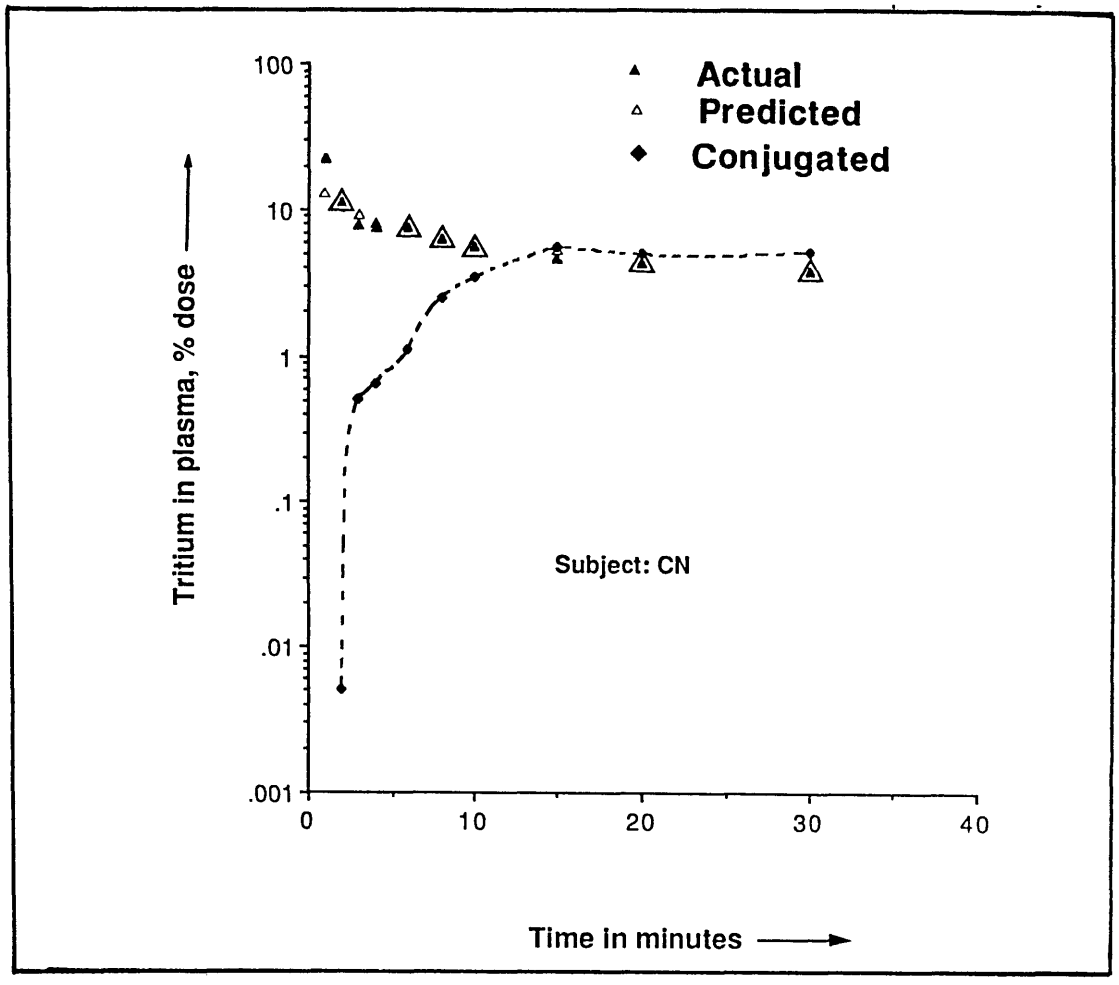


Figure 26

Kinetics of tritiated 18OHB (▲) and its conjugate (♦) in the plasma after a single rapid intravenous injection of the tritiated steroid in subject CN. The disappearance of the tritiated steroid is described by a bi-exponential equation :

% dose $= 7.02e^{-0.25t} + 5.7e^{-0.013t}$ where the half lives derived are 2.8 and 51.7 minutes and the predicted data (△) from this equation correlated with the observed data with a correlation coefficient of 0.991.



Inset of Figure 26 to illustrate the rapid decline of the injected radioactivity on first sampling after 1 minute and the discrepancy between the actual and predicted value from the double exponential equation at this time.

follows:-

A linear regression equation was computed for log [^{3}H]18OHB concentration and time over the period 30 - 180 minutes. From this equation concentrations were calculated for the earlier sampling times. These calculated values were subtracted from the observed values and the differences were found to be highly correlated (exponentially) with time. Thus the decline in plasma tritiated 18OHB concentration (Y) could be described by a biexponential equation of the form $Y = Ae^{-\alpha t} + Be^{-\beta t}$ where α and β are the slopes of the two regression lines. Half life ($t_{1/2}$) for each component was calculated from ln 2/slope. When values of Y corresponding to actual sampling time were predicted from this biexponential equation the predicted and observed values were very highly correlated over the period 2 - 180 minutes ($r > 0.98$) showing the biexponential equation is an adequate description of the observed data over this period.

When the notional quantity of tritiated 18OHB in the plasma at zero time (t_o) was calculated from this biexponential equation the value was considerably less than 100% (18.6 % in subject JF and 12.7% in subject CN). This is suggestive that most of the clearance of tritiated 18OHB was due to an even faster process which was missed by sampling even at 1 minute. This is consistent with the finding in both subjects that the observed value at 1 minute was greater than that predicted from the biexponential

equation. This anomaly could also be caused by incomplete mixing of the tracer in the plasma pool. However, in both subjects the difference between observed and predicted values was in the same direction.

Study 2:

Metabolic clearance rate (MCR) of 18OHB

The metabolic clearance rate (MCR) was calculated from the expression $R/t = MCR \times P_c$ where R is the total radioactivity administered in time t and P_c is the level of radioactivity in the plasma after corrected for procedural loss (see Figure 25) . This is only valid under steady state condition. Although only a short period of infusion was used (30 minutes) the plasma levels of tritiated 18OHB at 10, 20 and 30 minutes were 1104, 1282 and 970 dpm/ml (**Figure 30**) plasma respectively which I believe indicates an approximation to steady state conditions.

II Saliva:

The cleaned saliva (0.5 ml) was used for liquid scintillation counting. Excess saliva was pooled, extracted and the radioactive isotope was identified by paper chromatography. No ICG was detected in saliva samples.

III Glucuronide hydrolysis using ß-glucuronidase

The amount of ß-glucuronidase used was $\overset{a}{\wedge}$ nominal 1000 U per ml of urine. The protocol used was shown in Figure 27a for urine and plasma (Figure 27b).

IV Descending Paper chromatography

Two solvents systems (T_{50} and E_2B) were used. The development time used was 16 hours and the position of the 18OHB marker was located by ultra-violet absorption and blue tetrazolium reaction. The whole length of the paper chromatogram was cut into one cm strips, and eluted with methanol for radioactivity measurement.

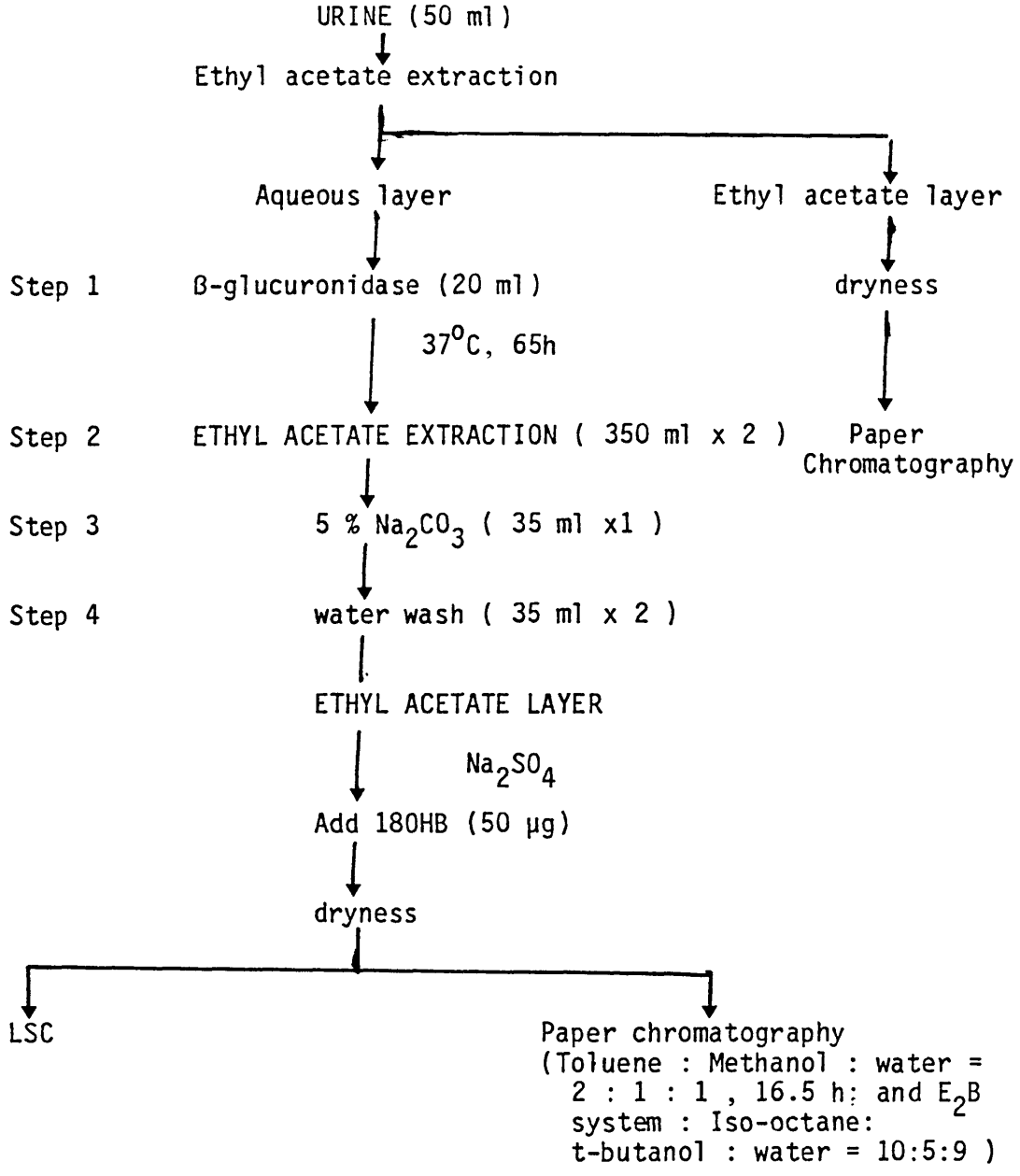


Figure 27a

Method for the identification of radioactive steroid in urine before and after hydrolysis with ß-glucuronidase.

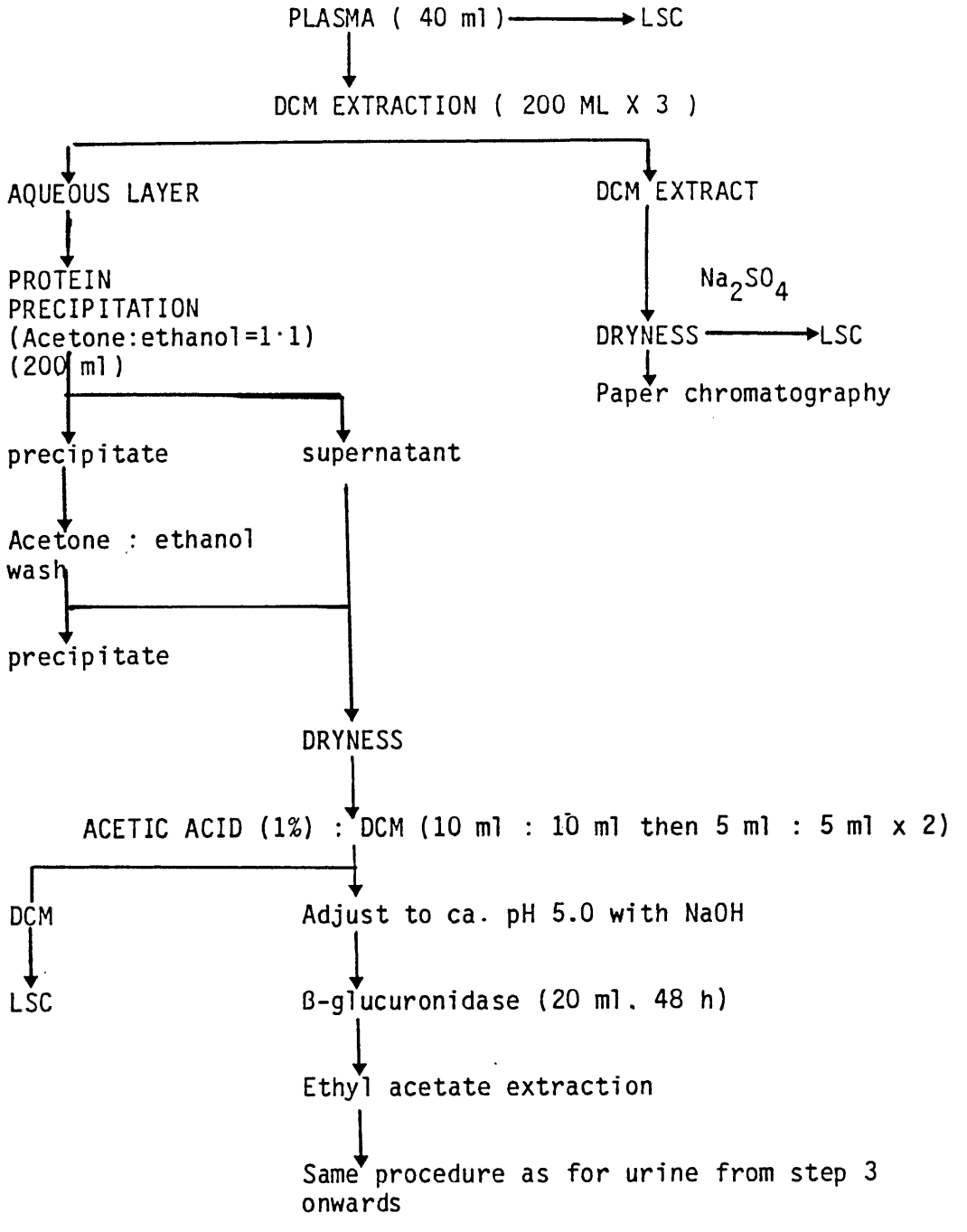


Figure 27b Method for the identification of radioactive steroid in plasma before and after hydrolysis with ß-glucuronidase by paper chromatography. This method was also adopted for the pooled saliva from the intravenous study.

Results and Discussion

I Disappearance curve of ICG

This is shown in Figure 28. The results of the fractional disappearance of ICG (^{ICG}k), the half-life of ICG ($^{ICG}t_{1/2}$) and the volume of distribution of ICG (VD_{ICG}) and the EHPF are shown in Table 10.

Table 10 Kinetic data of ICG in two healthy subjects:

Subjects	CN	JF
Body mass (kg)	73	76
Age (years)	30	50
Expected plasma volume (ml)+	3358	3268
Single exponential equation	lnY=-0.219t+2.04	lnY=-0.192t+1.82
ICG_k (min-1) *	0.219	0.192
$ICG_{t1/2}$ (min)	3.17	3.61
VDICG (ml) (% expected)	3275 (97.5%)	4099 (125%)
EHPF (ml/min)	1024	1124
Clearance (ml/min/kg)*	9.8	10.4

* Normal ^{ICG}k and clearance values obtained by Martin, Mikulecky et al., 1975 were 0.205 ± 0.005 (mean ± SE) and 8.9 ± 0.3 (mean ± SE) respectively when a dose of 0.5 mg ICG/Kg body mass was used.

+ based on body mass and age (Documenta Geigy, 1970)

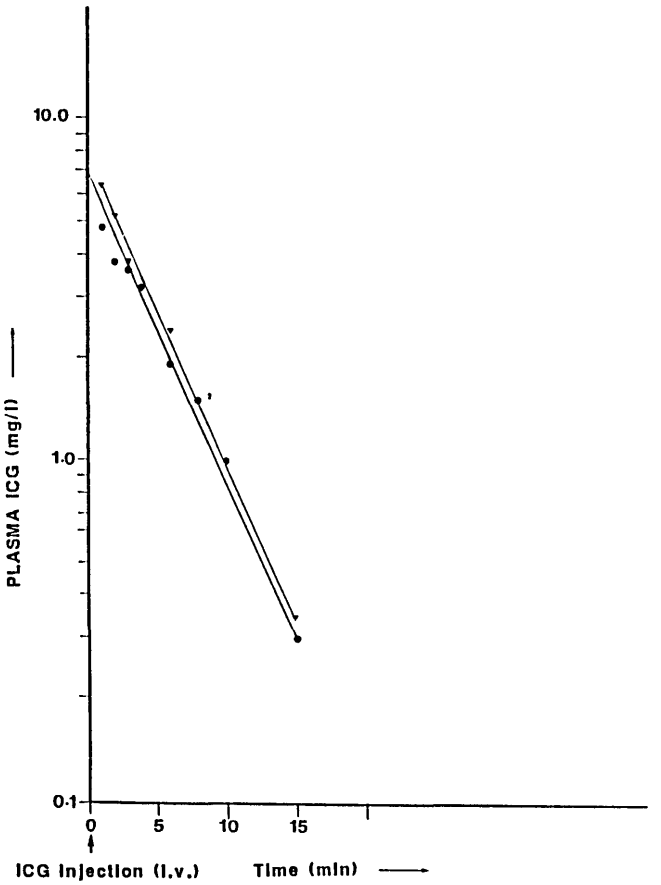


Figure 28

Disappearance curves of plasma indocyanine green (ICG) in two healthy subjects.

Subject	Keys	Line of linear regression (r)	$^{ICG}t_{1/2}$(min)
JF	•	$\log_e$ Y= -0.19t + 1.82 (0.9939)	3.62
CN	▼	$\log_e$ Y= -0.22t + 2.04 (0.9993)	3.17

Y = plasma ICG measured (mg/l)

t = Time in minutes after ICG administration

The dose used in our experiment averages 0.33 mg ICG/kg body mass and the values of ^{ICG}k and clearance of the two subjects indicated that they have normal liver function. The average value of EHPF from these two subjects was 1074 ml/min. Hence, within experimental error this again showed that the two subjects have normal liver function. This is important because the liver is the main organ responsible for conjugation of steroid and clearance depends on the efficiency of hepatic extraction and hepatic blood flow.

II Characterisation of the radioactive 18OHB in saliva, plasma and urine

(i) Saliva

DCM extracted all the radioactivity from saliva and the extracted material had the chromatographic mobility of 18OHB marker in both paper chromatographic systems (T-50 and E_2B) used. No other radioactive spot was found.

(ii) Plasma

(a) DCM extractable

The only radioactive material present corresponds to the authentic 18OHB.

(b) After ß-glucuronidase hydrolysis

The paper chromatographic data showed a radioactive spot having a lower mobility in T-50 than 18OHB but a higher mobility than 18OHB in E_2B system was present. It would be speculative that the radioactive compound is tetrahydro-18OHB or tetrahydro-18OHA from the chromatographic data on the tetrahydro-steroids and their parent steroids of cortisol, cortisone and aldosterone (Table 11).

Table 11 Relative mobilities of some tetrahydro-steroids and their parent steroids to cortisol in two paper chromatographic systems (T-50 and E_2B):

STEROIDS	T-50	E_2B
Cortisol	1.00	1.00
THcortisol	0.38	1.45
Aldosterone	1.51	0.73
THaldosterone	0.55	1.55
Cortisone	1.94	1.27
THcortisone	0.72	1.84

(iii) Urine

(a) Ethyl acetate extractable fraction

Four radioactive spots were observed in T-50 solvent system. One

of which corresponded to the chromatographic mobility of 18OHB and confirmed by using solvent system E_2B. Another had a lower mobility and the other two had a higher mobility than 18OHB.

(b) After ß-glucuronidase hydrolysis

The only radioactive spot observed had a chromatographic mobility of 18OHB in T-50 solvent system. However, when this radioactive spot was rechromatographed in the E_2B solvent sytem, two radioactive spots equidistant (5cm) from the authentic 18OHB spot were obtained. The urine analysed was from the urine of one of the two subjects in the single intravenous injection study and was collected 360 minutes post injection.

III Kinetics following a single intravenous injection of tritiated 18OHB into 2 subjects

SALIVA

Tritium appeared in the saliva rapidly after the isotope was injected intravenously into two healthy subjects (**Figure 29a** and **Figure 29b** for subject JF and subject CN respectively; they were the same subjects as shown in Figure 25 and 26 respectively). The chemical form of this salivary tritium was 18OHB. The rapid appearance of the isotope probably implies that the transfer of 18OHB from the blood to the saliva was by trans-cellular diffusion, which is the most likely mechanism for lipid-soluble

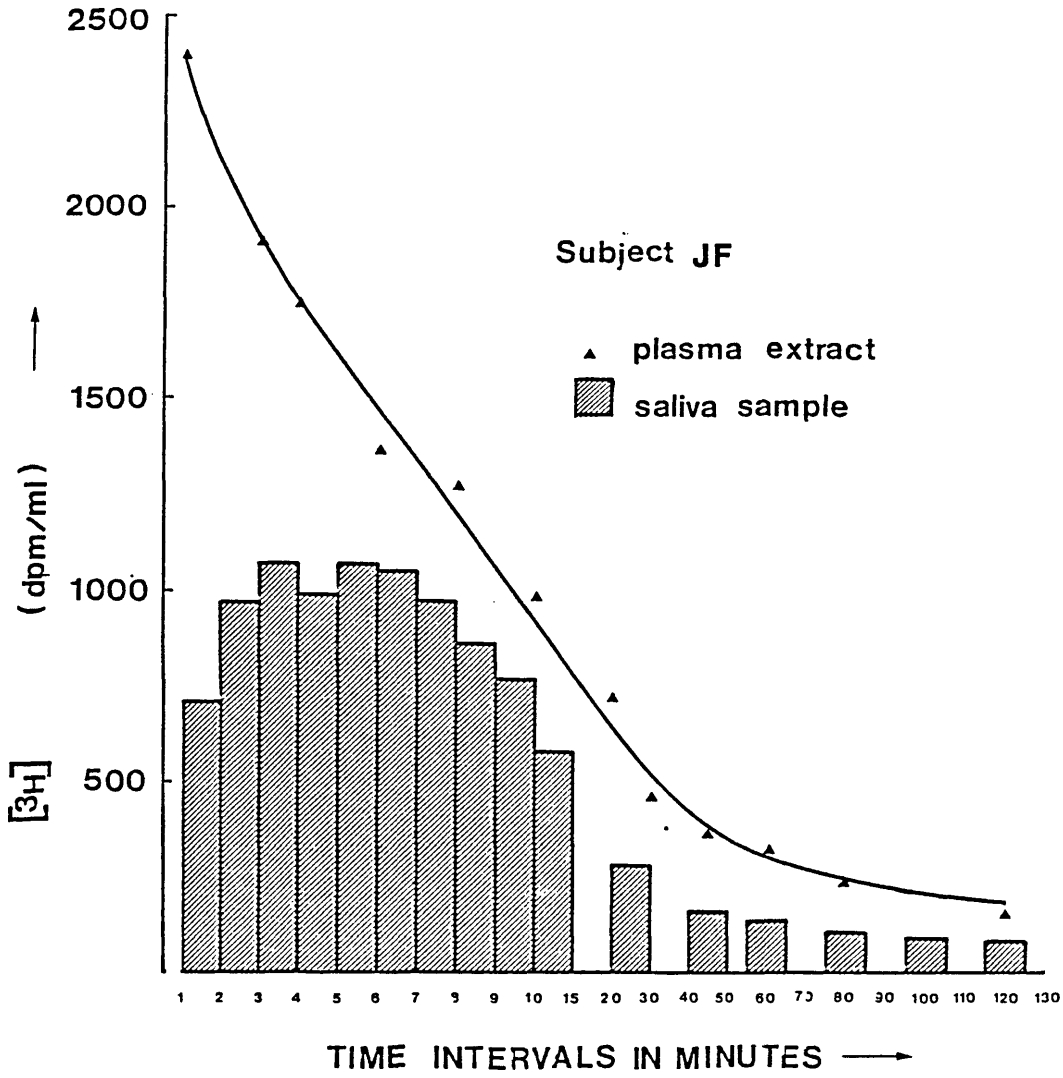


Figure 29a

Appearance of tritiated 18OHB in saliva after a single intravenous injection of tritiated 18OHB (20μCi) into a male subject (Subject JF).

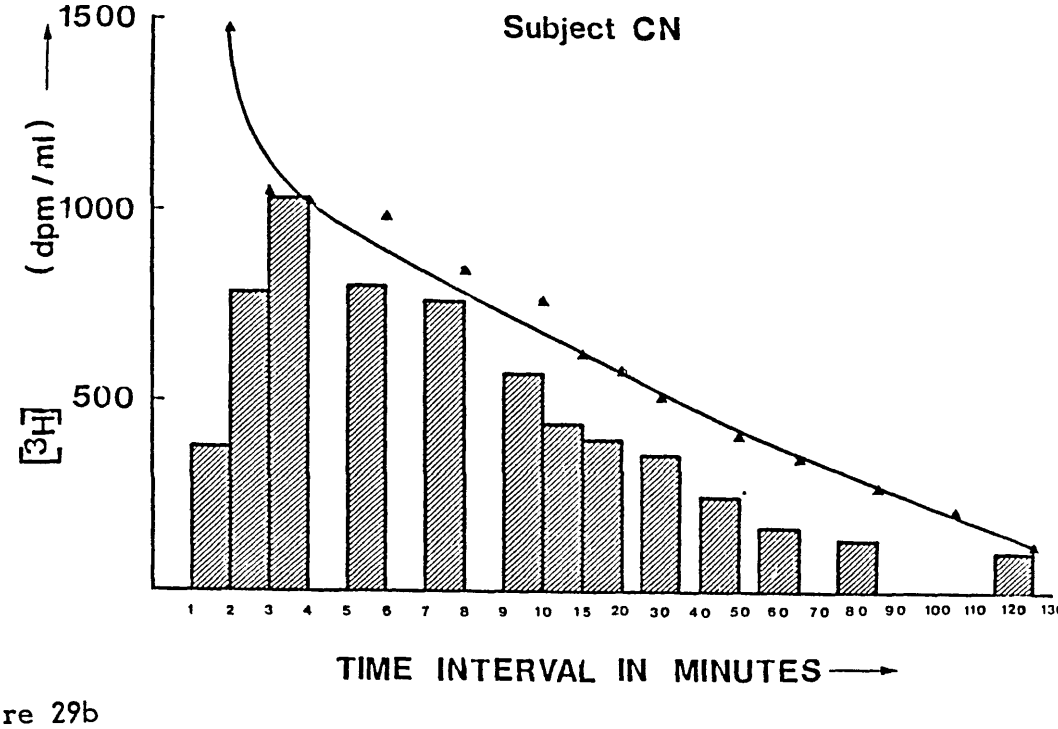


Figure 29b

Appearance of tritiated 18OHB in saliva after a single intravenous injection of tritiated 18OHB (20μCi) into a male subject (Subject CN).

steroids. The tritiated 18OHB in the saliva reached a plateau between 3 and 8 minutes post injection. It appears from Figure 29a and 29b that the ratios of saliva ^{3}H:plasma extractable ^{3}H are about 65% in subject JF and 80% in subject CN. These are higher than would be expected from the known values for % plasma free 18OHB (35 $\pm$ 3%; see pp157). This discrepancy could be due to non-steady state conditions at this stage of the experiment. These non-steady conditions are probably due to the following reasons:

(i) incomplete mixing of label and plasma pool

(ii) rapid disappearance of plasma tritiated 18OHB

(iii) non-equilibrium between free and bound tritiated 18OHB

(iv) temporal artefact due to time of transfer of tritiated 18OHB to saliva, collection of saliva and blood sampling.

Nevertheless, the detection of tritiated 18OHB in saliva following injection i.v. justifies measuring 18OHB in saliva and the use of saliva to investigate the dynamics of circulating free plasma 18OHB concentration.

Although the experiment was solely designed for investigating the suitability of using saliva for measuring 18OHB, the kinetics of tritiated 18OHB in plasma were studied simultaneously. However, no attempt was made to study the relationship between secretion and plasma 18OHB concentration by measuring the changes in the specific activity of 18OHB injected as was done for cortisol (Hellman et al., 1970). This was mainly because at the time of the

study, the method for measuring 18OHB concentration was not available and hence the specific activity of the steroid could not be determined.

PLASMA

The disappearance curves of the plasma tritiated 18OHB in two healthy subjects are shown in Figures 25 and 26 (pp126 - 129) and are best described by a two compartmental model. The average half life of the fast component was 4 minutes and that of the slow component was 54 minutes.

The rapid disappearance of the injected radioactive 18OHB (60 % of injected dose in subject JF and 80% in subject CN by 1 minute post injection) implies the rapid uptake by an extravascular compartment, which can be a combination of various anatomic sites and/or biological fluids inaccessible to sampling (see inset of Figure 25 & Figure 26). Such sites possibly include the interstitial fluids including lymph and/or adipose tissues and may even include the liver and the kidney.

The half life of the ICG was found on average to be 3.34 minutes, which is approximately equal to the half life of the fast component of plasma 18OHB (4 minutes). Since ICG is completely protein-bound (Cherrick et al, 1960) and solely taken by the liver (Wheeler et al., 1958; Cherrick et al., 1960), it is very likely that the disappearance of ICG is proportional to the liver blood

flow (see calculation of estimated hepatic blood flow on pp 124 & 135). The hepatic extraction rate of ICG is approximately 70% (Cherrick et al., 1960; Caesar et al., 1961) but that of 18OHB is not known. However, the hepatic extraction of aldosterone is approximately 90% (Bougas et al., 1964). Since the binding of 18OHB to proteins (42% to albumin and 26% to corticosteroid binding globulin (CBG) as reported by Zager et al., 1986) is similar to that of aldosterone (Dunn et al., 1981), its clearance by the liver will probably be similar to that of aldosterone and ICG. That is the fast component may be due to liver clearance, and this is supported by the rapid appearance of conjugates.

However, the fast component may not be due to uptake and clearance by the liver alone. The blood flow$_s$ through the liver and the kidneys are similar and hence this raises the possibility of uptake by the two organs simultaneously. No distinction was made between hepatic and renal conjugate produced owing to the lack of authentic markers.

The slow component indicates the return into the plasma of 18OHB which had disappeared into the extravascular compartment during the first minute after injection. Hence, the extravascular compartment might be acting as a reservoir for 18OHB. Such deduction of 'dwelling in organs or fluids of the extravascular compartment was derived from the data of the rapid disappearance of the radioactivity and the material unaccounted for as shown in

Table 12a. There was negligible radioactivity associated with red blood cells during the study and hence this further supports the disappearance of the radioactivity into the extravascular compartment.

Table 12a Radioactivity unaccounted for after an intravenous injection of tritiated steroid:

TIME	PLASMA		URINE	Total *	RADIOACTIVITY
(min)	18OHB	Metabolite			UNACCOUNTED
			% DOSE		
Subject : CN					
60	2.60	5.20	28.2	36	64.0
135	0.96	3.50	10.9	43.6	56.4
180	0.70	2.90	10.9	53.6	46.4
			Total=50.0		
Subject : JF					
65	2.60	8.30	14.2	25.1	74.9
160	0.80	6.00	13.1	24.1	65.9
300			7.9		64.8
			Total=35.2		

* Cumulative excretion in urine + plasma 18OHB and its metabolite

Table 12b Appearance of radioactivity in urine after a continous infusion of tritiated 18OHB into a healthy subject:

Time after infusion (h)	% dose in urine
1	5.5
2	4.5
3	3.0
4	1.5
8	2.8
9 - 12	2.9
13 - 24	0.7
	Total=20.9

IV Kinetics during a continuous infusion of tritiated 18OHB The infusion study was pursued in order to collect sufficient saliva for identification of the tritiated 18OHB by paper chromatography. The result confirmed that all the tritiated 18OHB present in saliva has the mobility of authentic 18OHB.

The appearance of radioactivity in the saliva and plasma is shown in Figure 30. This again demonstrates the presence of 18OHB in saliva and justifies the development of an assay for measuring

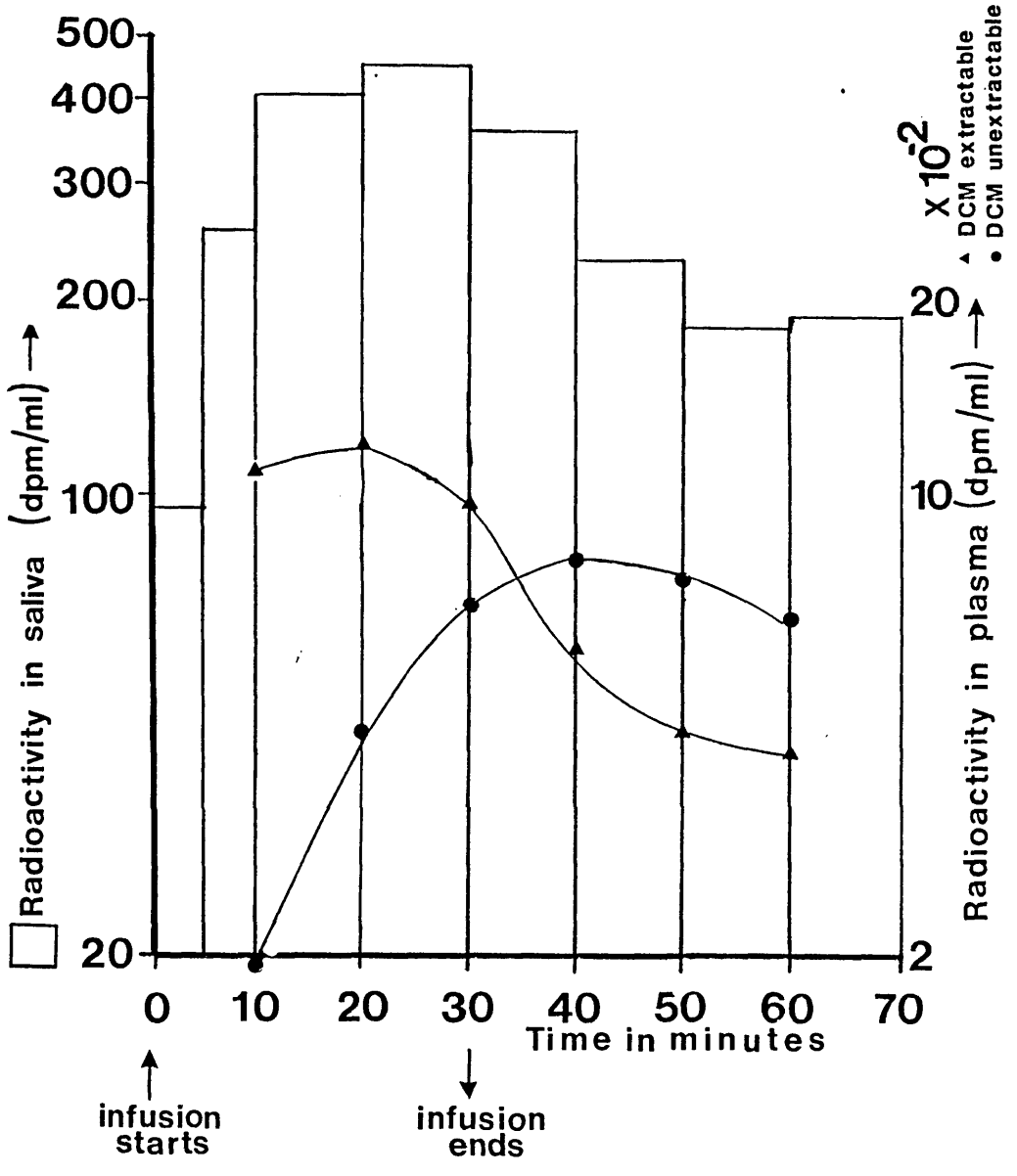


Figure 30

Appearance of tritiated 18OHB in saliva and the disappearance in plasma during a continuous infusion of [1,2-^{3}H]18OHB into a male subject (Subject MH).

18OHB in saliva. The metabolic clearance rate of tritiated 18OHB was found to be 1064 ml/min. This is approximately equivalent to the mean MCR of aldosterone in normal supine man (1125 ml/min, Tait et. al., 1961). This is also approximately equal to the estimated hepatic plasma flow from the ICG injection study described above (Study 1).

The radioactive isotope excreted into the urine is shown in Table 12b. The total amount excreted is less than the amount excreted in the single intravenous injection for the corresponding time period after the injection.

3.2. Saliva and Plasma 18OHB

INTRODUCTION

The rapid appearance % of tritiated 18OHB in the saliva after an intravenous administration of the tritiated steroid into two healthy subjects described in the previous section indicates the feasibility of using saliva as a biological fluid other than plasma and urine for physiological and clinical studies. In addition it will be interesting and important to study the relationship between 18OHB in saliva and blood. For such a study matched saliva and blood samples from healthy subjects under physiological conditions and after stimulation with corticotrophin were used. The percentage plasma free 18OHB (%PFB) was measured by equilibrium dialysis using the 'dianorm' machine. The validation of the equilibrium dialysis for measuring the percentage of plasma free 18OHB is presented here.

The total 18OHB concentration (PB) in saliva and plasma was measured by RIA after chromatographic purification. The plasma free 18OHB concentration (PFB) was calculated from %PFB and PB where PFB = %PFB X PB.

3.2.1. Measurement of non-protein bound 18OHB in plasma by equilibrium dialysis using a "Dianorm" machine.

The principle of the technique is to dialyse plasma against tritiated 18OHB until equilibrium is reached. Each steroid has its own characteristics. The pre-requisite of using the technique is to investigate the time required for equilibrium to be reached and the amount of tritiated 18OHB required for the dialysis over a wide plasma 18OHB concentration.

Procedure

Dialysis tubing (Visking VT-31, FSA) was cut into 41 mm squares and soaked in normal saline overnight at $4^{o}C$. The tubing was then unfolded by cutting the two edges and the four corners so that they would cover the dialysis cell. The membrane was then rinsed in normal saline by roller mixing it for 30 minutes or at least three changes of saline solution if being left in the cold room. The membrane was finally soaked for another 30 minutes with two changes of saline solution.

One ml of tritiated 18OHB (0.1 µci/ml) was injected into one half of the dialysis cell and plasma (1.0 ml) was injected to the other half. The dialysis cells were dialysed at $37^{o}C$ at a rotation speed of 12 r.p.m..

% Plasma Free 18OHB by Equilibrium Dialysis

Calculation

The calculation of the % plasma free 18OHB (% PFB) and the % bound were as follows:-

$$\% \text{ PFB} = \frac{\text{cpm on saline side}}{\text{(Total count in cpm - cpm on saline side)}}$$

$$\% \text{ bound} = \frac{\text{dpm on the plasma side - dpm on the saline side}}{\text{Total count in dpm}}$$

(Check for leakages:

Total count in dpm = (dpm on the plasma side + dpm on the saline side)

Either the % free or the % bound can be used but initially the % bound is calculated because the data are used for checking leakages.

RESULTS

A Validation of the method of equilibrium dialysis using the 'Dianorm' machine for measuring plasma free 18OHB concentration

(a) Time of dialysis

A pool of plasma was used for the investigation. Duplicate samples were dialysed for varying period of time. The results are shown in Figure 31. After 4 h of dialysis, equilibrium was reached. Therefore, the time of dialysis used was 5 h in all future experiments.

(b) Amount of tritiated 18OHB required for dialysis

The plasma pool used in experiment (a) was spiked with varying amounts of authentic 18OHB. In this experiment the amount of tritiated 18OHB used in experiment (a), that is 0.1 µCi/ml, was used. The results were shown in Table 13.

The results showed that by increasing the amount of 18OHB, the method did not detect a change in the % free. This could either be due to the binding of 18OHB to excess albumin available or the amount of tritium added was insufficient to detect any change. The latter was tested by varying the amount of tritium from 0.01 µCi/ml to 0.2 µCi/ml. The calculated percentage free remained constant, independent of the amount of tritiated 18OHB added on the saline

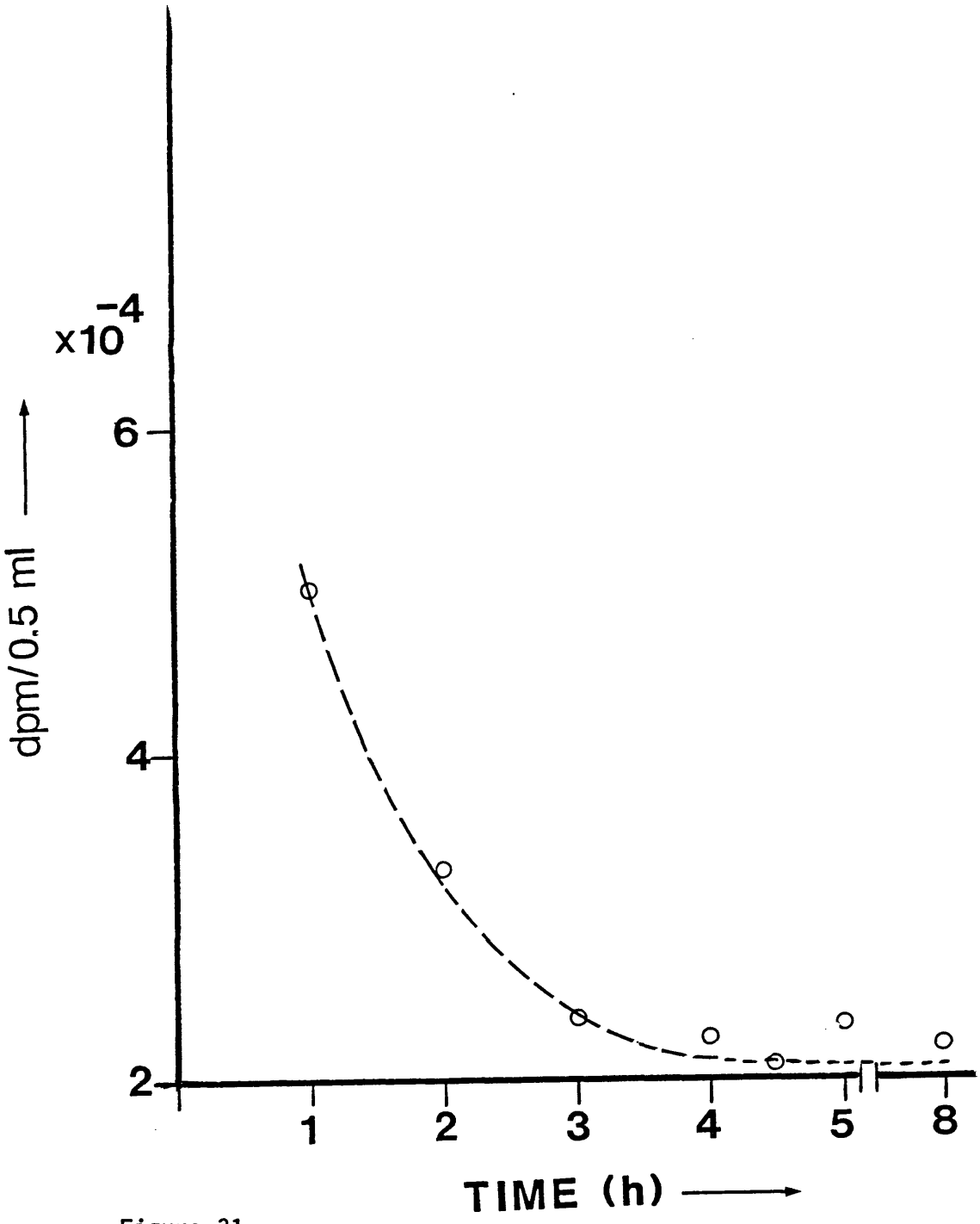


Figure 31

To study the time required to reach equilibrium using the "Dianorm machine" dialysing tritiated 18OHB in saline (1.0 ml) on one side of the dialysis cell and undiluted plasma (1.0 ml) on the other side. Total count used at time zero was 0.1 μCI.

side or of the amount of cold 18OHB on the plasma side. Therefore, for all future experiments, 0.1 μCi/ml was used so that the statistical error of the count rates in 0.5 ml of the saline dialysate was low.

Table 13 Effect of increasing dose of 18OHB on % plasma free 18OHB:

Plasma + 18OHB (pg/ml)	% free	
	mean of duplicates except *	SD of duplicates
0	27.0	2.4
125	27.5	1.3
250	29.4	0.35
500	28.6 *	
750	28.1	3.7
1000	22.2 *	
1250	28.5	0.28

(c) Quality control (QC) samples

The plasma pool used in the above experiments was used as a QC sample for all subsequent assays. It was not possible to use more than one pool of samples since it was not possible to find plasma where the % PFB was spreaded over a wide range. The within and

between batch variation using this QC samples is shown in Table 14. Ten replicates were assessed in one batch and the coefficient of variation was calculated (within-batch precision). Duplicates were then repeatedly measured in 10 different batches and the coefficient of variation was calculated using the formulae below:

CV % = SD x 100 %/ mean

where SD, standard deviation = square root of variance.

Variance = sum of squares of the difference between duplicate divided by 2n

where n= number of pairs of duplicates and

mean = sum of all the individual values / 2n

Table 14 Within and between batch precision of equilibrium dialysis using quality control samples:

mean ± SD	PRECISION (cv%)	
(n = 10)	within-batch	between-batch
26.8 ± 3.4	12.7	
28.0 ± 4.0		14.3

3.2.2. Determination of the relationship between salivary 18OHB and plasma concentration in normal subjects

RESULTS

(a) % PFB in normal subjects

Healthy subjects aged between 22 to 63 years old collected saliva over 10 minutes and a correponding blood sample was collected 5 minutes after the beginning of salivation. The blood was collected into lithium heparinised tubes and plasma was obtained by centrifuging the blood at $4^{o}C$ and 1500g for 15 minutes. Plasma was separated as quickly as possible and stored frozen until use. The plasma was stored for less than one month prior to the measurement and was only thawed immediately before assay. The results from 20 healthy subjects aged between 24 and 65 are shown in Table 15a.

Table 15a The %PFB of normal subjects going about their daily business:

	mean ± SD	n
Male	35.7 ± 2.5	13
Female	33.6 + 3.0	7
Student t	1.7335	
p >	0.1(N.S.)	

There was no difference in the % PFB between the sexes (Table 15a). The % PFB of the 20 healthy subjects was therefore 35.0 ± 2.8 % (mean ± SD) at a mean ± SD value of 904 ± 621 pmol/l plasma 18OHB and there was a narrow range of distribution of the % PFB (28.9% to 39.7%).

(b) Matched saliva level versus free plasma 18OHB concentration

The plasma free 18OHB concentration (PFB) was calculated using the formula :

PFB = % PFB x PB where PB was the total plasma 18OHB concentration determined by radioimmunoassay after TLC. The saliva 18OHB levels were also determined by radioimmunoassay after TLC. The relationship between saliva 18OHB (SB) and PFB concentrations in the 20 healthy subjects studied in experiment (a) is shown in

Figure 32 where SB=0.29PFB + 64 (n=20, r=0.7229, t=4.43, $p < 0.001$). Therefore, there was a highly significant correlation between the concentration of 18OHB in saliva and the plasma free 18OHB. The range of the saliva and plasma free 18OHB concentration measured and the % of saliva to plasma free 18OHB concentration is shown in **Table 15b**. The frequency of distribution is shown in **Table 15c**. Although the salivary 18OHB concentration ranged from 21 to 106 % of plasma free 18OHB concentration, there is an approximately equal frequency of distribution.

Table 15b The concentration of immunoreactive salivary 18OHB (SB) and plasma free 18OHB concentrations (PFB):

	Concentration (pmol/l)	
	Range	mean ± SD
SB	39 - 376	157 ± 94
PFB	43 - 944	322 ± 237
% SB/PFB	21 - 106	58 ± 28

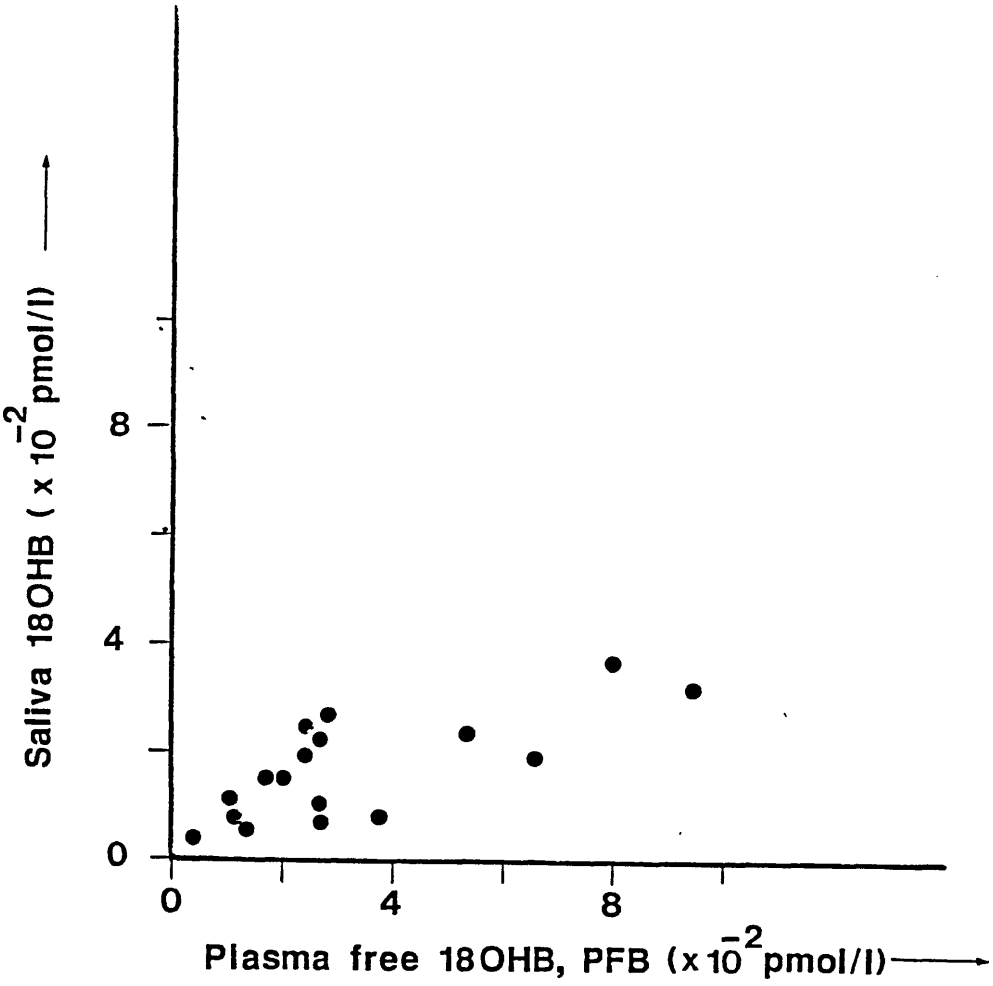


Figure 32

The relationship between salivary 18OHB concentration and plasma free 18OHB concentration in normal subject going about their daily business. Matched saliva and plasma were collected for such studies. The saliva concentration ranged from 39 pmol/l to 276 pmol/l with a mean $\pm$ SD (n=20) of 157 $\pm$ 94 pmol/l. The plasma free 18OHB concentration ranged from 43 pmol/l to 944 pmol/l with a mean $\pm$ SD of 322 $\pm$ 237 pmol/l. The line of linear regression between salivary 18OHB (SB) and plasma free 18OHB concentration (PFB) is : SB=0.29 PFB + 64 (n=20, r=0.7229, $p < 0.001$).

Table 15c The distribution frequency of the percentage of SB corresponding to the PFB:

% saliva /PFB	number	Frequency (%)
20 - 40	7	35
41 - 61	4	20
62 - 82	4	20
exceed 82	5	25

(c) Matched saliva versus total plasma 18OHB concentration

The relationship between saliva 18OHB and total plasma 18OHB concentration (PB) of the 20 healthy ambulatory subjects studied above is shown in **Figure 33** where SB=0.10PB + 59 (n=20, r=0.68,p < 0.001). The salivary 18OHB concentration expressed as a mean $\pm$ SD of plasma 18OHB concentration is 20.7 $\pm$ 10.5 %. This is very similar to the relationship between salivary aldosterone and plasma aldosterone concentration, which was found to be 25.8 $\pm$ 17.3 % (Few et al., 1986b).

(d) Relationship between 18OHB in saliva and plasma after corticotrophin stimulation (for details, see pp 223 - 234).

It has been shown that salivary 18OHB concentration correlates with plasma 18OHB concentration under basal condition. Therefore, this relationship was further examined under the stimulatory

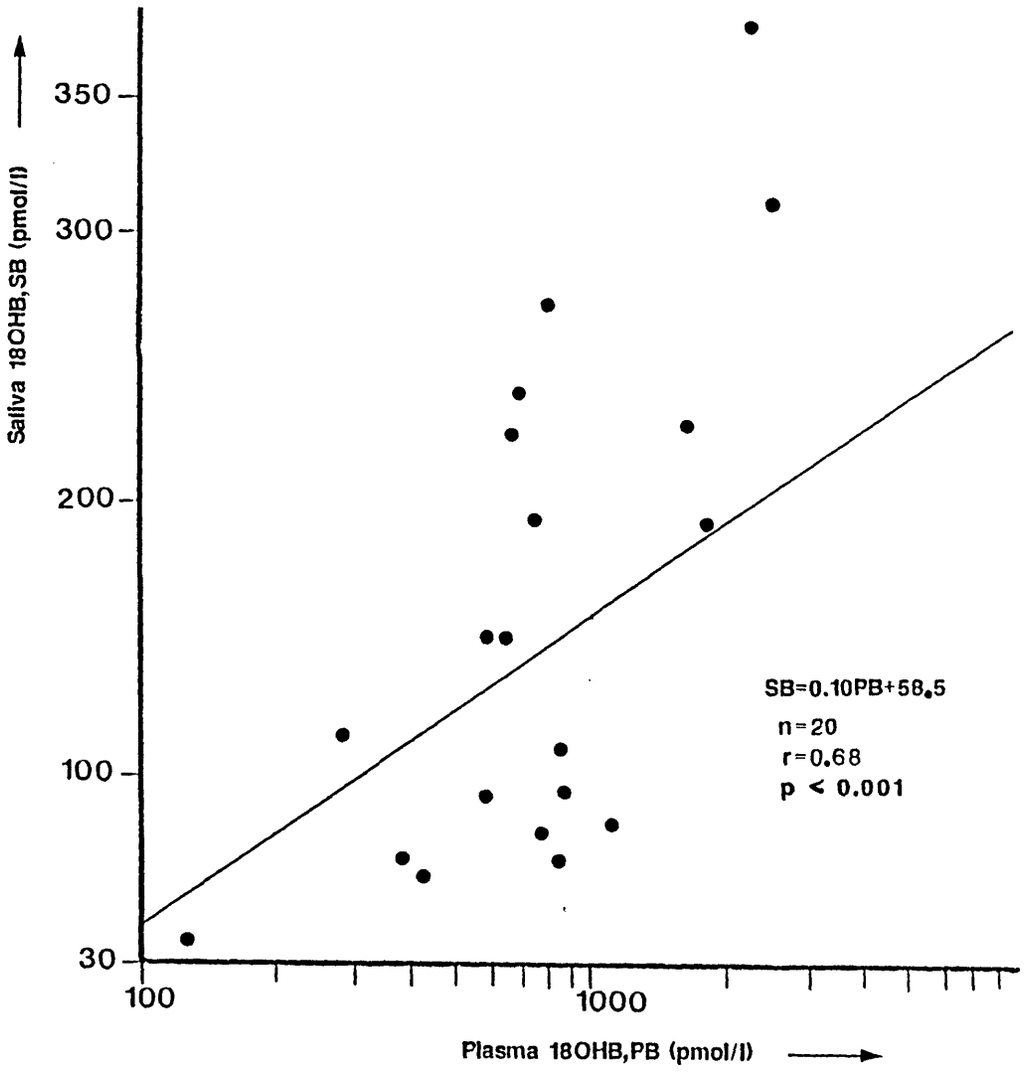


Figure 33

The relationship between salivary 18OHB concentration and total plasma 18OHB concentration in normal subjects going about their daily business. Note: 18OHB concentration are plotted on logarithmic scale so as to enable the individual datum point to be shown clearly; the line of linear regression was computed using the concentration of 18OHB in arithmetic scale.

action of corticotrophin. The %PFB remained unaltered despite the 5 fold increase of plasma 18OHB concentration. However, the increase of plasma 18OHB concentration was accompanied by a parallel increase in salivary 18OHB concentration. Hence, these data support the measurement of salivary 18OHB as a reflection of plasma 18OHB in healthy subjects under basal and stimulated conditions as was shown by the intravenous study using tritiated 18OHB described in the previous chapter.

DISCUSSION

Salivary 18OHB concentration (SB) was found to have a mean value of 169.0 ± 91.7 (SD) pmol/l and the plasma 18OHB concentration (PB) had a mean value of 904.1 ± 621.5 (SD) pmol/l which on the average were comparable with those of other authors (Appendix IIIa for values). The percentage plasma free 18OHB (%PFB) measured by equilibrium dialysis has a mean value of 35.0 ± 2.8 (SD)%, similar to the value reported for plasma free 18OHB (Zager *et al.*, 1986) and aldosterone (Few *et al.*, 1986b) measured by equilibrium dialysis. From the data on % PFB, it was calculated that the salivary 18OHB concentration was 20.1 ± 10.5 (mean ± SD) % (range from 8.1 % to 41.4%) of total plasma 18OHB concentration and 58± 28% (ranged from 21% to 106%) of plasma free 18OHB concentration (PFB). Therefore, in quantitative terms, saliva 18OHB concentration does not totally represent plasma free 18OHB concentration. The

correlation of SB with PFB (r=0.72) and SB with PB (r=0.68) is not very different. This again implies that SB reflects PB but does not necessarily represents PFB. This is probably related to the mechanism of transport of 18OHB from the blood into the saliva. Another reason is probably related to subject variations since the percentage of SB/PFB varied from 21 to 106% and the frequency of distribution is fairly evenly spread .

Studies in the two subjects stimulated with corticotrophin showed % SB/PFB under basal and stimulated conditions was constant in one subject but elevated in the other subject. In subject JF prior to corticotrophin stimulation, the %SB/PFB was 52.9 $\pm$ 13.8 % (mean $\pm$ SD, n=6) and was 43.5 $\pm$ 1.8 % (mean $\pm$ SD, n=5) after corticotrophin stimulation. In subject CN, it was 37.1 $\pm$ 5.1 % before and approaching 100% after stimulation with corticotrophin. The increase was due to the increase of plasma 18OHB concentration. Corticotrophin did not affect the % PFB despite 4,7 and 2 fold increase in plasma glucocorticoid, 18OHB and aldosterone concentration respectively in subject JF and 2, 5 and 3 fold in subject CN.

3.2.3. Relationship between 18OHB and other adrenocortical steroids such as aldosterone and glucocorticoids in matched saliva and plasma samples in healthy subjects

The concentrations of aldosterone and glucocorticoids in the healthy ambulating subjects described in the previous section were measured.

RESULTS

The correlation of 18OHB and aldosterone with glucocorticoids in these subjects and the concentration of these three steroids are summarised in **Table 16a** to **Table 16d** with graphical representations of the relationship between 18OHB and aldosterone in saliva and plasma in **Figure 34a** and **Figure 34b** respectively. The data show that there is a correlation between saliva and plasma 18OHB with aldosterone but not glucocorticoids in these subjects. The concentration of salivary 18OHB concentration is 20 $\pm$ 10.5 % (mean $\pm$ SD, n=20) of that in the plasma and for aldosterone it is 21 $\pm$ 9.4 % (mean $\pm$ SD, n=17).

18OHB, Aldosterone and Glucocorticoids --- Healthy Ambulatory Subjects

Table 16a Correlation matrix between 18OHB and two other adrenocorticosteroids (aldosterone and glucocorticoids):

	saliva aldosterone	saliva glucocorticoids
saliva 18OHB	0.70	0.22
Saliva glucocorticoids	0.26	

Table 16b Lines of linear regression between 18OHB and aldosterone in saliva and plasma:

	Line of linear regression	n	r	p <
1. SALIVA	SB = 1.5 SA + 56.4	19	0.70	0.001
2. PLASMA	PB = 4.0 PA - 268	19	0.87	0.001
3.	SB = 0.10PB + 59	20	0.71	0.001
4.	SA = 0.20PA + 109	17	0.84	0.001

Table 16c 18OHB:aldosterone ratios in saliva and plasma:

	Ratio of 18OHB : Aldosterone	
	Range	mean ± SD
SALIVA	0.77 - 6.1	2.74 ± 1.51
PLASMA	0.92 - 15.8	4.24 ± 3.72

Table 16d Concentration of 18OHB and aldosterone in healthy ambulatory subjects:

	Concentration (pmol/l)	
	18OHB (n=20)	Aldosterone (n=17)
	mean ± SD :	
SALIVA	168.9 ± 91.7	68.4 ± 46.2
PLASMA	904.1 ± 621.5	294.4 ± 138
	RANGE :	
SALIVA	39.5 - 376.9	9.9 - 193.6
PLASMA	125.3 -2427 (*)	70 - 550

* the values of three of these subjects were abnormally high (1779, 2209 and 2427 pmol/l). These values were from subjects who have just returned from mountain climbing; their salivary aldosterone values were corresponding high.

DISCUSSION

Since the discovery of 18OHB in 1957 (Ulick and Lieberman), its role as an intermediate of aldosterone biosynthesis has only been confirmed recently by means of the 'trapping' experiment (Kojima et al., 1984). None of the earlier in vitro experiments unequivocally established that 18OHB is a precursor of aldosterone

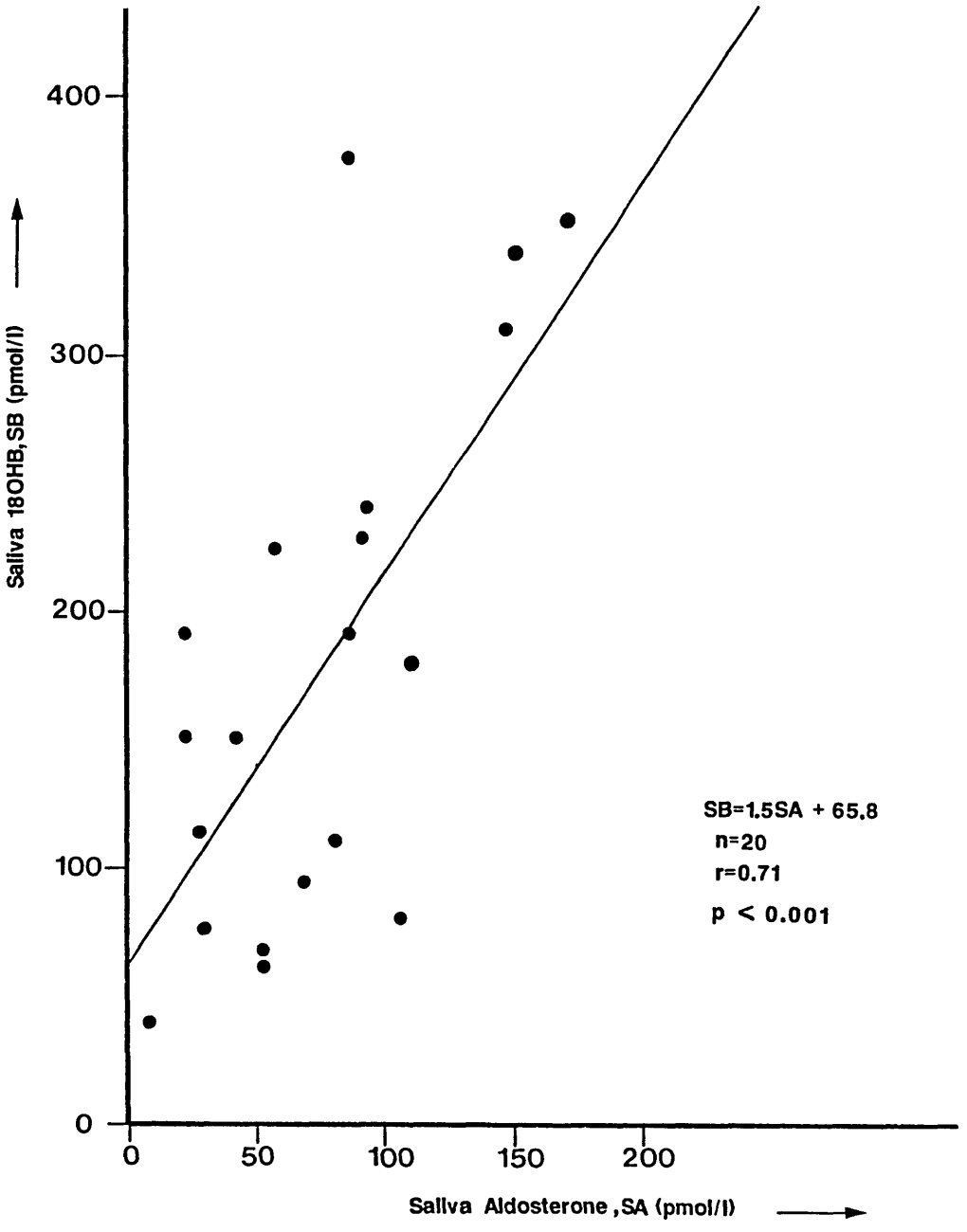


Figure 34a

The relationship between salivary 18OHB concentration and salivary aldosterone concentration in normal subjects going about their daily business.

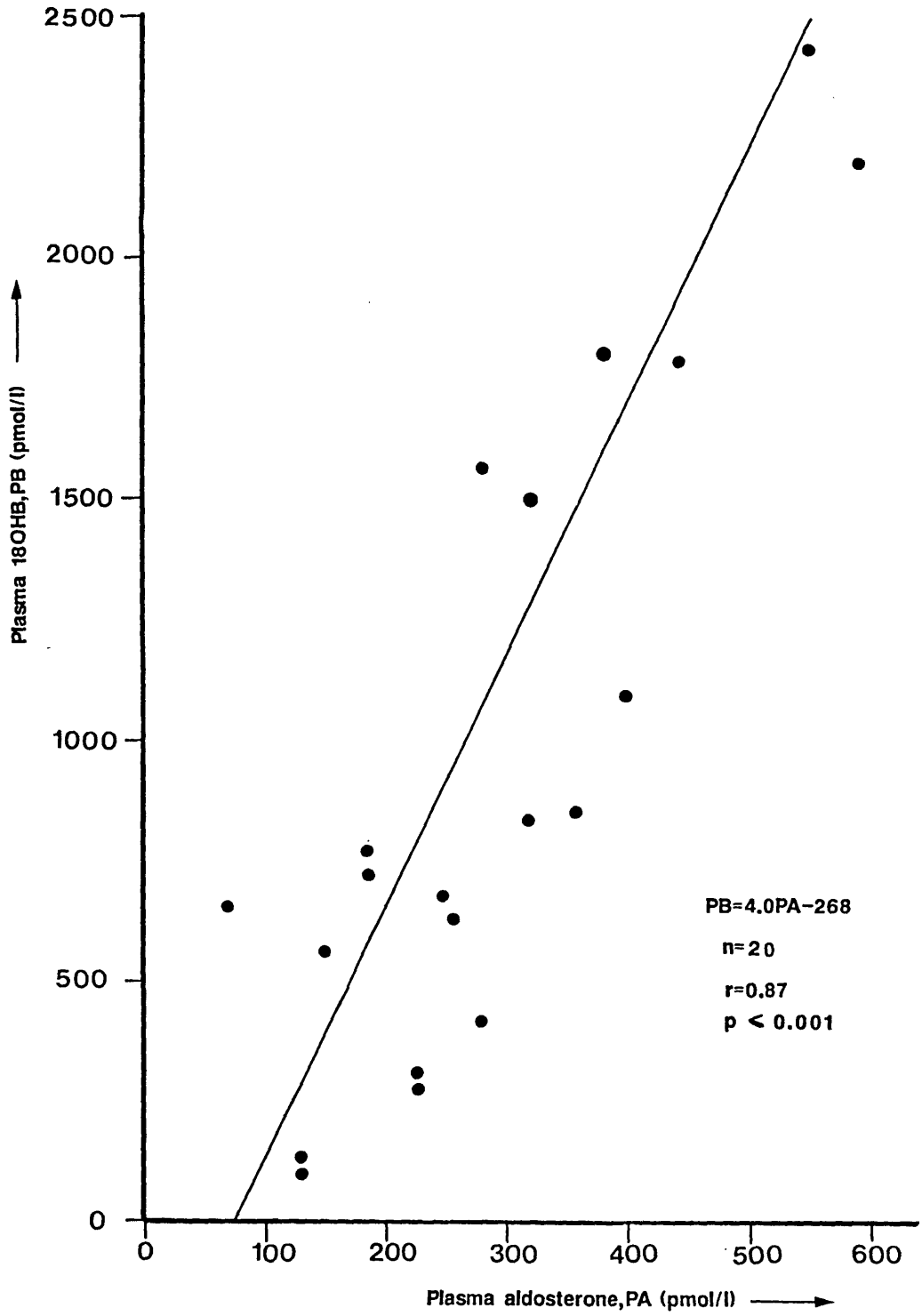


Figure 34b

The relationship between plasma 18OHB concentration and plasma aldosterone concentration in normal subjects going about their daily business.

biosynthesis. The postulation then was largely based upon the assumption that 18OHB has chemical structure favourable for the conversion to aldosterone, and observation that the secretory rates of 18OHB and aldosterone bore a constant ratio of 2 (Ulick et al., 1965). Later, plasma concentration of the steroids were frequently measured. It was found that the plasma concentration of the two steroids was approximately equal (Wilson et al., 1976; Mason et al., 1977; Biglieri et al., 1979) or greater than two and less than three (Martin et al., 1975; Witzgall et al., 1981) or greater than 3 (Kem et al., 1985). The latter authors reported that the ratios ranged from 2.6 to 103.7 with a mean $\pm$ SD (SEM) of 18.3 $\pm$ 32.8 (10.0). Such a variation of the ratio was consistent with my findings. This may be attributed to inter-subject variations or the blood samples were taken under a condition in which the subjects were not in an established supine posture (i.e. the subjects were not in a state of established recumbency during the period prior to blood sampling. Such a protocol was not followed in this study of mine because the experiment was designed to study the relationship between salivary 18OHB and plasma 18OHB concentrations under physiological condition). The fluctuation of the ratio could also be due to the constantly changing concentrations of the two steroids but not in an equal-proportion. The latter is a likely reason as indicated by the study of the diurnal profile of healthy subjects (pp173, 176 & 179).

Often the ratio of 18OHB:aldosterone has been described as an index of the 18-hydroxylase activity in particular the enzyme which converts 18OHB to aldosterone and has also been named as carboxymethyl oxidase Type I enzyme (Ulick et al., 1964b; Biglieri et al., 1979). There has been no rigorous proof of this and no such assumption has been made in the presentation of the results as a ratio in this thesis.

Previous investigations have reported a very wide range of normal values for plasma 18OHB (Appendix IIIa) and the values for plasma 18OHB in the subjects reported here are in agreement with some of these (Kem et al., 1985; Martin et al., 1975; Belkien et al., 1980; Witzgall et al., 1981; Watanabe et al., 1984; Sowers et al., 1983) but higher than others (Wilson et al., 1976; Biglieri et al., 1979a; Nagahama et al., 1983). There have been no previous reports on saliva 18OHB concentration.

It was found that there was no correlation of 18OHB or aldosterone with glucocorticoids in normal ambulatory subjects. However, it was found that salivary and plasma 18OHB was highly correlated with aldosterone and glucocorticoids concentrations in subjects stimulated with corticotrophin ($r > 0.90$). The reason was probably related to the narrow range of plasma glucocorticoids concentrations under basal conditions.

3.3. Diurnal variations of 18OHB, aldosterone and glucocorticoids in healthy subjects and patients with primary hyperaldosteronism and essential hypertension

INTRODUCTION

The diurnal fluctuation of salivary aldosterone (Few et al., 1987a) and circadian rhythm of plasma cortisol (Weitzman et al., 1966) have been documented. A 24h recumbent plasma 18OHB concentration of healthy subjects with half-hourly (Sowers et al., 1982) and 2 hourly (Sowers et al., 1983) sampling have been reported. However, the short term fluctuations studied by using a half-hourly sampling protocol of saliva in a physiological condition and situation such as in the usual habitat of the subjects have not been investigated. Hence, this was studied using saliva samples which were collected by the subjects while going about their daily business in their own homes. The patients were studied in the hospital and were ambulatory most of the time.

The first sample was collected after overnight recumbency while the subject remained supine. The short term fluctuations were studied using a frequent sampling protocol in order to exceed the secretory episodes of the steroid. This frequent sampling protocol was used so as to enable a derivation of a suitable protocol for collecting saliva with respect to the actual times

and the frequency of sampling for future physiological and clinical studies.

Aldosterone and glucocorticoids were measured in all these samples in order to study indirectly the influence of the renin-angiotensin system and ACTH respectively on 18OHB secretion.

3.3.1. Diurnal variations of 18OHB with respect to aldosterone and glucocorticoids in healthy subjects

RESULTS and DISCUSSION

(a) Frequency of sampling

The salivary 18OHB and aldosterone profiles of three subjects (two males and a female) who collected saliva every 30 minutes (subject CS and SW) and approximately every hour (subject JF) are shown in Figures 35a, 36a and 37 respectively. From the data of two of the subjects who sampled saliva every 30 minutes it was found that the sampling frequency (Table 17) did not affect the mean value and range of 18OHB, aldosterone and glucocorticoids measured. Therefore, less frequent sampling may be used for the estimation of the mean diurnal level but not if the temporal pattern of 18OHB is required.

The ratio of 18OHB : aldosterone was changing continuously

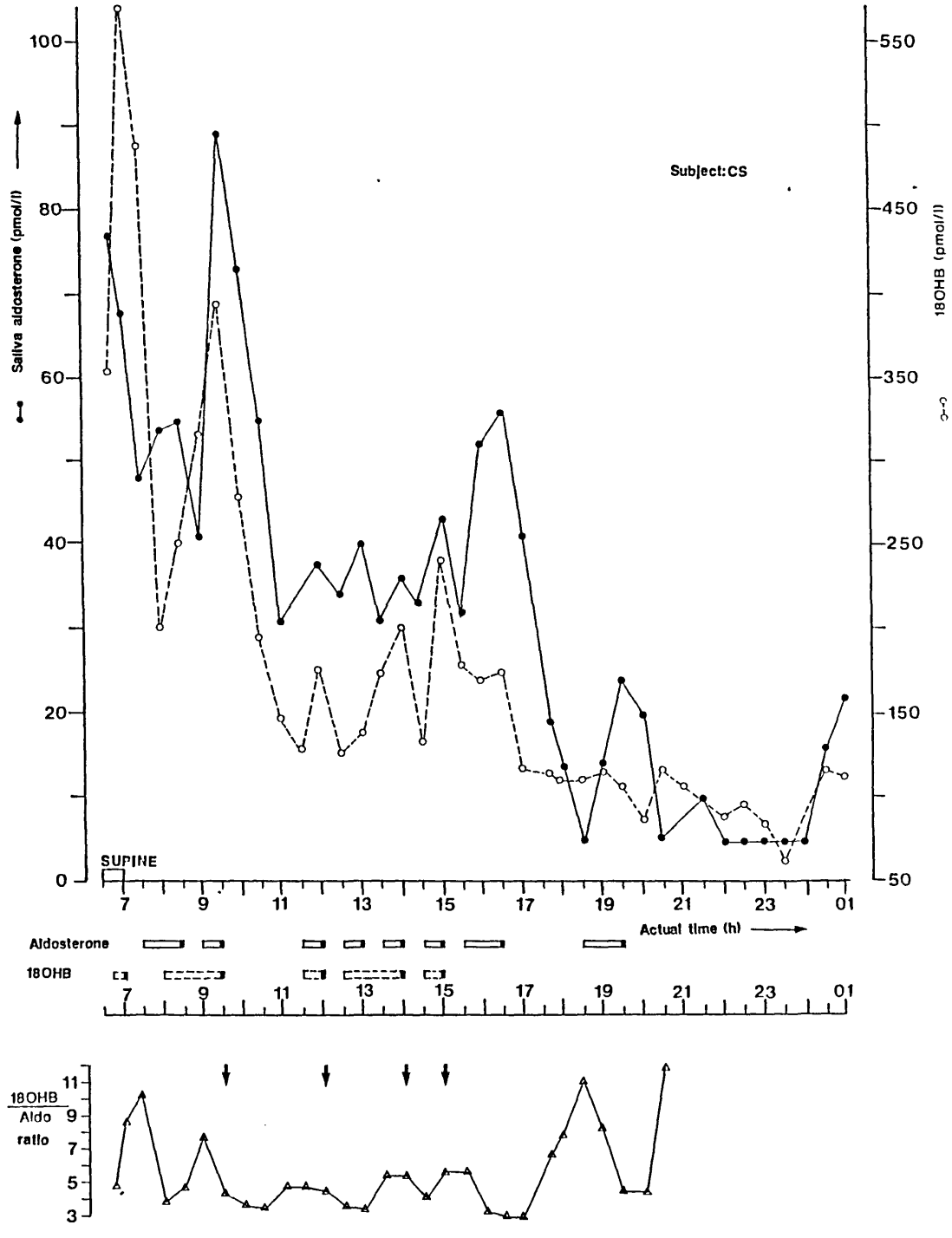

Subject: CS
Saliva aldosterone (pmol/l)
18OHB (pmol/l)
100
80
60
40
20
0
550
450
350
250
150
50
SUPINE
7
9
11
13
15
17
19
21
23
01
Actual time (h)
Aldosterone
18OHB
18OHB / Aldo ratio
11
9
7
5
3

Figure 35a

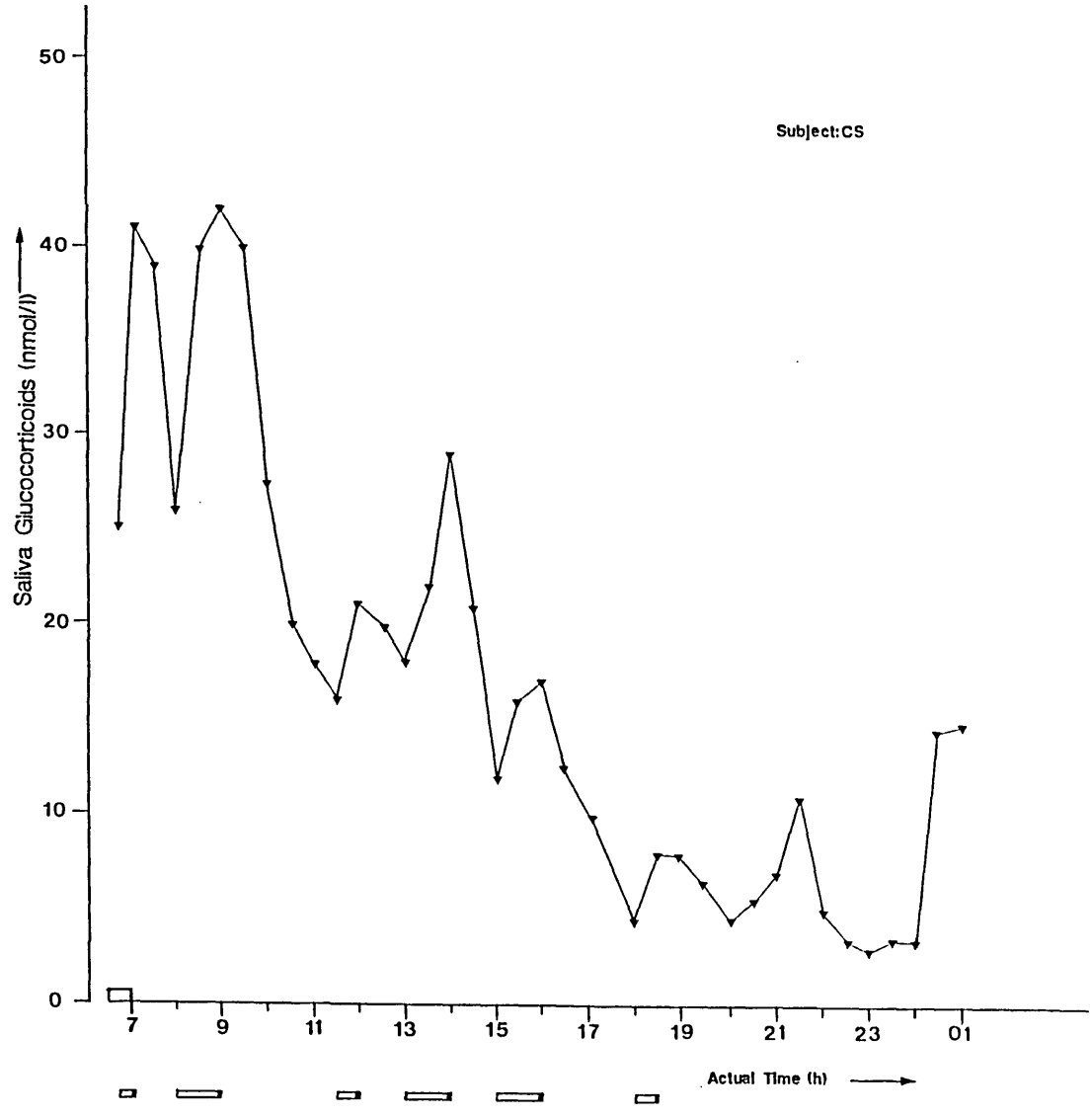
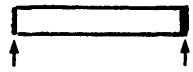
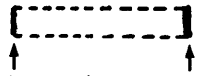

Subject:CS
Saliva Glucocorticoids (nmol/l)
50
40
30
20
10
0
7
9
11
13
15
17
19
21
23
01
Actual Time (h)

Figure 35b

Figure 35a & 35b

Saliva 18OHB, aldosterone and glucocorticoids concentrations of a healthy male subject aged 35 (Subject :CS). The saliva samples were collected every 30 minutes for a day while going about his normal daily activities. The first sample was collected after overnight recumbency while in the supine position. The peaks and troughs of the steroid concentrations were represented by  for aldosterone and  for 18OHB in Figure 35a. An arrow (↓)marked the positions of the coincident peaks of 18OHB and aldosterone. B, L and D indicated breakfast , lunch and dinner respectively. The range and mean 18OHB and aldosterone concentrations were as follows:

	Concentration (pmol/l)		
	Range	mean ± SD	CV %
18OHB	64 - 571	178 ± 113	63.5
Aldosterone	5 - 89	33 ± 23	69.7
18OHB:Aldosterone ratio	3 - 11	5 ± 2	40.0
Glucocorticoids (nmol/l)	3 - 49	17 ± 12	70.0

The saliva 18OHB (SB) concentration was correlated with the saliva aldosterone (SA) concentration by the equation:-

SB = 3.8 SA + 52.8; r= 0.76, $p < 0.001$ (n=38)

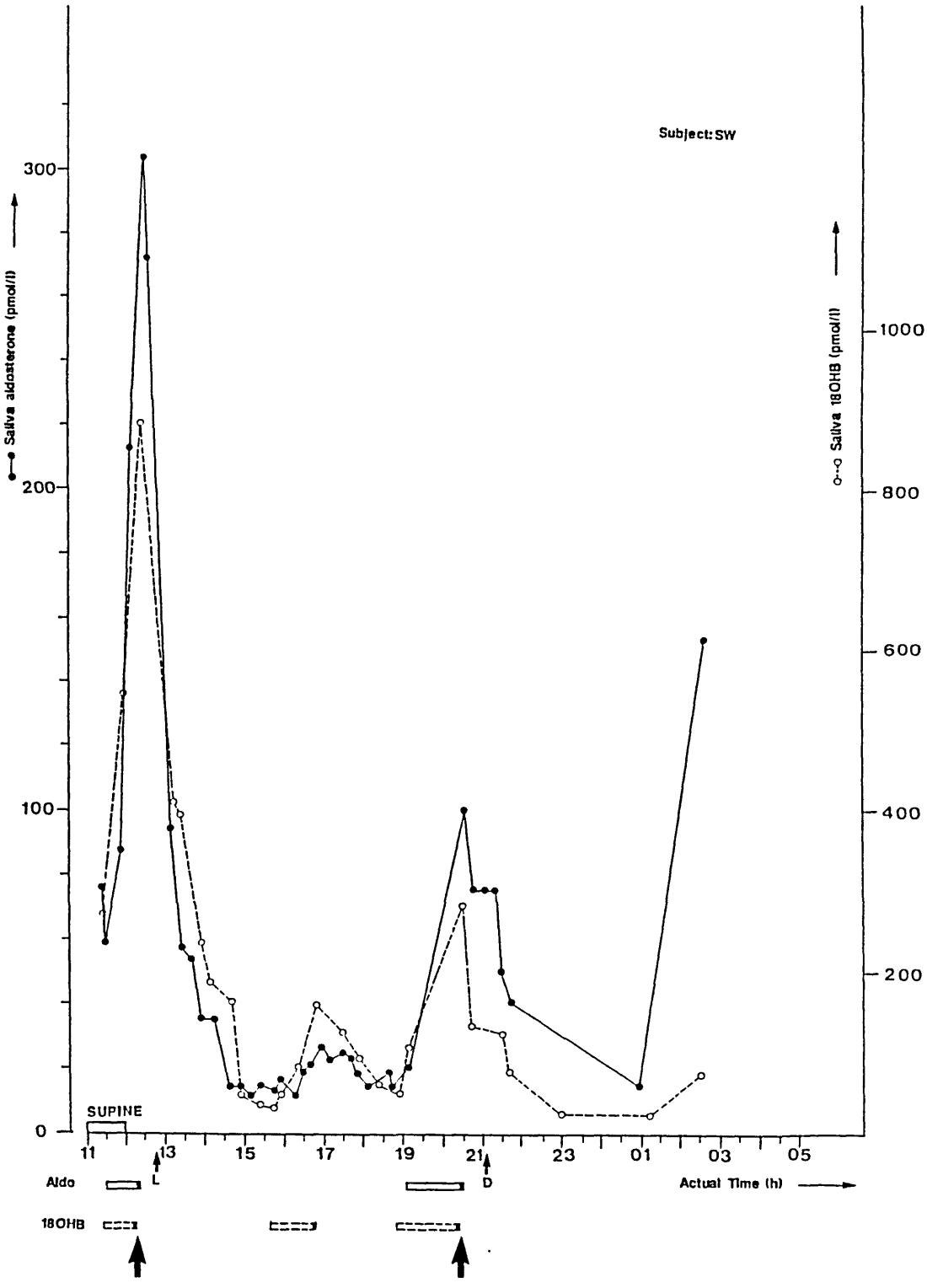

Subject: SW
Saliva aldosterone (pmol/l)
Saliva 18OHB (pmol/l)
300
200
100
0
1000
800
600
400
200
SUPINE
11
13
15
17
19
21
23
01
03
05
L
D
Aldo
18OHB
Actual Time (h)

Figure 36a

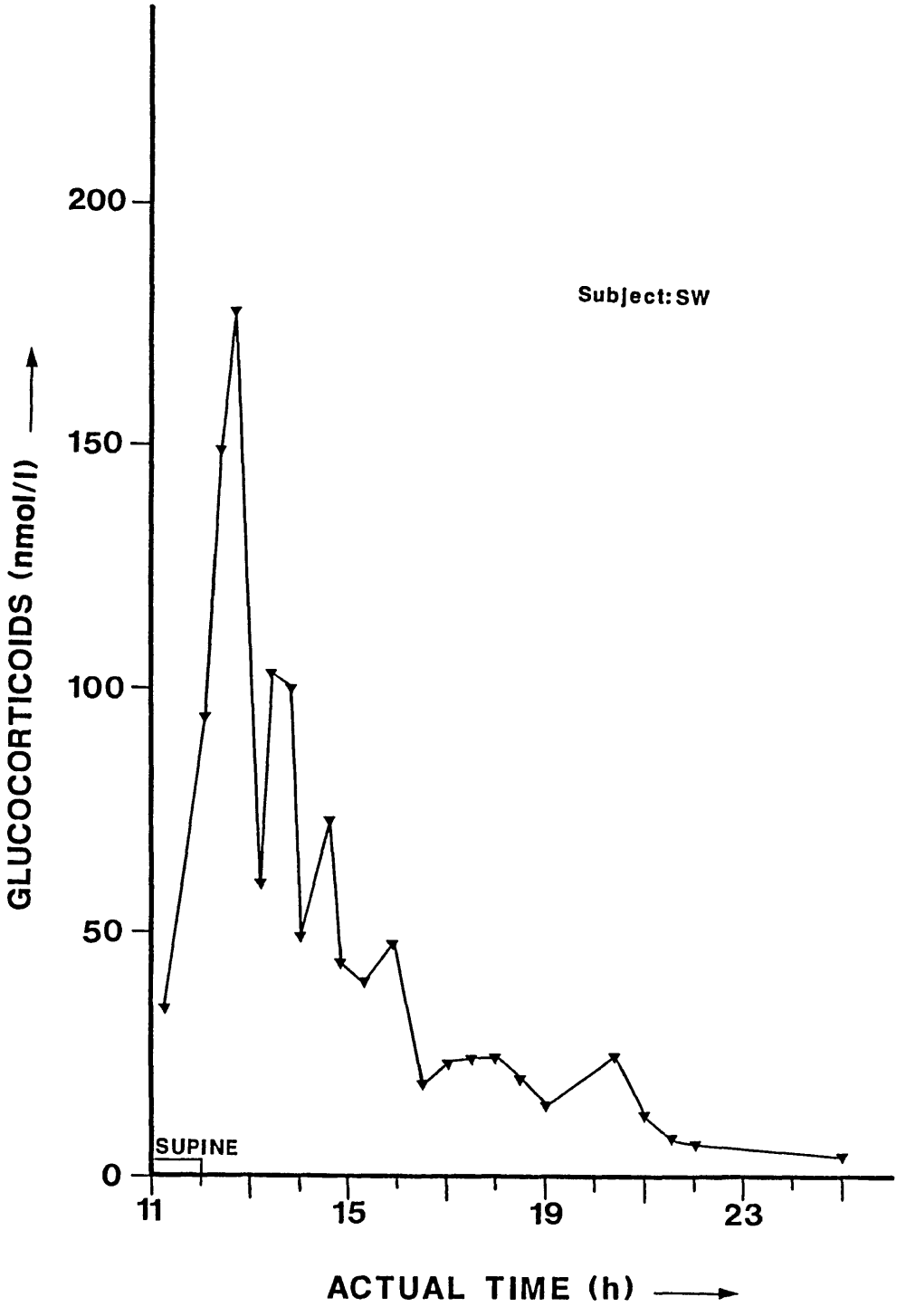

Subject: SW
200
150
100
50
0
GLUCOCORTICOIDS (nmol/l)
SUPINE
11
15
19
23
ACTUAL TIME (h)

Figure 36b

Figure 36a & 36b

Saliva 18OHB, aldosterone and glucocorticoids concentrations of a female subject aged 22 and of Chinese origin (Subject:SW). Saliva samples were collected every 15 minutes for 24 hours while going about her normal daily activities. The first sample was collected after overnight recumbency while still in the supine position. Her waking up time was 1100h. The peaks and troughs of the steroids were denoted by horizontal bars similar to that shown in the Figure 35a. The 18OHB and aldosterone peaks coincided at 1220h, 1650h and 2025h, and the ratio of 18OHB:aldosterone ratio at these times were 2.9, 5.9 and 2.8 respectively. The range and mean 18OHB and aldosterone concentration are as follows:-

	Concentration (pmol/l)		
	Range	Mean ± SD	CV%
18OHB	22 - 885	213 ± 230	108
Aldosterone	11 - 303	56 ± 74	132
18OHB/Aldosterone ratio	2 - 11	5 ± 2	40

The line of linear regression of saliva 18OHB (SB) and saliva aldosterone (SA) for the day was:-

SB = 2.9 SA + 48.9; r= 0.93, $p < 0.001$ (n= 26)

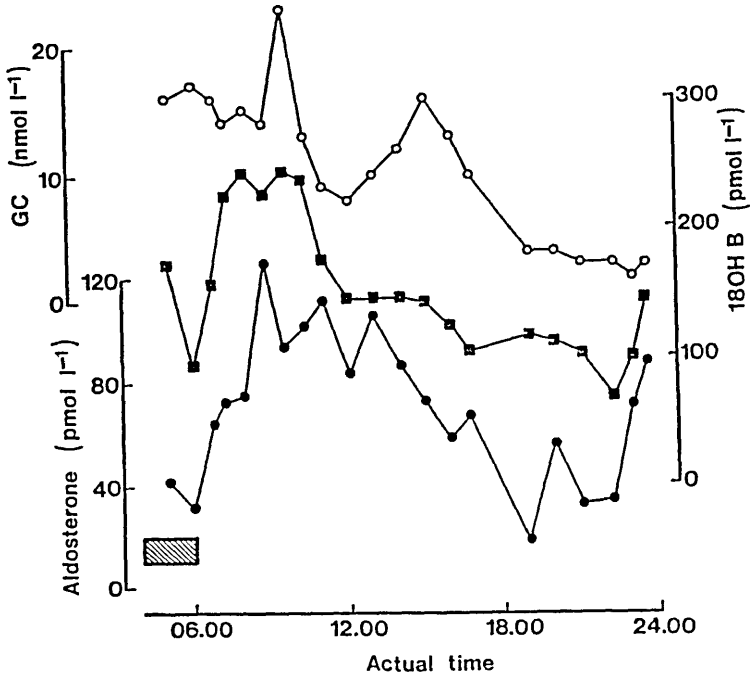


Figure 37

Diurnal fluctuation of the saliva concentrations of 18OHB, aldosterone and glucocorticoids in subject JF.

Squares = 18OHB (SB); Closed circles = aldosterone (SA); open circles = glucocorticoids (GC).

	Range	mean ± SD	CV%
18OHB (pmol/l)	67 - 241	152 ± 53	35
Aldosterone (pmol/l)	18 - 126	72 ± 29	40
18OHB:Aldosterone ratio	1.5 - 6.3	2.4 ± 1.2	48
Glucocorticoids (nmol/l)	3 - 23	11 ± 6	54

SB = 1.1 SA + 70; r=0.614, $p < 0.005$ (n=21)

Frequency of sampling	n	Concentration (pmol/l) Range	mean ± SD
Subject: CS			
Every 30 min	36	64 - 571	175 ± 112
60	18	75 - 571	180 ± 119
120	9	85 - 571	205 ± 115
180	6	87 - 571	227 ± 180
240	5	85 - 571	232 ± 198
Subject: SW			
Every 30 min	20	22 - 885	215 ± 214
60	10	62 - 885	225 ± 259
120	6	35 - 392	190 ± 150
240	3	35 - 368	168 ± 176

Table 17

Effect of sampling frequency upon the range and mean 18OHB concentration in saliva collected in a day. Subject CS (male) and subject SW (female) were two healthy subjects. Comparison of hourly and four hourly sampling by student t-test showed that they were not significantly different. CS: $t = 0.6469$, p is less than 0.1; SW: $t = 0.52$, p less than 0.1).

with time. This is probably related to the secretory phase of the steroids not being synchronous. During the peak secretion of both steroids, the ratio appeared to be constant as denoted by the arrows in Figure 35a , Figure 36a and Figure 37. The fluctuations of the ratio at other times therefore probably accounts for the wide ratio range in the normal ambulatory subjects discussed in section 3.2.3. (pp 164 - 170). Therefore, multiple sampling is essential to accurately determine the ratio and to avoid artifactually high values due to the level of aldosterone at its physiologically low level. Hence, in clinical studies it might be necessary to collect multiple samples, for example every 30 minutes for a period of time chosen for the study (probably 1 to 2h) instead of just one plasma sample. Unlike studies for the assessment of circadian rhythm or diurnal fluctuations, it might not be economical or of clinical benefits to measure samples collected throughout the whole day.

(b) Diurnal fluctuations

It has not been shown that changes of salivary 18OHB concentrations reflect changes in biosynthetic activity. Therefore, it has to be assumed that a peak (at least two successive increases in concentration) corresponds to a secretory episode. A quiescent phase occurs after a secretory episode (consistently two successive falls of 18OHB levels).

The secretion of 18OHB, like those of aldosterone and glucocorticoids, was episodic (Figure 35a to 37). The number of episodes varied from one subject to the other. There were a few coincident 18OHB and aldosterone peaks, indicated by the rectangular boxes shown in the figures. The relationship between 18OHB and aldosterone in these subjects was significant with lines of linear regression shown in the respective figures. At any point in time, the concentration of 18OHB was higher than that of aldosterone. One of the coincident 18OHB and aldosterone peaks was at the time when the subjects had assumed an upright posture soon after overnight recumbency. This was also the time in which the levels of both 18OHB and aldosterone were higher than the rest of the samples collected after that time. Similarly, the level of glucocorticoids was highest in the morning. Although glucocorticoids levels increased after a meal, it was not apparent for the other two steroids.

It must be mentioned that the salivary glucocorticoids concentration in subject SW was abnormally high. This was probably related to the contamination of the saliva sample with hydrocortisone cream, which the subject was applying onto the face at the time of the study. This was only realised after the study.

Glucocorticoid concentration was determined rather than cortisol because the antibody used for the assay has a strong cross reactivity with cortisone. This would not affect the

interpretation of the results because there was a good correlation between true cortisol and glucucorticoid levels (Few et al., 1987a).

The inter-subject correlation of saliva 18OHB or aldosterone with glucocorticoids in normal ambulatory subjects was 0.22 and 0.26 respectively (Table 16a, pp 165). However, the intra-subject correlation was considerably higher considering only the data of subject CS and JF using concentrations of the steroids in all the samples collected throughout the day (Table 18). The discrepancy between the inter-subject and intra-subject correlation is probably due to the biological variation of an individual. The significant parallelism of 18OHB, aldosterone and glucocorticoids in subject CS and JF might therefore suggest the influence of ACTH on the secretion of these 3 steroids.

The concentration of 18OHB was highest in the morning and lowest before bed time in addition to be significantly correlated with time (Table 19). This probably implies that the secretion of 18OHB could be displaying a circadian rhythm (Sowers et al., 1983).

The correlation of 18OHB with glucocorticoids (r=0.83) was found to be higher than aldosterone (r=0.76) using the data of subject CS. Similarly it was found subject JF (r=0.45 versus r=0.26). It is therefore probable that the secretion of 18OHB is under the influence of ACTH (Tuck et al., 1981; Wilson et al.,

Table 18 Correlation coefficient matrix of salivary 18OHB, aldosterone and glucocorticoids in two healthy subjects:

	glucocorticoids		
	r	n	p <
Subject : CS			
18OHB	0.83	37	0.001
Aldosterone	0.77	37	0.001
Subject : JF			
18OHB	0.45	21	0.05
Aldosterone	0.26	21	N.S

Table 19

Regression of corticosteroids with time (t) in healthy subjects:

Subjects	Equations	r	n	p <
18OHB:				
CS	Y = - 16.5t + 323	0.754	37	0.001
SW	Y = - 44.0t + 448	0.665	26	0.001
JF	Y = - 6.0t + 204	0.659	21	0.001
Aldosterone:				
CS	Y = - 3.3t + 63	0.814	37	0.001
SW	Y = - 9.8t + 108	0.455	26	0.02
JF	Y = - 0.8t + 73	0.143	21	N.S
Glucocorticoids:				
CS	Y = - 1.8t + 34	0.854	37	0.001
JF	Y = - 0.9t + 18	0.840	21	0.001

1976) and AII (Mason et al., 1976 & 1977: Belkien et al., 1980; Fraser et al., 1981).

The mean salivary aldosterone levels of subject CS were within the limits of normal (33 ± 23 pmol/l; range 5-89 pmol/l). His 18OHB levels were consistently higher than his aldosterone levels. His salivary 18OHB:aldosterone ratio varied between 3.0 to 11.0. The high ratios were sometimes due to the level of aldosterone being at its lowest (e.g. between 1830h and 2030h). There were only 4 coincident aldosterone and 18OHB peaks in this subject. During such time, the ratio of 18OHB:aldosterone was constant. It was not possible to determine from such a study the exact time at which the two steroids were being secreted, whether one precedes the other or simultaneously. The variation of the ratio could also be due to the kinetics of 18OHB being slightly different from that of aldosterone where it's disappearance into the extravascular compartment was more rapid than aldosterone. The constantly changing ratios of the two steroids means that a single point plasma sample used for the determination of 18OHB:aldosterone ratio may be misleading. Moreover, the salivary 18OHB concentration could change within half an hour of sampling especially in the morning after waking up.

The concentration of 18OHB or aldosterone reached its peak concentration 30 minutes after assuming an upright posture after overnight recumbency, the concentration after 2h or 4h of

ambulation did not result in an increase level of the steroids. The same results were confirmed in a two point study where the subjects collected saliva after overnight recumbency while remaining recumbent followed by 4h ambulation (see pp 211-218). It is therefore concluded that a rise in 18OHB or aldosterone concentration in healthy subjects (Biglieri et al., 1979) on assuming an upright posture after overnight recumbency (Appendix IIIb) could have been missed by sampling 4h after overnight recumbency in my study.

Despite subject SW being an 'atypical' subject and her waking up time was different from subject CS, her 18OHB levels displayed a similar diurnal profile to that of subject CS in term of relative clock time. The highest 18OHB level occurred soon after waking up, lowest before bed time, the low afternoon level occurred 4 hours post waking up compared to 4.5 hours for subject CS.

3.3.2. Diurnal variations of 18OHB, aldosterone and glucocorticoids in patients with primary hyperaldosteronism and essential hypertension

MEDICAL HISTORY

Preliminary diagnosis of the patients with primary hyperaldosteronism, was made by the Medical Unit at St. Mary's hospital Medical School and was based on medical history, examination including blood pressure (BP) measurement and biochemical measurements of a single blood sample taken when first seen. The patients remained supine for 15 minutes before blood was sampled and were on unrestricted salt intake. Primary hyperaldosteronism was indicated if the patients had elevated blood pressure, hypokalaemia, low plasma renin activity and elevated plasma aldosterone concentration. The data of the patients studied were as follows:

Table 20 Medical histories of patients with primary hyperaldosteronism and essential hypertension:

Subject code *	Sex 1-male 2-female	Age yrs	BP mm Hg	PA (pmol/l)	PRA (pg/ml/h)
M	1	54	168/93	270	72
R	2	35	193/95	577	600
W	2	49	205/109	352	226
F	1	62	176/99	197	2507
Normal Range +				150-500	500-2500

* Patients M and R have bilateral adrenal hyperplasia treated with anti-hypertensive drugs; patient W had aldosterone-producing adenoma, which was removed subsequently; patient F was diagnosed to have essential hypertension. + Values were obtained by the Medical Unit, St. Mary's Hospital Medical School. BP, blood pressure; PA, plasma aldosterone concentration; PRA, plasma renin activity.

RESULTS

Diurnal fluctuations of 18OHB and aldosterone in 3 patients with primary hyperaldosteronism were investigated using multiple saliva samples collected during the day (Figure 38a to Figure 40a). The concentration of 18OHB was highest during early hours of the

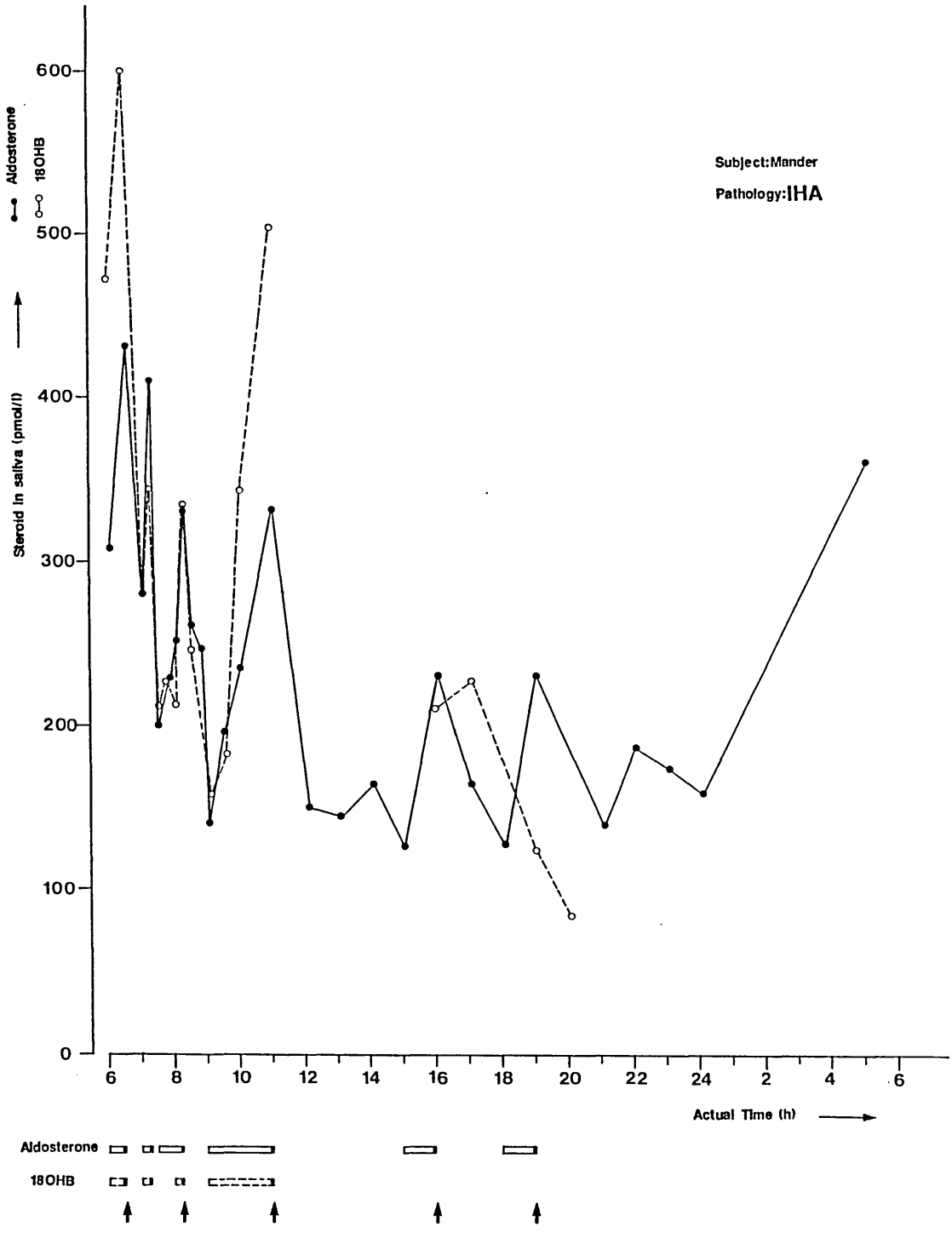


Figure 38a

Diurnal variations of salivary 18OHB and aldosterone levels of a male patient with primary hyperaldosteronism (bilateral adrenal hyperplasia, so called idiopathic hyperaldosteronism, IHA)(Subject M). Rectangular blocks representing peaks and troughs have been described in the legend of Figure 38a. The coincident peaks are denoted by the arrows.

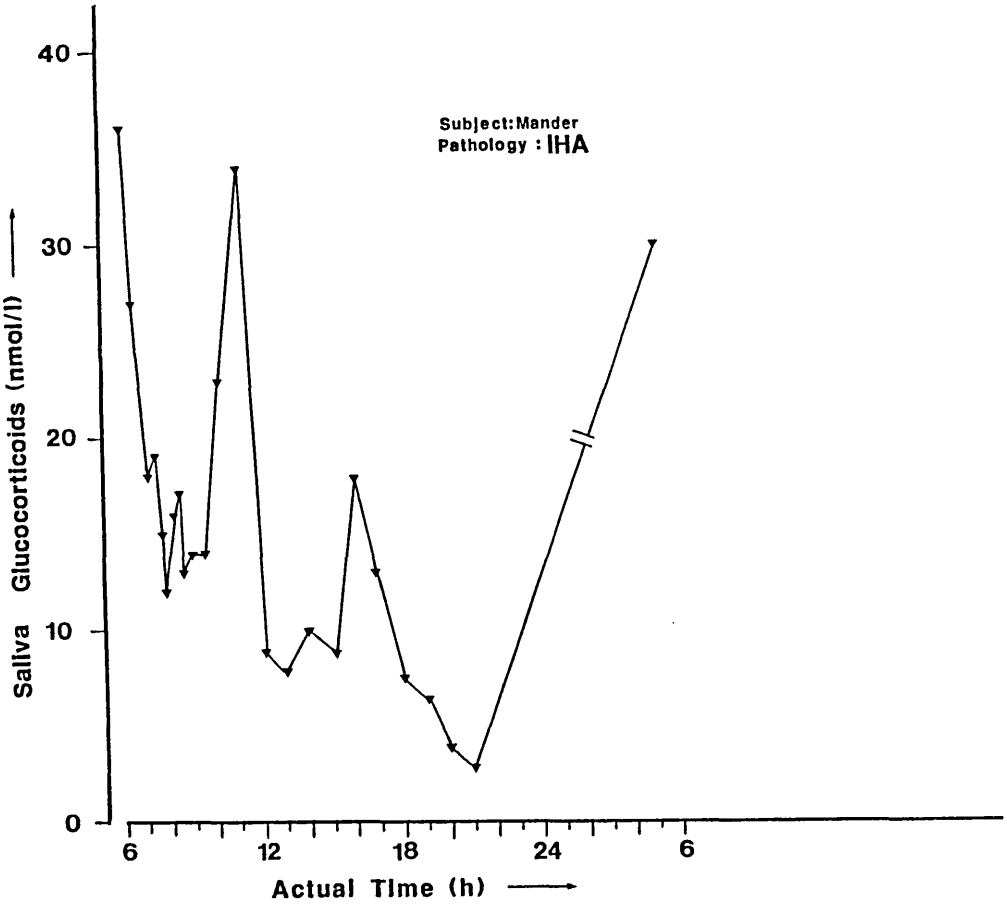


Figure 38b

Diurnal variations of salivary glucocorticoids of the male patient with primary hyperaldosteronism (bilateral adrenal hyperplasia, so called idiopathic hyperaldosteronism, IHA)(Subject M).

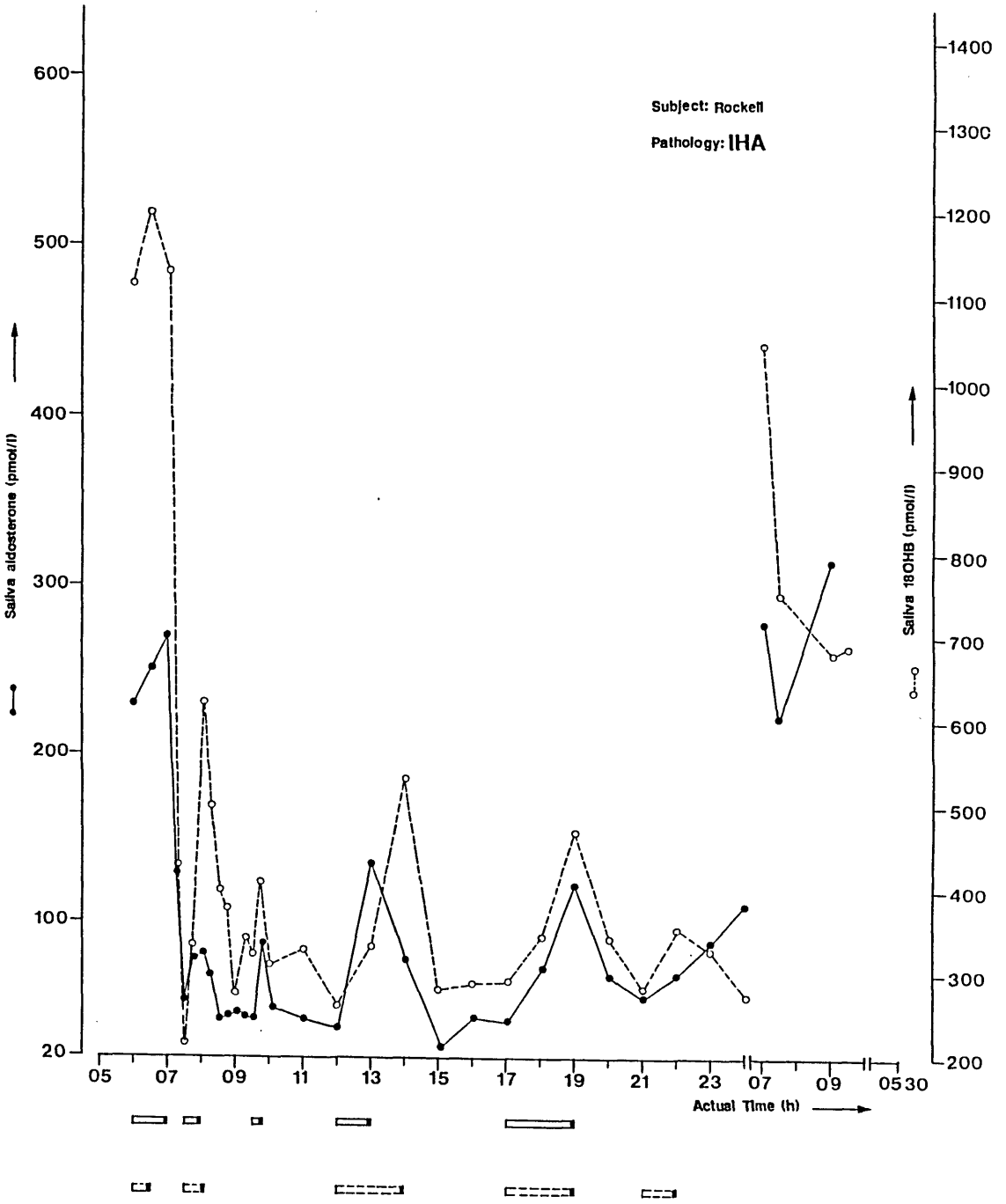


Figure 39a

Diurnal variations of salivary 18OHB and aldosterone levels of a male patient with primary hyperaldosteronism (bilateral adrenal hyperplasia, so called idiopathic hyperaldosteronism, IHA)(Subject R). Rectangular blocks representing peaks and troughs have been described in the legend of Figure 38a. The coincident peaks are denoted by the arrows.

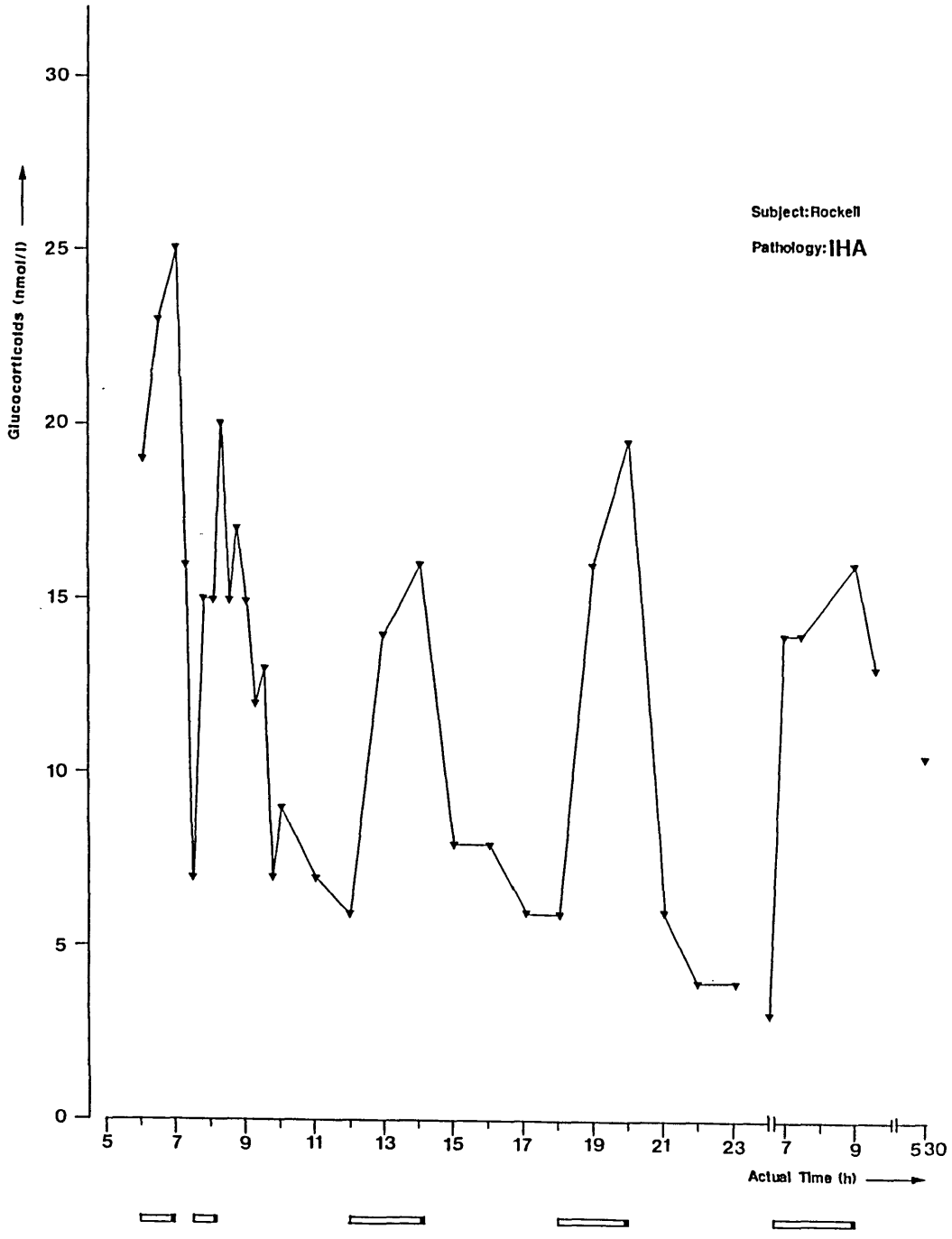


Figure 39b

Diurnal variations of salivary glucocorticoids of the male patient with primary hyperaldosteronism (bilateral adrenal hyperplasia, so called idiopathic hyperaldosteronism, IHA)(Subject R).

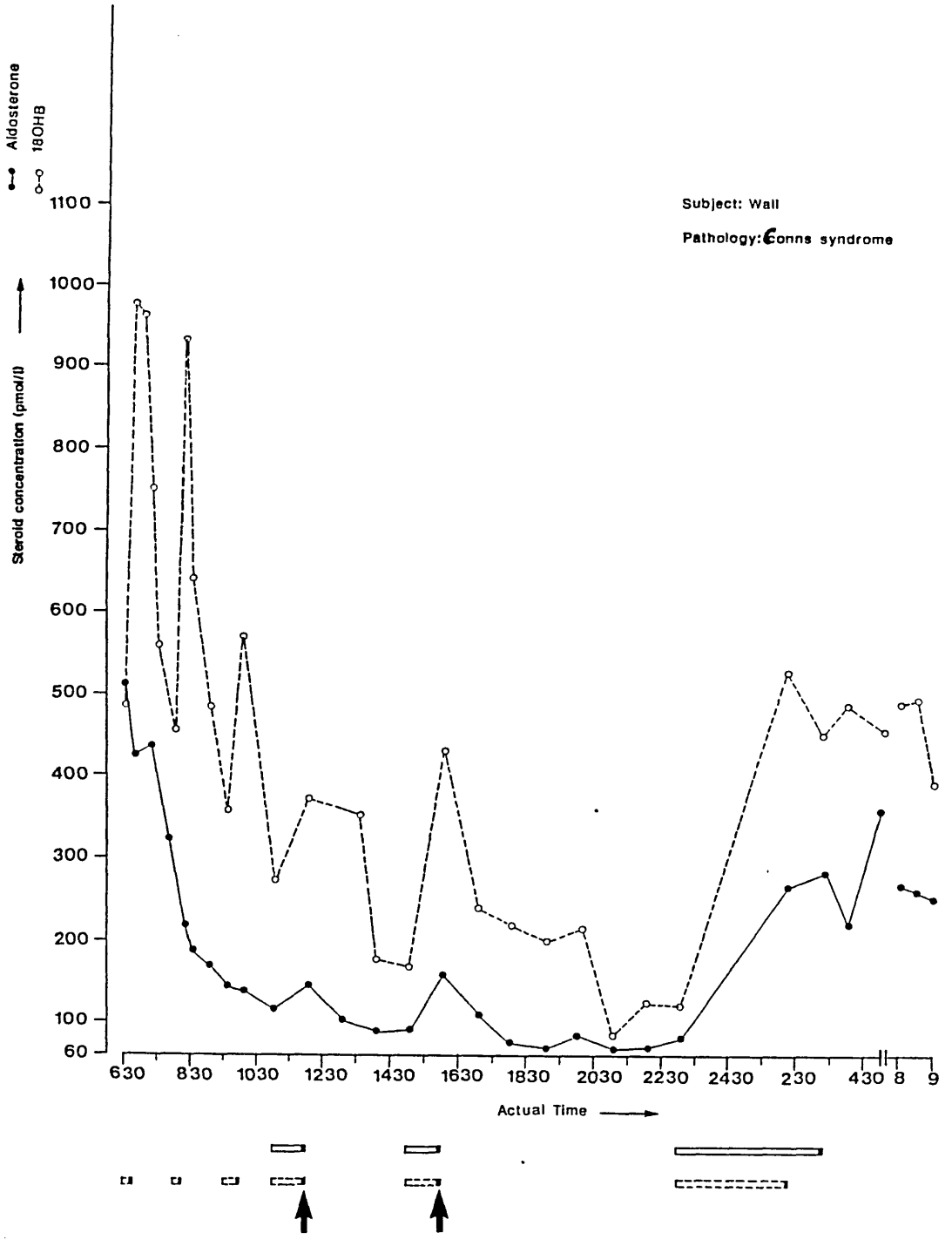


Figure 40a

Diurnal variations of salivary 18OHB and aldosterone levels of a male patient with aldosterone-producing adenoma (APA, so called Conn's syndrome)(Subject W). Rectangular blocks representing peaks and troughs have been described in the legend of Figure 38a. The coincident peaks are denoted by the arrows.

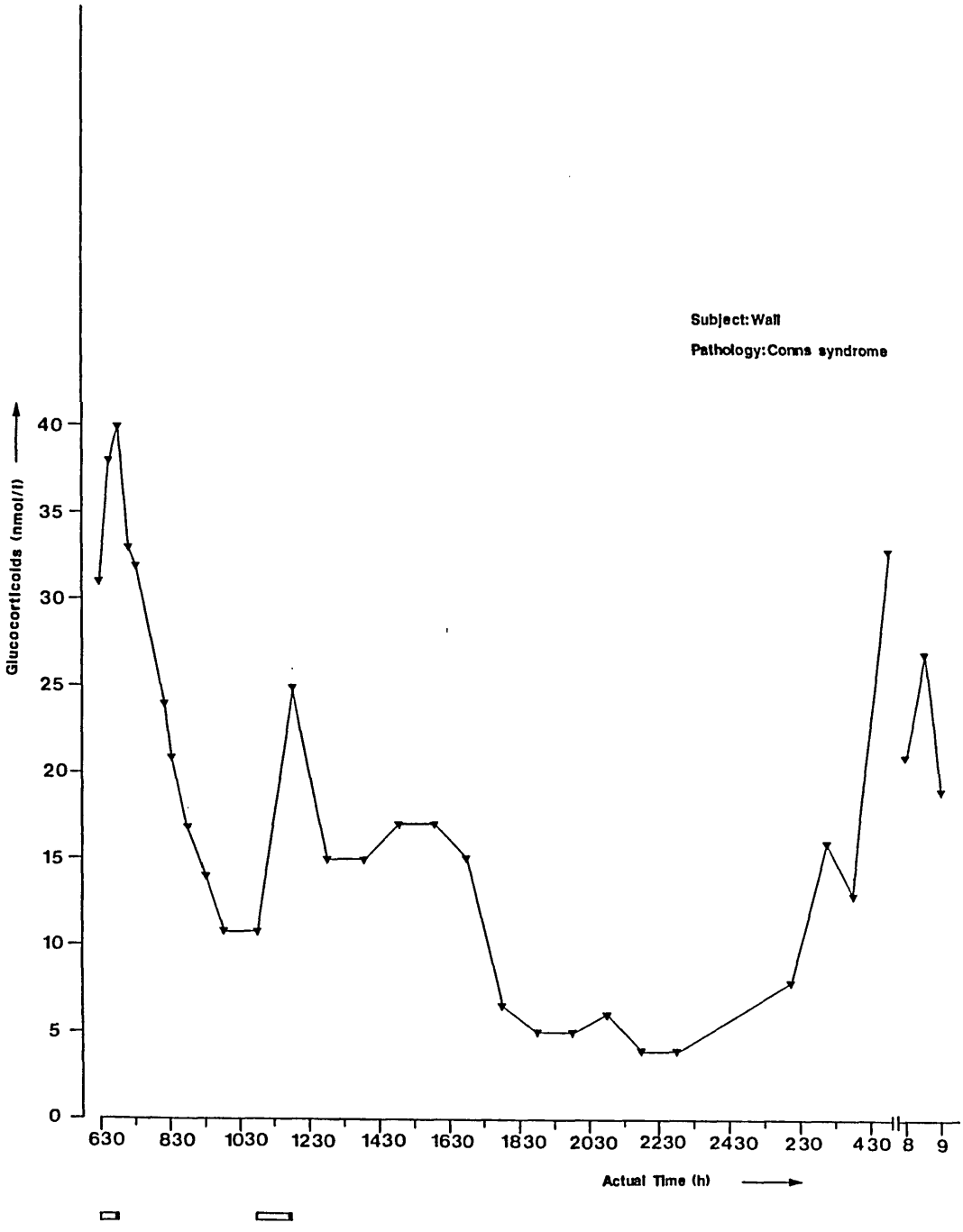


Figure 40b

Diurnal variations of salivary glucocorticoids of the male patient with aldosterone-producing adenoma (APA, so called Conn's syndrome)(Subject W).

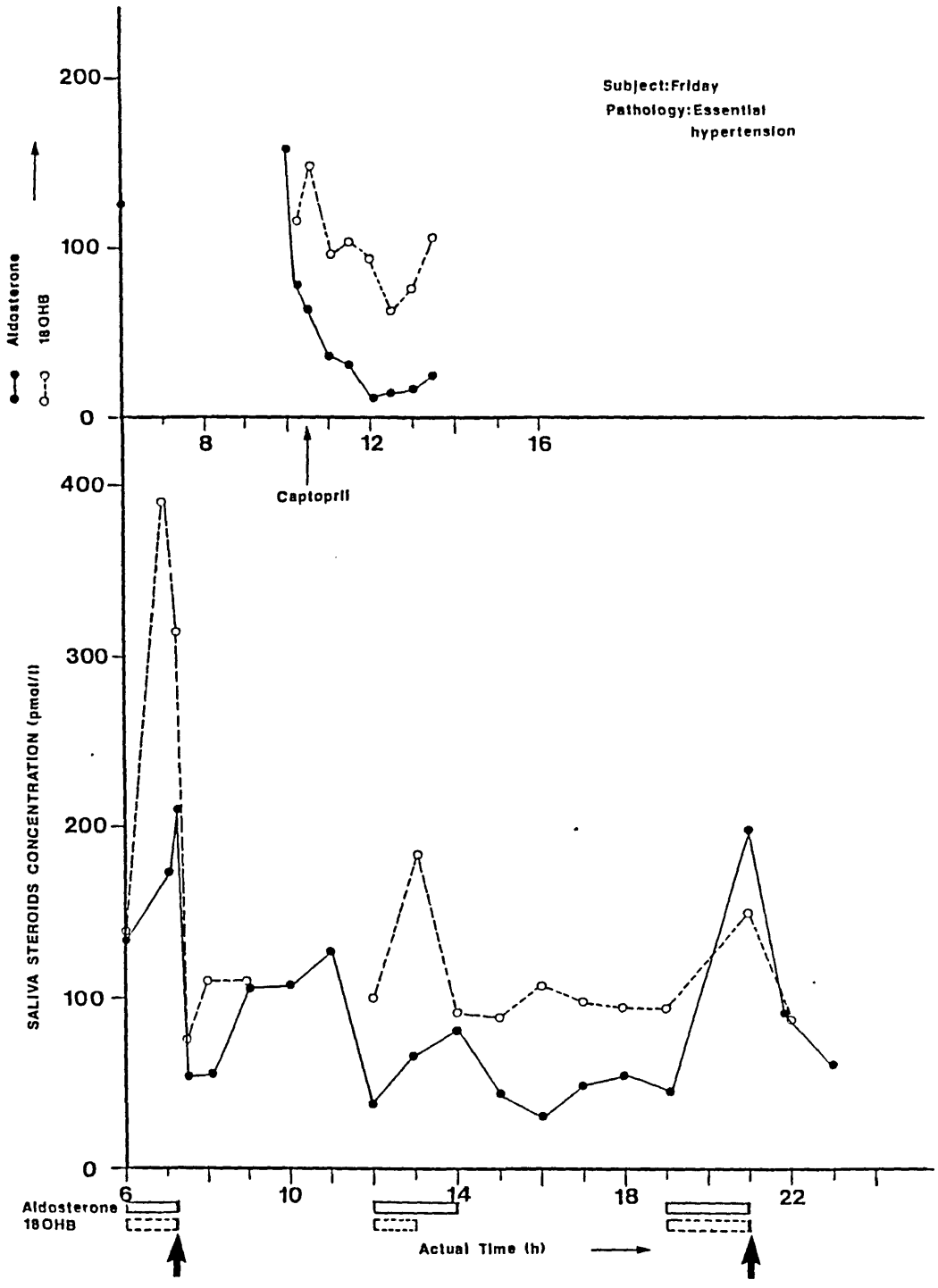


Figure 41a

Diurnal variations of salivary 18OHB and aldosterone levels of a male patient with essential hypertension (Subject F)

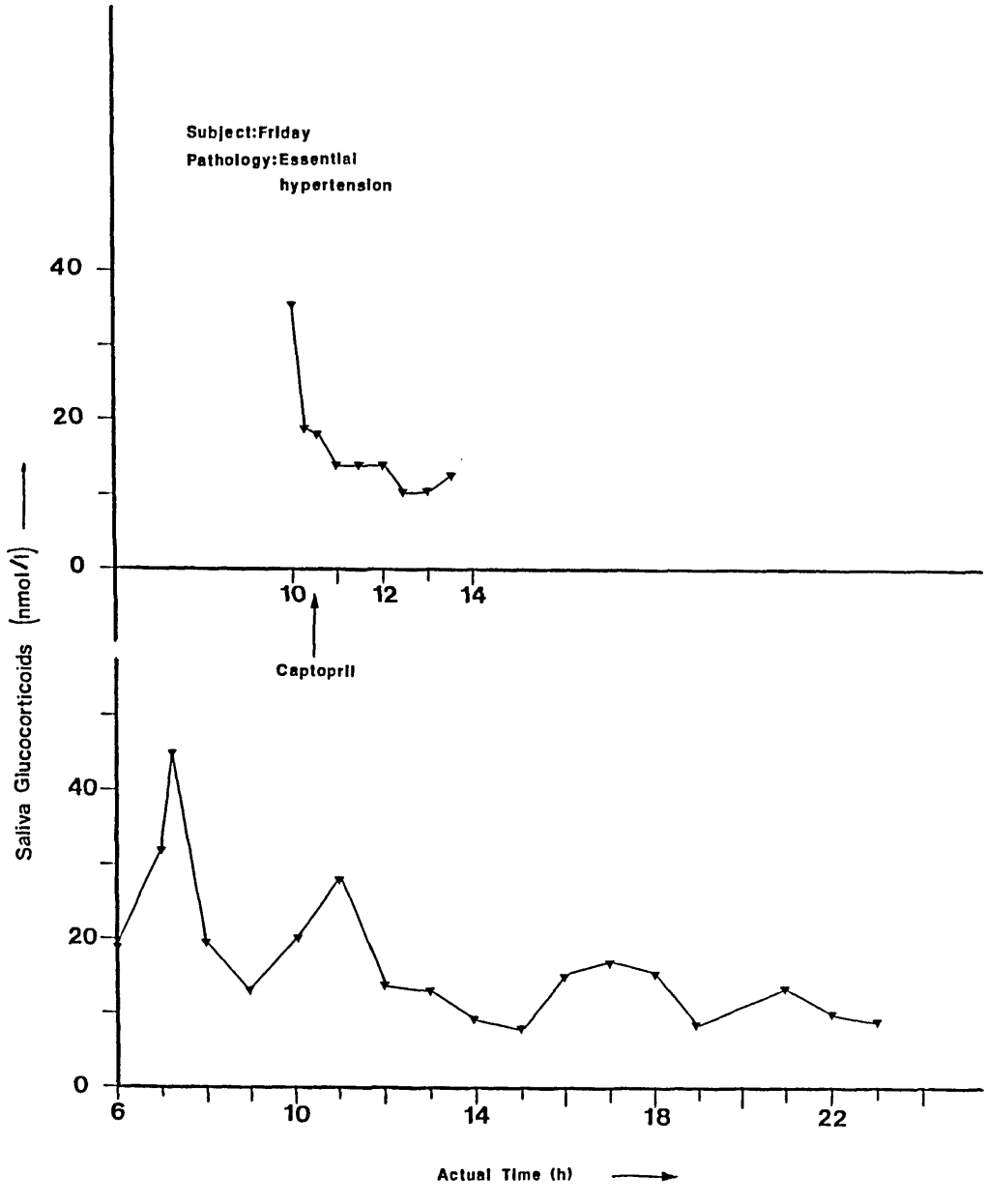


Figure 41b

Diurnal variations of salivary glucocorticoids of the male patient with essential hypertension (Subject F).

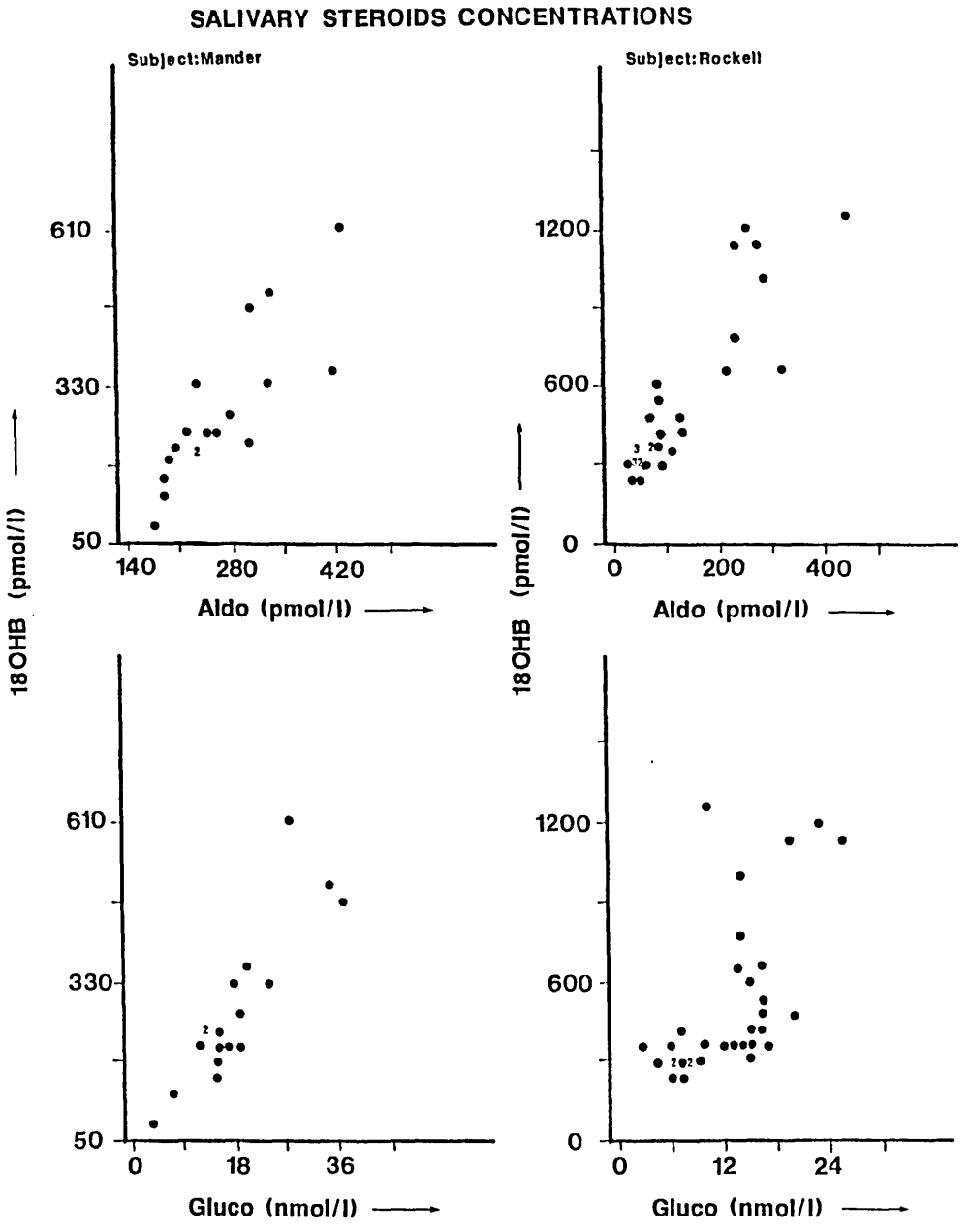

SALIVARY STEROIDS CONCENTRATIONS
Subject:Mander
Subject:Rockell
18OHB (pmol/l)
Aldo (pmol/l)
Gluco (nmol/l)
610
330
50
140
280
420
1200
600
0
200
400
18
36
12
24

Figure 42a

Figure 42a

The salivary 18OHB, aldosterone and glucocorticoids concentrations of patients (M & R) with primary hyperaldosteronism are plotted as scattergrams. The correlation coefficients of the steroids are shown as follows:-

	Aldosterone	Glucocorticoids
Subject:Mander		
Glucocorticoids	0.633	
18OHB	0.829	0.887
Subject:Rockell		
Glucocorticoids	0.397	
18OHB	0.894	0.591

The line of linear regression between salivary 18OHB (SB) and aldosterone (SA) concentrations in these subjects are:

Subject M:

SB = 1.5 SA - 114; r= 0.82, $p < 0.001$ (n=18)

Subject R:

SB = 2.7 SA + 195; r= 0.89, $p < 0.001$ (n=33)

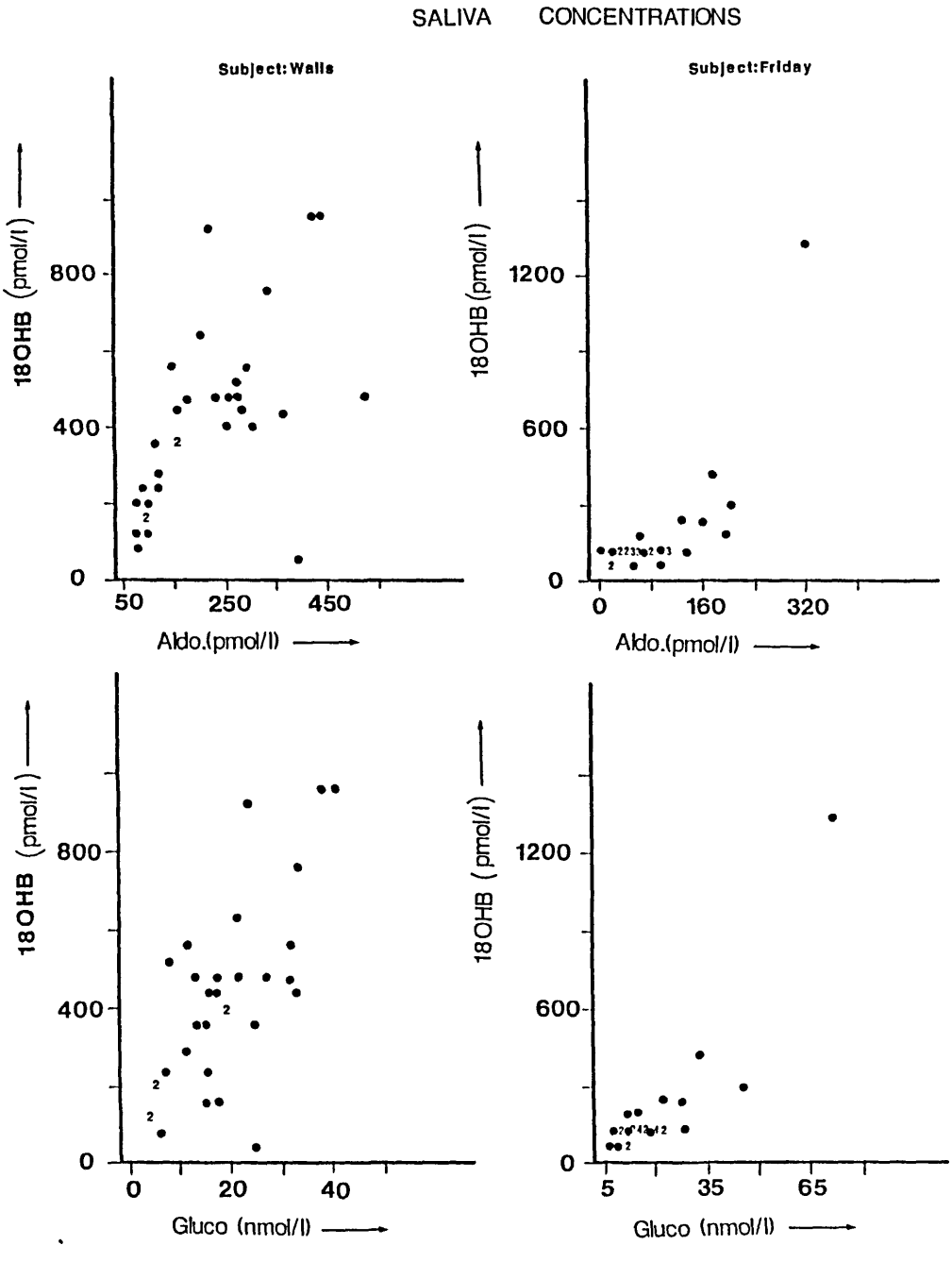

SALIVA CONCENTRATIONS
Subject: Wells
Subject: Friday
18OHB (pmol/l)
Aldo.(pmol/l)
Gluco (nmol/l)
0
400
800
1200
600
50
250
450
160
320
20
40
5
35
65

Figure 42b

Figure 42b

The saliva concentrations of 18OHB, aldosterone and glucocorticoids of the patient with Conn's syndrome (Subject W) and the patient with essential hypertension (Subject F) were plotted as scattergrams. The correlation coefficients of saliva 18OHB, aldosterone and glucocorticoids of these patients are as follows:-

	Aldosterone	Glucocorticoids
Subject:Wall		
Glucocorticoids	0.828	
18OHB	0.592	0.686
Subject:Friday		
Glucocorticoids	0.809	
18OHB	0.790	0.903

The line of linear regression between 18OHB (SB) and aldosterone (SA) in these subjects are:-

Subject:Wall

SB = 1.2 SA + 171; r= 0.58, $p < 0.001$ (n=32)

Subject:Friday

SB = 2.6 SA - 52; r= 0.78, $p < 0.001$ (n=31)

Table 21a

Regression of corticosteroids with time in patients with primary hyperaldosteronism (M,R &W) and essential hypertension (F):

Subjects	Equations	r	n	p <
18OHB:				
M	Y = - 15.8t + 349	0.521	18	0.05
R	Y = - 19.0t + 571	0.409	29	0.05
W	Y = - 40.7t + 709	0.819	23	0.001
F	Y = - 7.5t + 195	0.448	16	N.S
Aldosterone:				
M	Y = - 0.9t + 304	0.543	18	0.02
R	Y = - 2.4t + 99	0.202	29	N.S
W	Y = - 17.6t + 300	0.766	23	0.001
F	Y = - 2.7t + 110	0.244	16	N.S
Glucocorticoids:				
M	Y = - 1.0t + 22	0.515	18	0.05
R	Y = - 0.7t + 17	0.684	29	0.001
W	Y = - 1.7t + 30	0.847	23	0.001
F	Y = - 0.9t + 22	0.487	16	0.05

morning and was lowest in the evening with the possibility that 18OHB is secreted with a diurnal rhythm (**Table 21a**). The diurnal variations of 18OHB and aldosterone were also observed in a patient with essential hypertension (**Figure 41a**). In this patient, 18OHB or aldosterone secretion did not seem to exhibit a diurnal rhythm (**Table 21a**). The changes of saliva 18OHB and aldosterone were synchronous. The relationship between saliva 18OHB and aldosterone is shown in **Table 21b**. The scatter plots of the data are shown in **Figure 42a** and **Figure 42b**.

Table 21b The line of linear regression between salivary 18OHB and aldosterone concentrations in the patients with primary hyperaldosteronism and essential hypertension:

Subjects	Line of linear regression	r	n	p <
M	SB = 1.5 SA - 114	0.82	18	0.001
R	SB = 2.7 SA + 195	0.89	33	0.001
W	SB = 1.2 SA + 171	0.58	32	0.001
F	SB = 2.6 SA + 52	0.78	31	0.001

The 18OHB:aldosterone ratios of the subjects with primary hyperaldosteronism (subjects M,R and W) had a mean $\pm$ SD (n) value of 0.99 $\pm$ 0.28 (18), 5.9 $\pm$ 1.99 (29), 2.39 $\pm$ 0.8 (25)

respectively and 1.76 $\pm$ 0.73 (16) in subject F with essential hypertension (Table 21c). The ratio of saliva 18OHB:aldosterone in the patients with primary hyperaldosteronism were fluctuating as widely as found in healthy subjects.

In the patient with essential hypertension (subject F) being given captopril (50 mg orally), salivary aldosterone level was decreased by four fold after 120 minutes whereas 18OHB level was reduced by two fold. This probably implies that synthesis of 18OHB in the zona glomerulosa was suppressed due to the inhibition of captopril on angiotensin converting enzyme but not that in the zona fasciculata. Hence, this probably suggests that 18OHB secretion is probably not solely under the influence of AII. The relationship between 18OHB (SB) and aldosterone (SA) during captopril administration was related by the SB = 1.27SA + 61 ($r=0.86$, $p < 0.02$). The relationship at the same actual time without the treatment was not available. However, the relationship on another day for this subject was SB = 2.6 SA + 52 ($r=0.78$, $p < 0.001$).

The multiple peaks which occur between the morning and evening are probably related to the change of posture or as a consequence of daily activities of the patients or is it related to the endogenous secretions of the steroids. It seems that there is only one period when the main secretion of the steroids (18OHB and aldosterone) occur and that is during the early hours of the

Actual Time (h)	Subjects:	18OHB : aldosterone ratios M	R	W	F
0600		1.54	4.90		1.03
0630		1.40*	4.80	0.95	
0645			2.30		
0700		1.01	4.20	2.22+B	2.25
0715		0.85*	3.30	2.32	1.50*
0730		1.06	4.20	1.98	1.40
0745		0.99	4.30		
0800		0.72*	7.50*		1.90
0815		1.02	7.40	4.24+B	
0830		0.96	9.30	3.28	
0845		1.02	8.60		
0900		0.80	6.00	2.86+B	1.00
0915			8.20		
0930			8.00	2.48	
0945			4.60		
1000		0.90	6.40	4.10	
1100		1.50*	7.70	2.30	
1200			6.80	2.60*	2.70
1300			3.10+A	3.40	2.80+B
1400			6.80+B	1.95	1.10+A
1500			10.80	1.88	2.00
1600		0.90+A	6.70	2.70*	3.30
1700		1.10+B	7.20	2.20	1.90
1800			4.70	2.90	1.70
1900		0.70	3.90*	2.90	2.00
2000		0.50	5.00	2.60	
2100			5.20	1.20	0.80*
2200			5.20	1.80	0.90
2300			3.70	1.40	
2400			3.10		
0100					
0200				2.00+B	
0300				1.50+A	
mean ± SD		0.99±0.28	5.9±1.99	2.39±0.8	1.76±0.73
n		17	29	25	16

Table 21c
The salivary 18OHB:aldosterone ratios in patients with primary hyperaldosteronism (Subject M and R have bilateral adrenal hyperplasia; Subject W has aldosterone-producing adenoma) or essential hypertension (Subject F). * denotes coincident peaks of 18OHB and aldosterone;
+A denotes aldosterone peak without being accompanied by 18OHB
+B denotes 18OHB peak alone.

morning.

Patients with IHA seemed to have more synchronous 18OHB and aldosterone peaks than a patient with APA. Therefore, it seems that the secretion of 18OHB is dissociating from the secretion of aldosterone in patient with APA but not in patients with IHA (see **Figure 38a to 40a**).

(b) Correlation of 18OHB and aldosterone with glucocorticoids

The profile of glucocorticoids levels in these four patients are shown in **Figure 38b to Figure 41b**. The correlation between saliva 18OHB and aldosterone with glucocorticoids is shown in **Table 21d.** Since the correlation of 18OHB with aldosterone and glucocorticoids was shown to be significant, these data might suggest that the secretion of 18OHB and aldosterone could both be ACTH-driven.

Table 21d Correlation coefficents of aldosterone or 18OHB with glucocorticoids:

	Subjects	Aldosterone	18OHB
Glucocorticoids	M	0.633 **	0.887***
	R	0.597 ***	0.591***
	W	0.828 ***	0.686***
	F	0.809 ***	0.903***

*** $p < 0.001$; ** $p < 0.01$

DISCUSSION

All these patients, irrespective of their underlying pathological state, have 18OHB profiles qualitatively similar to that of healthy subjects. However, the aldosterone profile in the one subject with aldosterone-producing adenoma (APA) consisted of a reduced number of secretory episodes as compared to the number of 18OHB secretory episodes. This is probably because patients with APA are less sensitive to the influence of the renin-angiotensin system (RAS) as compared to healthy subjects and patients with primary hyperaldosteronism due to bilateral hyperplasia (IHA). Hence it may be possible to distinguish these two types of patients by the use of the technique of diurnal profiling of the two steroids simultaneously.

Patients with APA have been reported to have higher overnight recumbent 18OHB levels than patients with hyperplasia (Biglieri et al., 1979a; Bravo et al., 1983; Kem et al., 1985). It was found that the 18OHB levels in the saliva sample collected after overnight recumbency had little discrimminatory value of patients with IHA (472 pmol/l and 1118 pmol/l in subject M and R respectively) from a patient with APA (489 pmol/l in subject W). But overnight salivary aldosterone level was found to be more discriminatory than overnight recumbent 18OHB level (515 pmol/l in subject W compared to 308 pmol/l and 230 pmol/l in subject M and R respectively). However, the early morning peak value of 18OHB was

far more discrim inatory (1000 pmol/l in subject W compared to 600 pmol/l and 520 pmoll/ in the two subjects with IHA; Normal salivary 18OHB peaked at less than 300 pmol/l).

In conclusion, with the restricted number of patients available for studies, the data suggested that measurement of 18OHB and aldosterone levels simultaneously gave more meaningful data for diagnosing patients with primary hyperaldosteronism. A diurnal profiling technique might give a better picture of the aetiology of the disease.

3.4. Effect of postural change on saliva 18OHB, aldosterone and glucocorticoids levels in healthy subjects, and patients with primary hyperaldosteronism and essential hypertension

The commonly used protocol for studying the effect of changes of posture on aldosterone and 18OHB secretion is to sample blood after an overnight recumbency at 0800h and after a period of ambulation, either 2h or 4h (Ganguly et al., 1973; Biglieri et al., 1979; Bravo et al., 1983). In normal subjects and patients with IHA, the levels of both steroids increased when the subjects changed from supine to upright posture. On the contrary, patients with APA exhibit an anomalous postural fall of the two steroids. Therefore, the effect of postural change from overnight recumbency and after 4 hours of ambulation were studied using saliva samples to support these results.

RESULTS AND DISCUSSION

The effect of postural change from supine to upright position on salivary 18OHB levels in healthy subjects is shown in Figure 43. The effect of the postural change on aldosterone and glucocorticoids levels in comparison with 18OHB levels in some of these subjects are shown in Table 22a, 22b and 22c. The effect

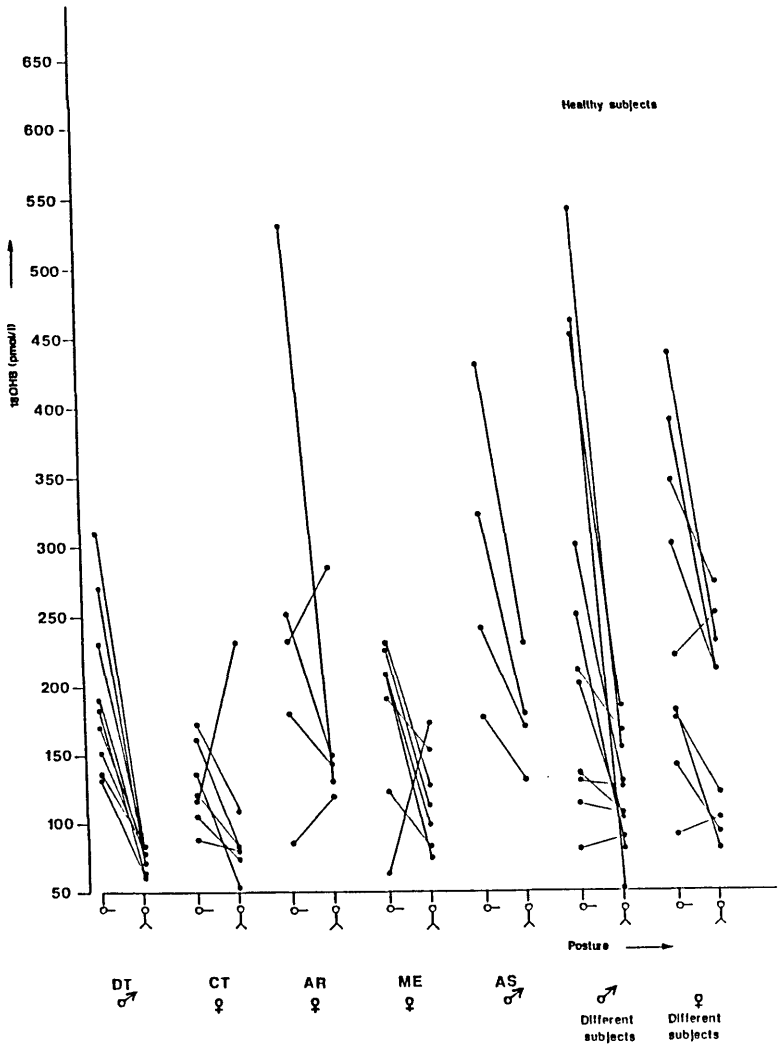


Figure 43

Effect of postural change from overnight recumbency to 4 h ambulation on salivary 18OHB levels in normal subjects (see **Table 21a to 21c** for supplementary notes)

Figure 43

Effects of overnight recumbency (o—) followed by 4 hours ambulation (χ) on salivary 18OHB concentration in healthy subjects. The overnight recumbent samples were collected between 0600h and 0800h while the ambulant samples were collected before lunch. Samples were collected daily by subjects (DT, CT, AR, ME and AS). The values of these subjects were excluded from the two groups of subjects classified as 'different subjects'. The mean ± SD of these different groups of subjects were as follows:-

Subjects	18OHB (pmol/l)		paired t-test		No. of
	Recumbent	Ambulant	t	p	Days
DT male	196 ± 60	75 ± 10	5.87	***	9
AS male	292 ± 109	176 ± 41	1.97	*	4
CT female	127 ± 30	100 ± 59	1.07	N.S	7
AR female	254 ± 166	161 ± 61	1.17	N.S	5
ME female	177 ± 62	114 ± 36	1.81	N.S	7
Different subjects (excluding those above):					
Male (n=11)	260 ± 157	116 ± 39	2.94	**	
Female (n=9)	240 ± 107	163 ± 75	1.73	*	

	ALDOSTERONE		GLUCOCORTICOIDS	
	Recumbent	Ambulant	Recumbent	Ambulant
Correlation Coefficients:				
18OHB	0.7292	0.8035	0.4378	0.3560
p	***	***	N.S	N.S

	ALDOSTERONE	GLUCOCORTICOIDS
Recumbent vs Ambulant	N.S	***

N.S means not significant; * $p < 0.05$; ** $p < 0.002$; *** $p < 0.001$

of postural change on the steroid concentrations of patients with Conn's syndrome and essential hypertension is shown in Table 23.

Table 22a The relative change in 18OHB and aldosterone concentrations when the subjects changed from overnight recumbency to 4 hours of ambulation:

		18OHB level		
	Changes	INCREASE	NO CHANGE	DECREASE
		No of subjects:		
A	INCREASE	2	2	2
L				
D	NO CHANGE	1	2	2
O				
	DECREASE	0	0	6

The Effect of Postural Change on 18OHB and Aldosterone levels

Table 22b The relative change in 18OHB and glucocorticoids concentrations when the subjects changed from overnight recumbency to 4 hours of ambulation:

		18OHB level		
	changes	INCREASE	NO CHANGE	DECREASE
		No of subjects:		
	INCREASE	1	0	0
F*	NO CHANGE	0	1	0
	DECREASE	2	3	10

F* = Glucocorticoids

Table 22c The concentrations of 18OHB, aldosterone and glucocorticoids levels in 20 healthy subjects:

	Concentration (pmol/l) except + (nmol/l)			
	Range (n = 20)		mean ± SD	
	Recumbent	Ambulant	Recumbent	Ambulant
18OHB	80 - 540	80 - 271	243 ± 137	153 ± 64 *
ALDO	11 - 188	16 - 185	60 ± 42	59 ± 51 N.S
F +	20 - 37	2 - 34	23 ± 7	10 ± 8 ***

Recumbent vs Ambulant : * $p < 0.05$; *** $p < 0.001$

The results obtained in this study using saliva are in agreement with the results obtained by Wilson et al. (1976) where the concentration of plasma 18OHB in healthy subjects decreased in response to postural change from overnight recumbency to 4h ambulation measured at 1200h. This is in contrast with the data obtained by other authors (see Appendix IIIb for data obtained by various authors). A peak secretion of the steroids could possibly be missed after 4 hrs of ambulation (see steroids diurnal profiles, Figures 38a and 39a). Therefore, it might be necessary to sample after 2 hrs of ambulation and preferably with multiple samplings.

The Effect of Postural Change on 18OHB and Aldosterone levels

A healthy subject who was supine for 2 hrs (from 2100h to 2300h) had a saliva 18OHB concentration of 60 pmol/l. On assuming an upright posture for 2 hrs without ambulation, her saliva 18OHB concentration rose to 130 pmol/l. The rise could possibly be due to the rigid protocol being used. This rigid protocol would be unsuitable for patients as it caused great discomfort to the healthy subject.

Although it is not possible to discrim inate healthy subjects from patients when the subjects changed from an overnight recumbency to 4 hrs of ambulation (Table 23), the absolute salivary 18OHB and aldosterone concentrations in the patients were higher than in normal subjects. The one patient with essential hypertension studied had a normal level of 18OHB but slightly higher than normal aldosterone level. With this limited number of patients studied, it is found that patients with primary hyperaldosteronism have abnormally high 18OHB levels in the morning after overnight recumbency.

Table 23 18OHB, aldosterone and glucocorticoids concentrations in patients with primary hyperaldosteronism and essential hypertension after overnight recumbency and 4h ambulation:

Steroid	Subj.	Range	mean ± SD (n)	OR	4A	18OHB: Aldosterone	
			Concentration (pmol/l):			OR	4A
18OHB	M	85- 602	280 ± 134 (18)	537	343	1.5	1.5
	R	216-1209	486 ± 274 (33)	1163	313	4.8	6.4
	W	22- 979	421 ± 245 (32)	734	574	1.6	4.1
	F	62-1132	172 ± 225 (31)	266	100	1.7	0.9
Aldo	M	175-430	264 ± 75 (18)	369	235		
	R	39-315	106 ± 83 (33)	240	49		
	W	70-515	210 ± 121 (32)	470	140		
	F	11-322	86 ± 69 (31)	155	110		
			Concentration (nmol/l)				
F*	M	4-36	17 ± 8 (18)	32	23		
	R	3-25	12 ± 6 (33)	21	9		
	W	4-40	18 ± 10 (32)	6	11		
	F	8-72	18 ± 12 (31)	25	20		

*Glucocorticoids ; OR, overnight recumbent values (0600h, supine); 4A, 4hours of ambulation (1000h)

3.5. Levels of 18OHB, aldosterone and glucocorticoids in children

There has been no report on the concentration of salivary 18OHB concentrations in children. It is known that the secretion of aldosterone is higher in children than adults (Kowarski et al., 1974) and hence may reflect the levels of aldosterone and 18OHB. Therefore, the experiment was designed for such a comparison. Saliva was collected from children, 10 females and 14 males, aged 5.5 ± 0.5 years, at 0900h and 1300h. Salivary 18OHB, aldosterone and glucocorticoids were measured.

RESULTS AND DISCUSSION

It was found that there was no difference in the concentrations of the three steroids between males and females. The mean concentrations of the three steroids are shown in Figure 44. The concentrations of the three steroids were higher in the morning than afternoon (Table 24a). Interestingly, the concentration of 18OHB correlates better with aldosterone in the 1300h samples than 0900h samples whereas the correlation with glucocorticoids was better in the 0900h samples than 1300h samples (Table 24b). Apparently, the absolute concentrations of 18OHB was higher in the children than the adults (a mean value of 250 pmol/l after

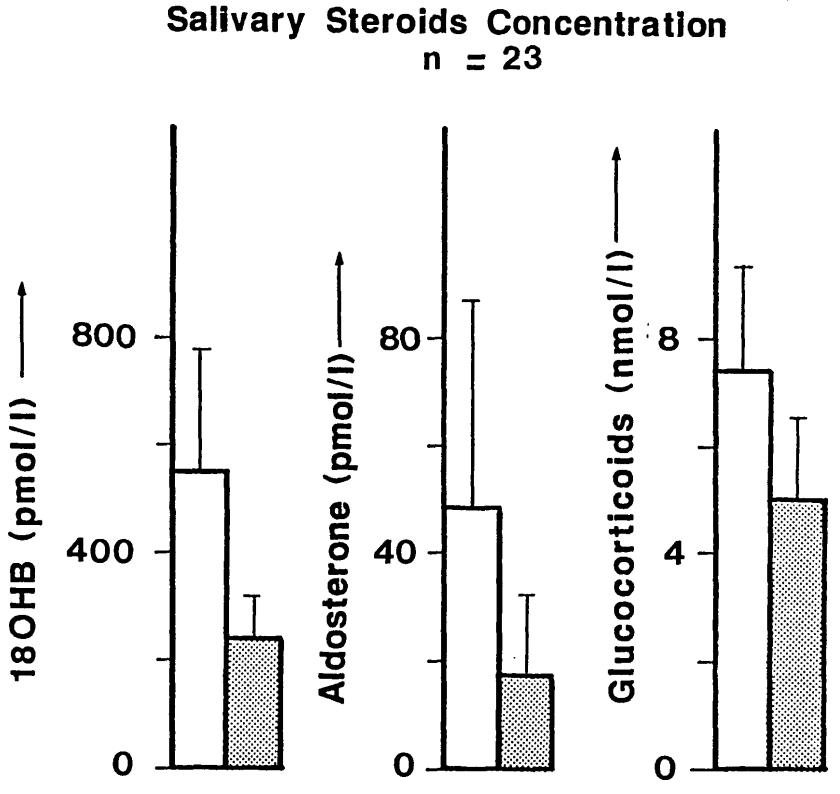


Figure 44

Salivary 18OHB, aldosterone and glucocorticoids concentrations in children aged 5.0 $\pm$ 0.5 years old (10 females and 14 males). Saliva was collected in the morning (0900h), being denoted by the open bars and in the afternoon (0100h) after lunch, being denoted by the hatched bars. It was found that the concentration of the three steroids was significantly higher in the morning than the afternoon ($p < 0.001$).

overnight recumbency at 0800h and 150 pmol/l at 1200h in the adults, see Table 22c pp 215). However, the concentrations of aldosterone in these children was apparently lower than adults in the 1300h samples but very similar in magnitude in the 0900h samples.

Table 24a The morning (0900h) and afternoon (1300h) 18OHB, aldosterone and glucocorticoids concentrations in children:

	Range	mean ± SD (n=24)
	18OHB (pmol/l)	
a.m	209 -1512	550.6 ± 274.0
p.m	159 - 476	242.0 ± 89.4
	Aldosterone (pmol/l)	
a.m	6.8 - 186	47.9 ± 39.8
p.m	6 - 53	17.4 ± 14.8
	Glucocorticoids (nmol/l)	
a.m	3.6 - 10.9	7.4 ± 2.0
p.m	2.2 - 9.6	5.0 ± 1.5
	18OHB : Aldosterone ratio	
a.m	5.2 - 73.2	18.1 ± 14.9
p.m.	7.1 - 29.1	19.4 ± 8.0

Table 24b Correlation between 18OHB, aldosterone and glucocorticoids levels in the morning and afternoon :

	ALDOSTERONE		GLUCOCORTICOIDS	
	correlation coefficients			
	a.m.	p.m.	a.m.	p.m.
18OHB(a.m.)	0.533		0.739	
18OHB(p.m.)		0.815		0.579

In this preliminary investigation, it is suggestive that 18OHB secretion as in the adults is ACTH-driven in the morning whereas the renin-angiotensin system has a greater influence on the secretion in the afternoon. Furthermore, the higher plasma renin activity in children compared to adults is probably due to the lower aldosterone concentration in children.

The remarkable correlation between 18OHB and aldosterone or glucocorticoids at different times of the day in these children was not observed in healthy ambulatory adults (section 3.2.3. pp 165). The reasons are probably because these children collected the saliva at the same time, shared the same physical activities and woke up around the same time.

3.6. Effects of corticotrophin on the secretion of 18OHB, aldosterone and glucocorticoids levels in two healthy male subjects

INTRODUCTION

The increase in plasma aldosterone and cortisol levels with pharmacological and physiological doses of corticotrophin (ACTH) is a well documented finding. Similarly, it has been shown that corticotrophin (both alpha$^{1-24}$ and ß$^{1-24}$ ACTH), administered in physiological or pharmacological doses stimulates the secretion of 18OHB irrespective of the method of admininistration (i.v., i.m. or infusion) (Wilson et al., 1976, Martin et al., 1975, Ojima & Kambegawa, 1979; Guthrie et al., 1981; Tuck et al., 1981; Watanabe et al., 1984; Lee et al., 1987).

In our study, two healthy subjects were injected with 5 doses of ß$^{1-24}$ACTH at a dose of 50µg in 200 µl saline per 15 minutes interval via an indwelling catheter. Blood was collected via another catheter from the other arm into lithium heparinised tubes. Heparin (ca. 200 µl) containing chlorbutanol (0.5%) as preservative was flushed in between each blood collection. Whole mixed saliva was collected for 10 minutes beginning 5 minutes before the corresponding blood collection. The study was done with the subjects seated from 0900h to 1230h. The subjects were on ad libitum sodium and potassium intake before the investigation.

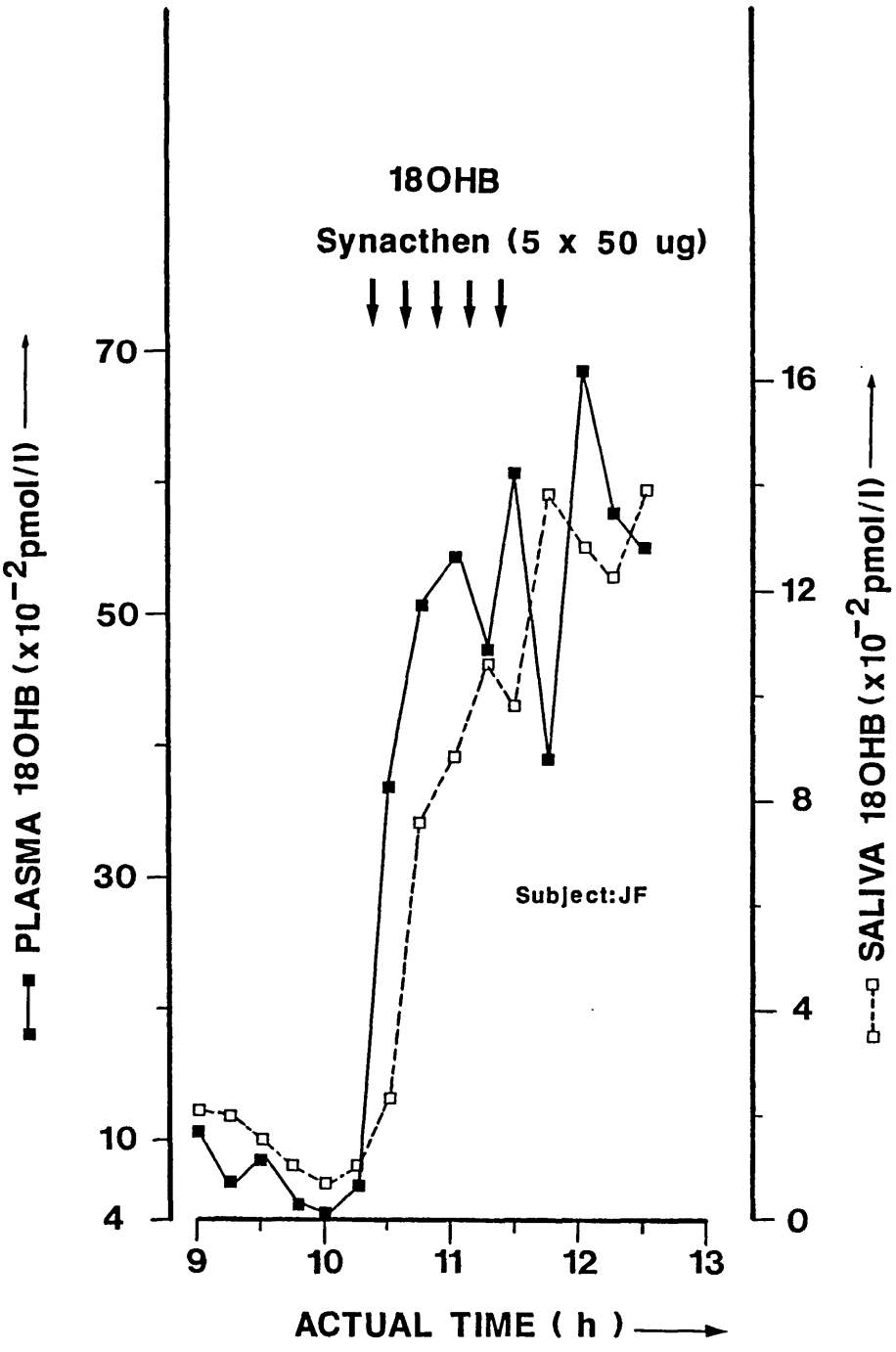


Figure 45a

Response of salivary and plasma 18OHB levels to corticotrophin in subject JF.

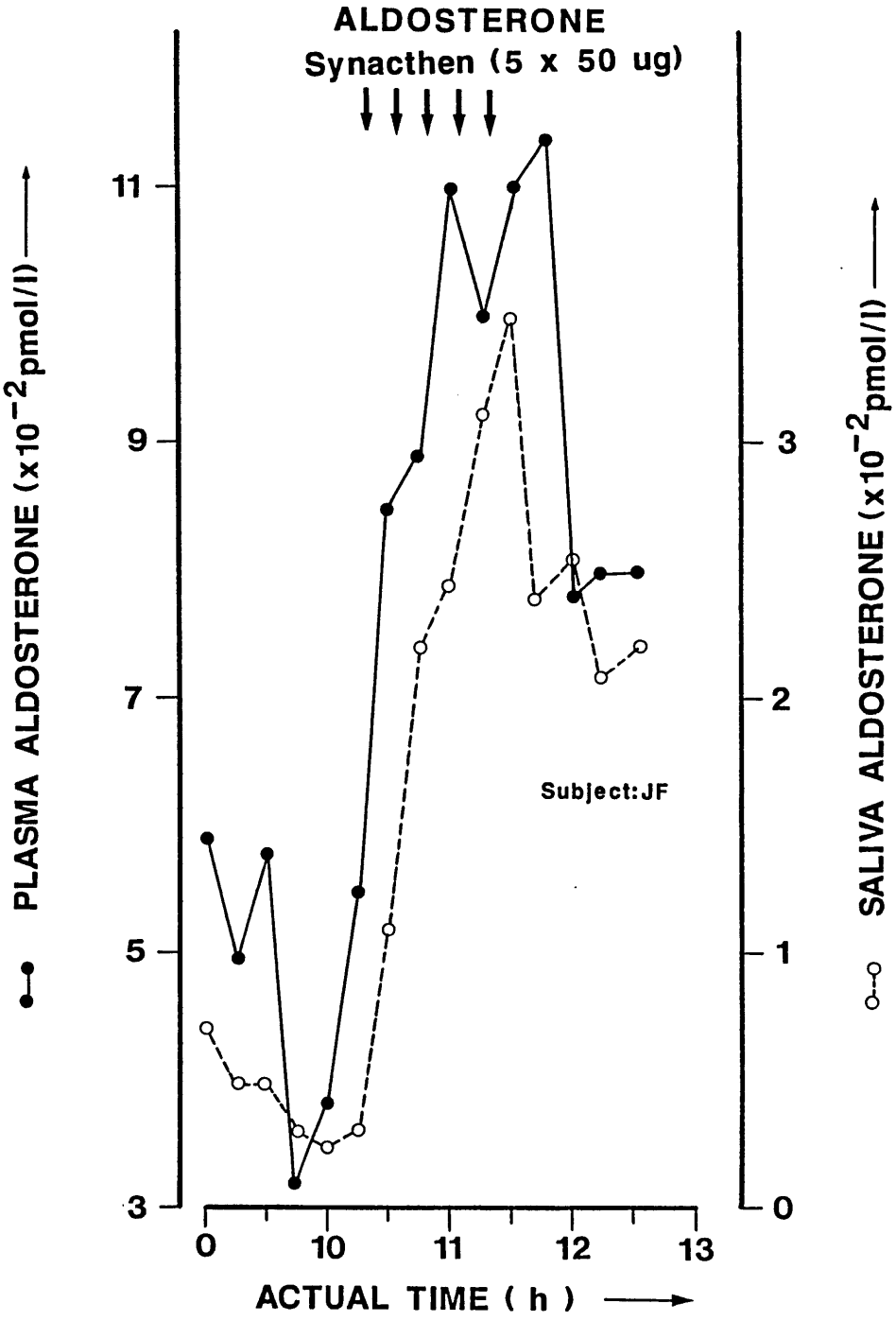


Figure 45b

Response of salivary and plasma aldosterone levels to corticotrophin in subject JF.

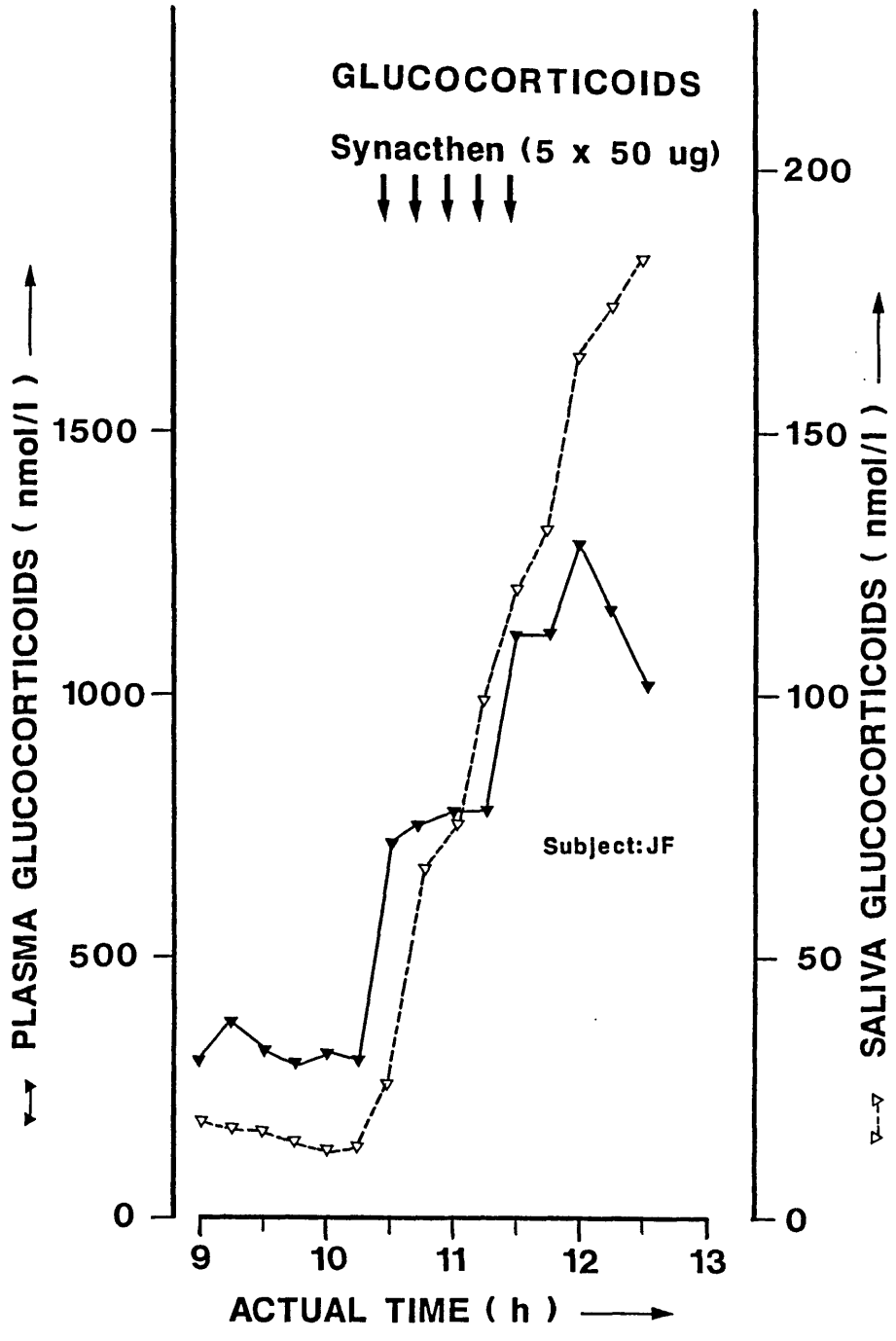


Figure 45c

Response of salivary and plasma glucocorticoids levels to corticotrophin in subject JF.

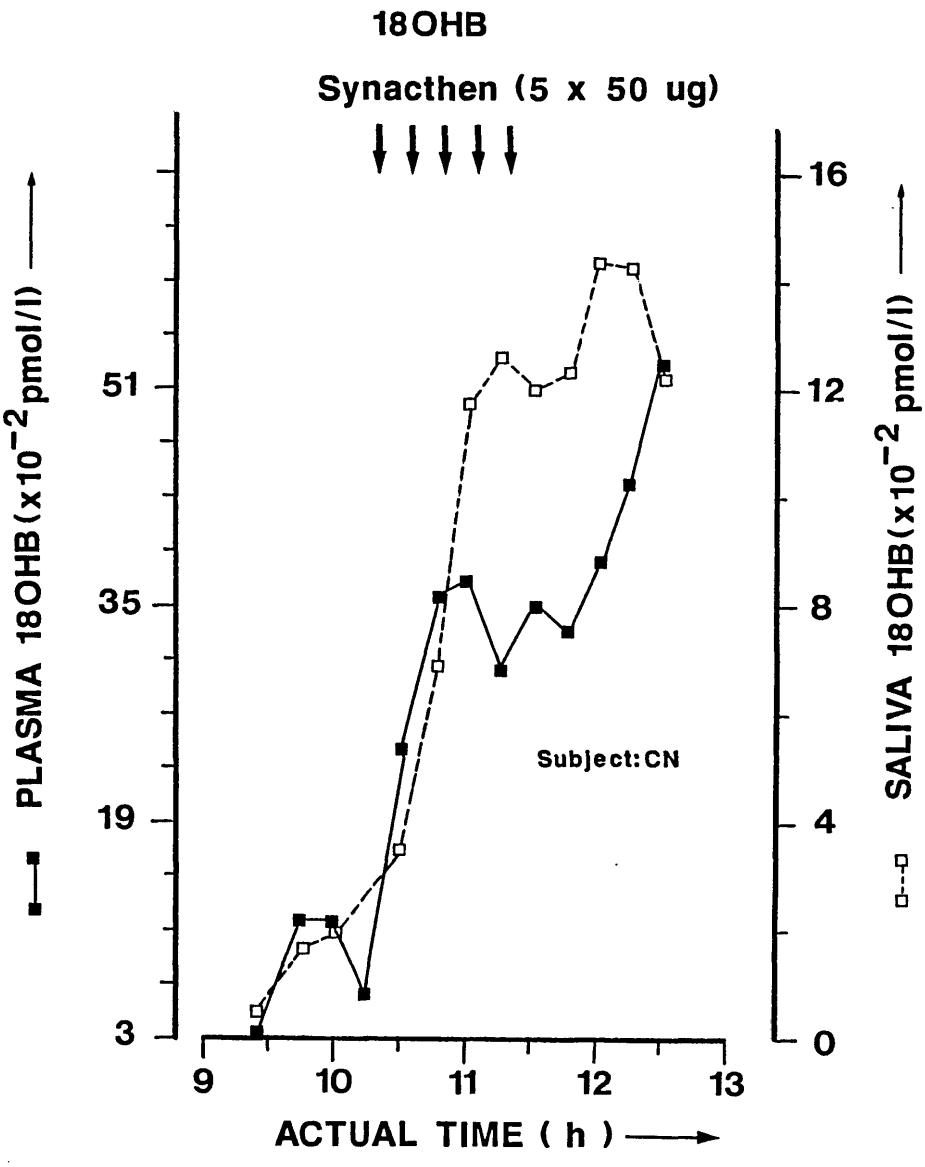


Figure 46a

Response of salivary and plasma 18OHB levels to corticotrophin in subject CN.

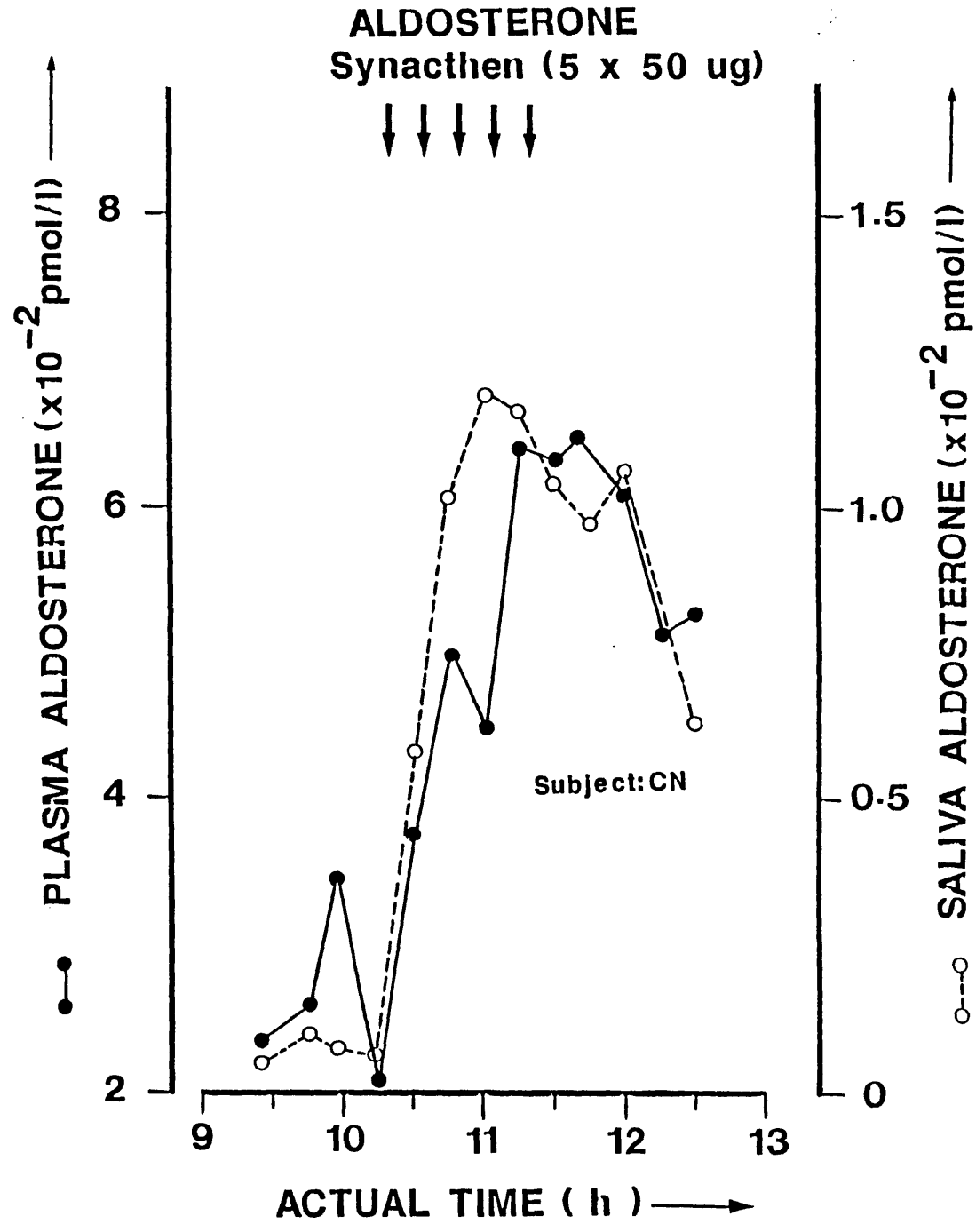


Figure 46b

Response of salivary and plasma aldosterone levels to corticotrophin in subject CN.

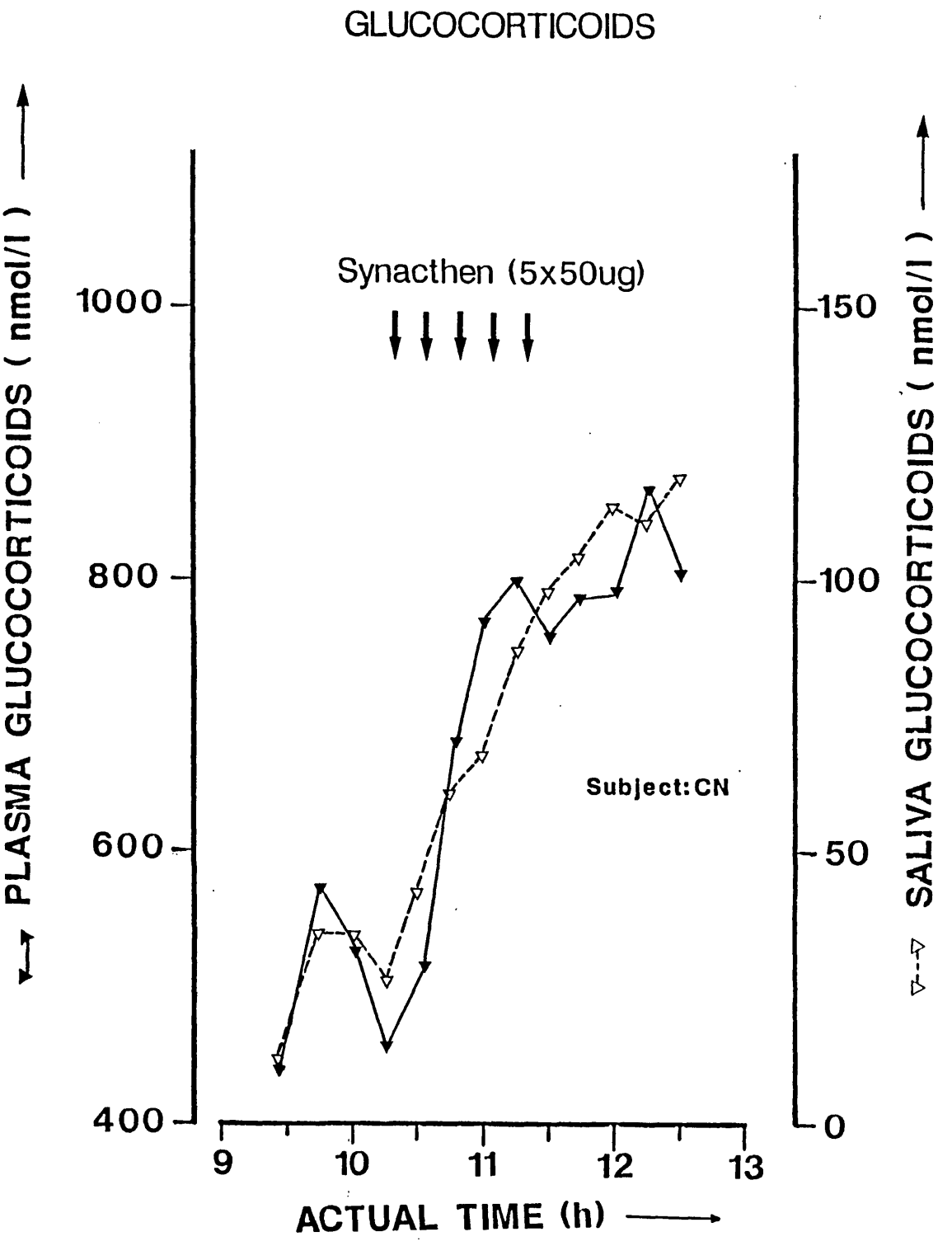


Figure 46c

Response of salivary and plasma glucocorticoids levels to corticotrophin in subject CN.

RESULTS AND DISCUSSION

The responses of saliva and plasma 18OHB, aldosterone and glucocorticoids to ACTH are shown in **Figure 45a** to **Figure 45c** in one subject and **Figure 46a** to **Figure 46c** in another subject. The increment of 18OHB, both in relation to the basal value and in absolute term was higher than aldosterone (**Table 25**). This difference between 18OHB and aldosterone persisted throughout the duration of the experiment. Moreover, the 18OHB:aldosterone ratio increased with time in both the saliva and plasma. In both subjects the saliva and plasma concentration of all the three steroids were highly correlated (r ca. 0.9) even though the saliva:plasma ratio of the three steroids increased as the plasma concentrations increased. The increasing saliva:plasma ratio was not reflected in a corresponding increase in the proportion of free plasma 18OHB measured by equilibrium dialysis which was 36.3 $\pm$ 5.4% (mean $\pm$ SD, n=13) for subject JF and 32.1 $\pm$ 7.6% (mean $\pm$ SD,n=8) for subject CN. In subject JF, the absolute concentration of salivary 18OHB was 52.0 $\pm$ 13.8% (mean $\pm$ SD, n=6) of plasma free 18OHB concentration prior to ACTH stimulation and was 43.5 $\pm$ 1.8 % (mean $\pm$ SD, n=5) post stimulation. However, there was a change in magnitude in subject CN from a basal level of 37.1 $\pm$ 5.1 % (n=3) to ca. 100% (n=9) post ACTH stimulation. This again probably implies that the difference in % salivary 18OHB concentration relative to plasma free 18OHB concentration is due to subject

Table 25

The basal concentration and increment of 18OHB, aldosterone and glucocorticoids in saliva and plasma in two healthy subjects in response to corticotrophin:

	Baseline values(mean ± SD , n=4) in pmol/l except * in nmol/l		
	18OHB	ALDOSTERONE	Glucocorticoids *
Subject : CN			
PLASMA	816 ± 368	252 ± 40	500 ± 62
SALIVA	124 ± 74	8 ± 2	20 ± 9
Subject : JF			
PLASMA	702 ± 215	487 ± 111	318 ± 31
SALIVA	137 ± 59	43 ± 17	15 ± 2

	Increment from baseline concentrations in pmol/l except * in nmol/l	
	SUBJECTS:	
STEROIDS	JF	CN
PLASMA		
18OHB	4500	3200
Aldosterone	650	400
Glucocorticoids *	900	300
SALIVA		
18OHB	1000	1000
Aldosterone	250	100
Glucocorticoids *	140	100

variation. It could not be related with the salivary flow rate because the flow rate of these two subjects were relatively similar (0.5 - 1.0 ml. min^{-1} throughout the experiment). The influence of salivary flow rate on 18OHB concentration was not undertaken because there was an indirect implication that salivary 18OHB concentration is not affected by the flow rate. This was because the 18OHB:aldosterone in saliva changed in parallel with the change in plasma 18OHB:aldosterone ratio and it had been reported that salivary aldosterone concentration did not vary with salivary flow rate (McVie et al., 1979).

In conclusion, our studies using a standard dose of β^{1-24} ACTH administered in pulses to simulate an infusion confirms the stimulating effect of ACTH on 18OHB and aldosterone secretion in healthy subjects. The increment of 18OHB in the plasma and saliva was found to be greater than aldosterone.This is probably related to the conversion of 18OHB to aldosterone being rate limiting.

Although pharmacological or low dose administration of synthetic ACTH stimulated the secretion of aldosterone, dexamethasone has no effect on basal aldoserone secretion measured throughout the 24 hour period whereas cortisol levels were blunted (James et al., 1978; Mason et al., 1975). Thus it is believed that corticotrophin does not play a physiological role in the control of aldosterone secretion. However, there is an alternative explanation to the lack of suppression of aldosterone secretion by

dexamethasone. It was reported only by one group of investigators that the suppression of cortisol due to the administration of dexamethasone (1 mg orally at 2300h and blood was collected at 0800h) was not accompanied by decrease of plasma ACTH (Lypka and Szczudlik, 1985). They found that only four out of the 10 subjects had decreased ACTH levels post dexamethasone. They hypothesised that the mechanism of exogenous glucocorticoid suppression of cortisol release was due to the inhibition of the adrenal cortex without the involvement of corticotrophin-releasing factor (CRF) and ACTH. Hence, this might explain the lack of influence of dexamethasone on aldosterone secretion.

Dexamethasone significantly depressed the secretion of 18OHB (Tuck et al., 1981; Guthrie 1981). The different response of 18OHB and aldosterone to dexamethasone could be due to the secretion of 18OHB from the zona fasciculata in addition to zona glomerulosa whereas aldosterone is secreted only from the latter zone.

ACTH administration has been tested on patients with APA or IHA (Guthrie 1981). The basal concentration of these steroids were abnormally high to begin with. It was found that both types of patients responsed to ACTH administration. According to the authors, no conclusion about the differential diagnostic utility can be made. Other authors found overlapping response to ACTH by these two types of patients (Kem et al., 1985). It is not surprising because aldosterone levels in APA patients are not only

ACTH dependent (Wisgerhof et al., 1981) but modulated by the renin-angiotensin system (RAS, Vetter and Vetter, 1975). Similarly, there is some evidence that aldosterone secretion in patients with IHA is under the control of both the RAS and ACTH but predominantly the former (Ganguly et al., 1973; Schambelan et al., 1976; Kem et al., 1976; Vetter et al., 1975). Hence, this might explain the overlapping results of 18OHB or aldosterone levels observed in these subjects after ACTH administration.

3.7. Relationship between 18OHB and aldosterone in a patient with long established ileostomy

INTRODUCTION

It is well-established that negative sodium balance is associated with an increase in aldosterone secretion. Thus it will be interesting to study the relationship between 18OHB and aldosterone in subjects who are sodium depleted either due to low sodium intake or loss due to diuretics, ileostomy or pyloric stenosis for example.

SUBJECT

A woman aged 53 with ileostomy 15 years ago was admitted with complaints of tiredness and lethargy. Her urinary sodium excretion on admission was less than 1 mmol/day.

METHOD

Saliva was collected the day after admission and subsequent days when she was given salt supplement$^{s}_{\lambda}$. The first saliva sample was collected after overnight recumbency when still supine. The other samples were collected without restriction of her activities.

RESULTS

The diurnal profiles of salivary 18OHB (SB) and aldosterone (SA) are shown in **Figure 47a** & **47b** with their respective salivary 18OHB:aldosterone ratios being shown in **Figure 47c** & **47d**. The line of linear regression between 18OHB and aldosterone throughout the 6 days of investigation are shown in **Table 26** below:

Table 26 The lines of linear regression between 18OHB (SB) and aldosterone (SA) in a patient with a 15 year old ileostomy:

Day	Lines of linear regression	n	r	p <
1	SB = 0.85 SA + 1938	5	0.694	N.S.
2	SB = 1.02 SA + 1201	5	0.597	N.S.
8	SB = -0.48 SA + 4519	5	0.239	N.S.
9	SB = 2.30 SA - 579	4	0.751	N.S.
10	SB = 1.50 SA + 349	10	0.640	0.05
11	SB = 0.69 SA + 800	9	0.422	N.S.
1 - 11	SB = 2.20 SA - 573	38	0.712	0.001

The scatter plot of all the results between 18OHB and aldosterone in this patient is shown in **Figure 47e**. The 18OHB and aldosterone level is found to have a mean $\pm$ SD value of 2174 $\pm$ 1733 pmol/l and 1286 $\pm$ 1109 pmol/l respectively. Salivary

18OHB:aldosterone is found to have a mean ± SD value of 2.29 ± 1.32 (n=30).

The levels of 18OHB and aldosterone reach a peak in the morning and this is similar to that shown by normal subjects. The absolute concentrations of the two steroids in this patient are abnormally high, even higher than patients with primary hyperaldosteronism or after corticotrophin stimulation. The peak concentration in the morning was 7000 pmol/l on day 1. Salt supplement appears to reduce the peak level to 3000 pmol/l. In the case of salivary aldosterone, it decreased from 4000 pmol/l to 1000 pmol/l.

DISCUSSION

Although the salivary 18OHB and aldosterone concentrations appear to change in synchrony, the correlation between these two steroids are not statistically significant when the day by day data were analysed separately except day 10 (Table 26). By using all the data available, the correlation between the two steroids are statistically significant ($p < 0.001$) where 50.8% of the variance in 18OHB concentration is due to the variance in aldosterone concentration ($r=0.712$). However, qualitatively the changes in 18OHB and aldosterone concentrations were similar.

In 36 out of 38 samples from this patient the concentration of 18OHB exceeded that of aldosterone. However, the

18OHB:aldosterone ratio varied considerably. This difference could be due, in part to differences in the kinetics of clearance of the two steroids. However, as shown earlier (pp 143 - 146 & Few et al., 1987b) overall the kinetics of 18OHB and aldosterone are similar. It is therefore possible that these data indicate that though 18OHB and aldosterone production are increased to a comparable degree, nevertheless there may be some temporal separation of the actual secretion of them.

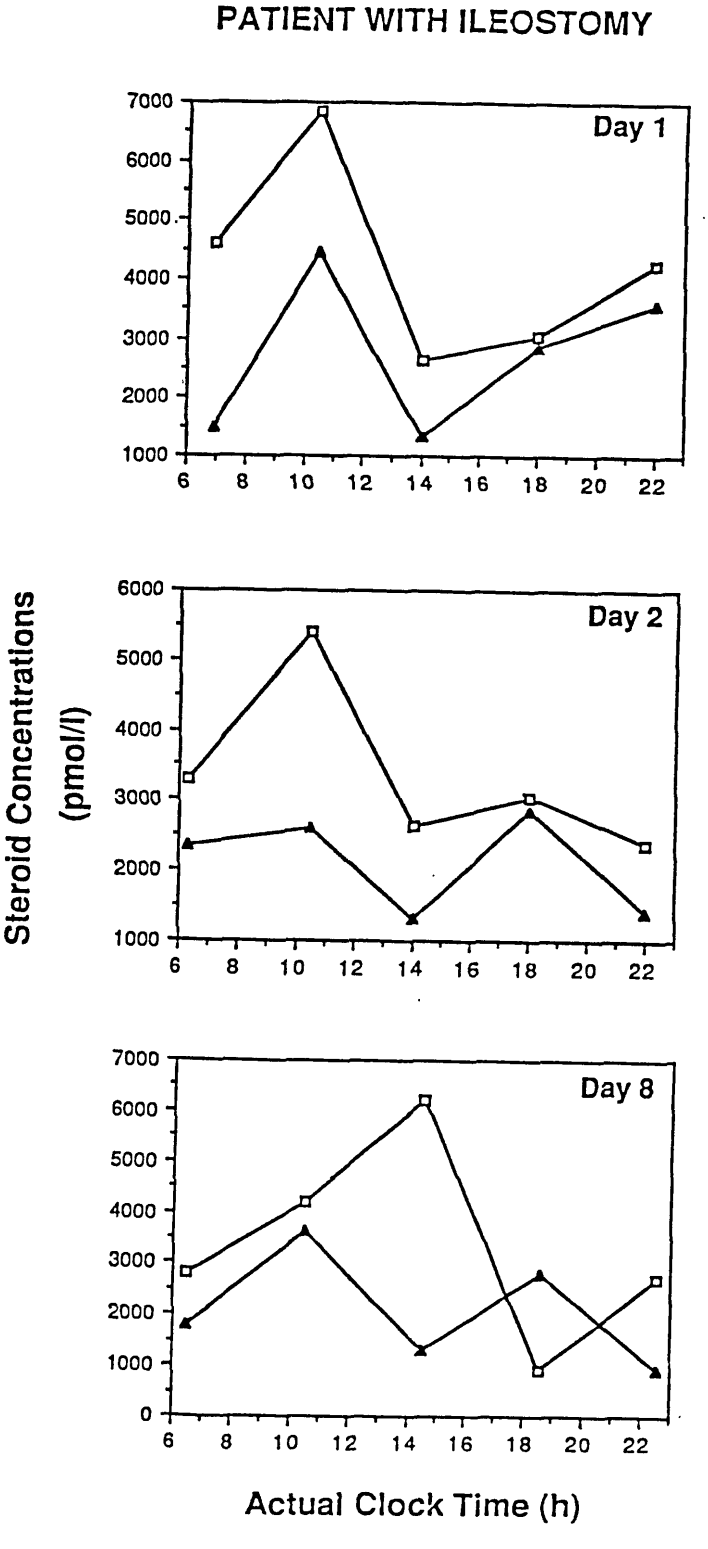


Figure 47a

Daily diurnal profiles of salivary 18OHB and aldosterone in a patient with established ileostomy (day1, day2, day8). Symbols: Bold triangles for aldosterone; open squares for 18OHB.

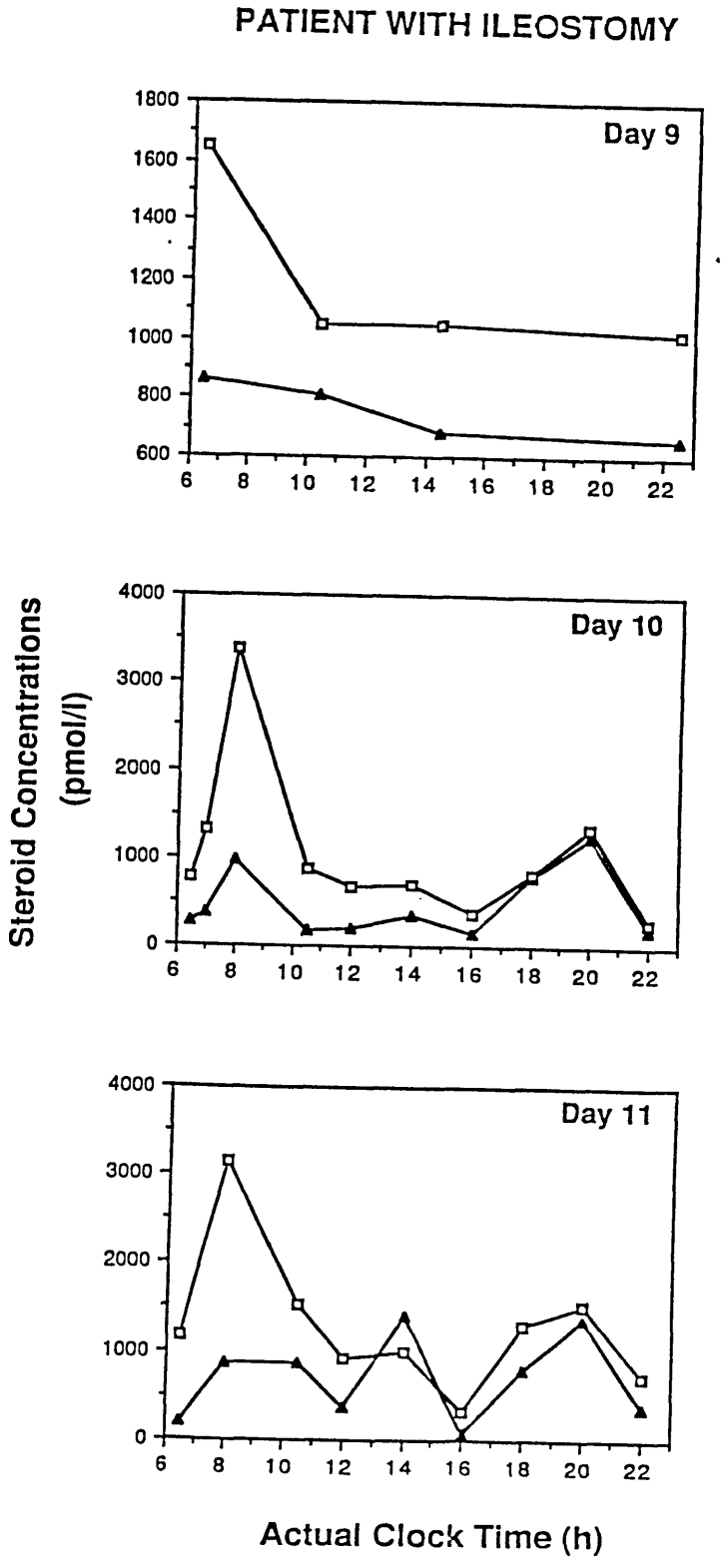


Figure 47b

Daily diurnal profiles of salivary 18OHB and aldosterone in a patient with established ileostomy (day9, day10, day11). Symbols: Bold triangles for aldosterone; open squares for 18OHB.

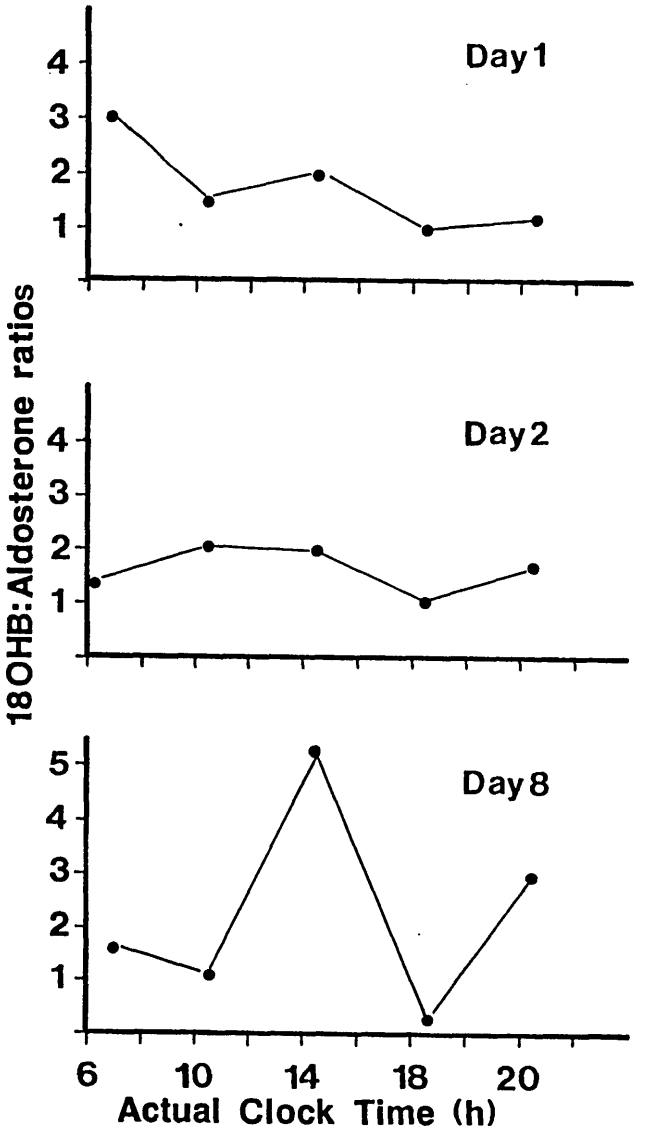


Figure 47c

Daily salivary 18OHB:aldosterone ratios in a patient with established ileostomy (day1, day2, day8). Symbols: Bold triangles for aldosterone; open squares for 18OHB.

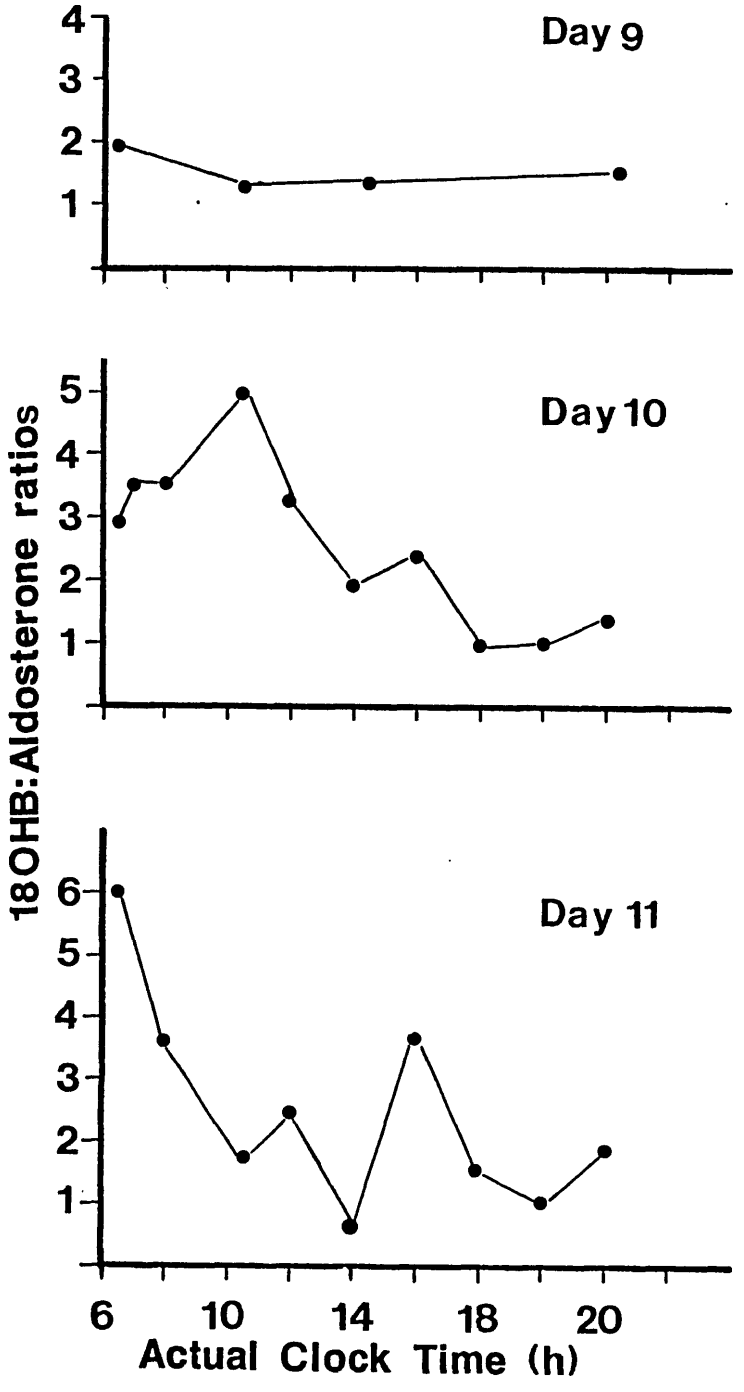


Figure 47d

Daily salivary 18OHB:aldosterone ratios in a patient with established ileostomy (day9, day10, day11). Symbols: Bold triangles for aldosterone; open squares for 18OHB.

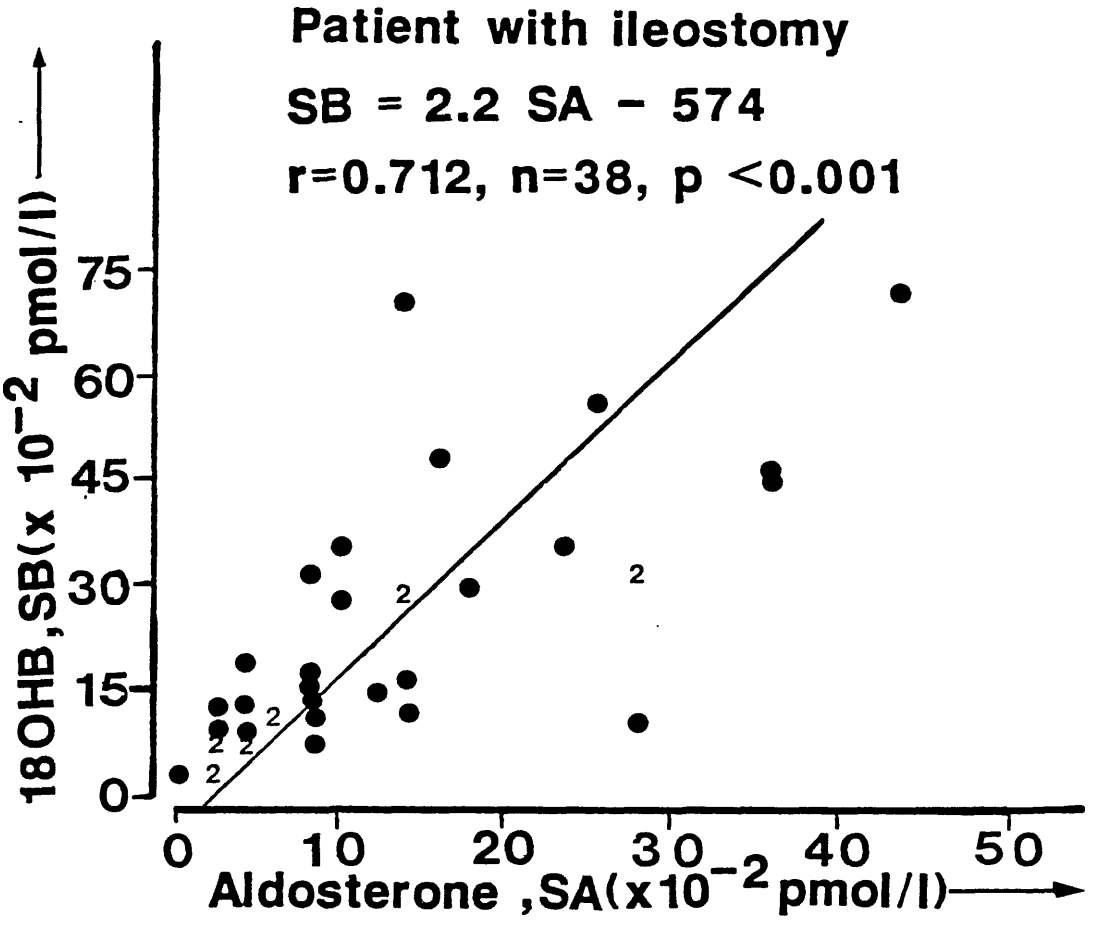


Figure 47e

Cumulative data (shown previously in Figure 47a & 47b) of a patient with long established ileostomy who collected saliva samples on 6 different days to show the relationship between salivary 18OHB and aldosterone levels in this patients (see **Table 26** for complementary data).

3.8. Relationship between 18OHB and aldosterone in the third trimester of pregnancy

INTRODUCTION

The increase of aldosterone levels during pregnancy is a well-known physiological response (for references see Few et al., 1986a). However, the precise mechanism for the increase is unknown. It has also been reported that urinary 18OHB excretion increased during pregnancy (Bauknecht et al., 1982). Therefore, women during their pregnancy will provide natural biological fluids high in aldosterone and 18OHB levels. Hence, the relationship between 18OHB and aldosterone levels in a pregnant woman in her third trimester of pregnancy was investigated using saliva with multiple sampling for exploring the diurnal fluctuations of the secretion of both steroids.

SUBJECT

A 23 years old primagravida collected saliva while going about her daily business during week 36 of a normal pregnancy. The first sample was collected after overnight recumbency while supine.

RESULTS

The salivary 18OHB (SB) and aldosterone (SA) diurnal profiles of the subject are shown in **Figure 48** (SB = 2.0 SA + 79, n=12, r=0.9720, $p < 0.001$) with 18OHB:aldosterone ratios shown in the same diagram. The concentration of 18OHB has a mean ± SD of 932 ± 562 (n=12) pmol/l (range: 188 - 2210 pmol/l) and that of aldosterone has a mean ± SD of 419 ± 268 pmol/l (range: 82 - 1000 pmol/l). Her 18OHB:aldosterone ratio has a mean ± SD value of 2.13 ± 0.24 (range: 1.6 - 2.4 if one value of 4.9 was excluded). If the latter value is included, the mean ± SD value is 2.35 ± 0.830.

DISCUSSION

The extremely high correlation (r=0.9720) between salivary 18OHB and aldosterone found in a woman at 36 weeks of a normal pregnancy has not been found before with saliva from other subjects with hyperaldosteronism (primary and secondary). This might be related to a closer relationship between plasma free 18OHB and salivary 18OHB levels in pregnant subjects than in normal subjects as has been found for aldosterone (Few et al., 1986). The remarkable correlation between 18OHB and aldosterone probably indicates that both the secretion and metabolism of these steroids are under the same influencing factors.

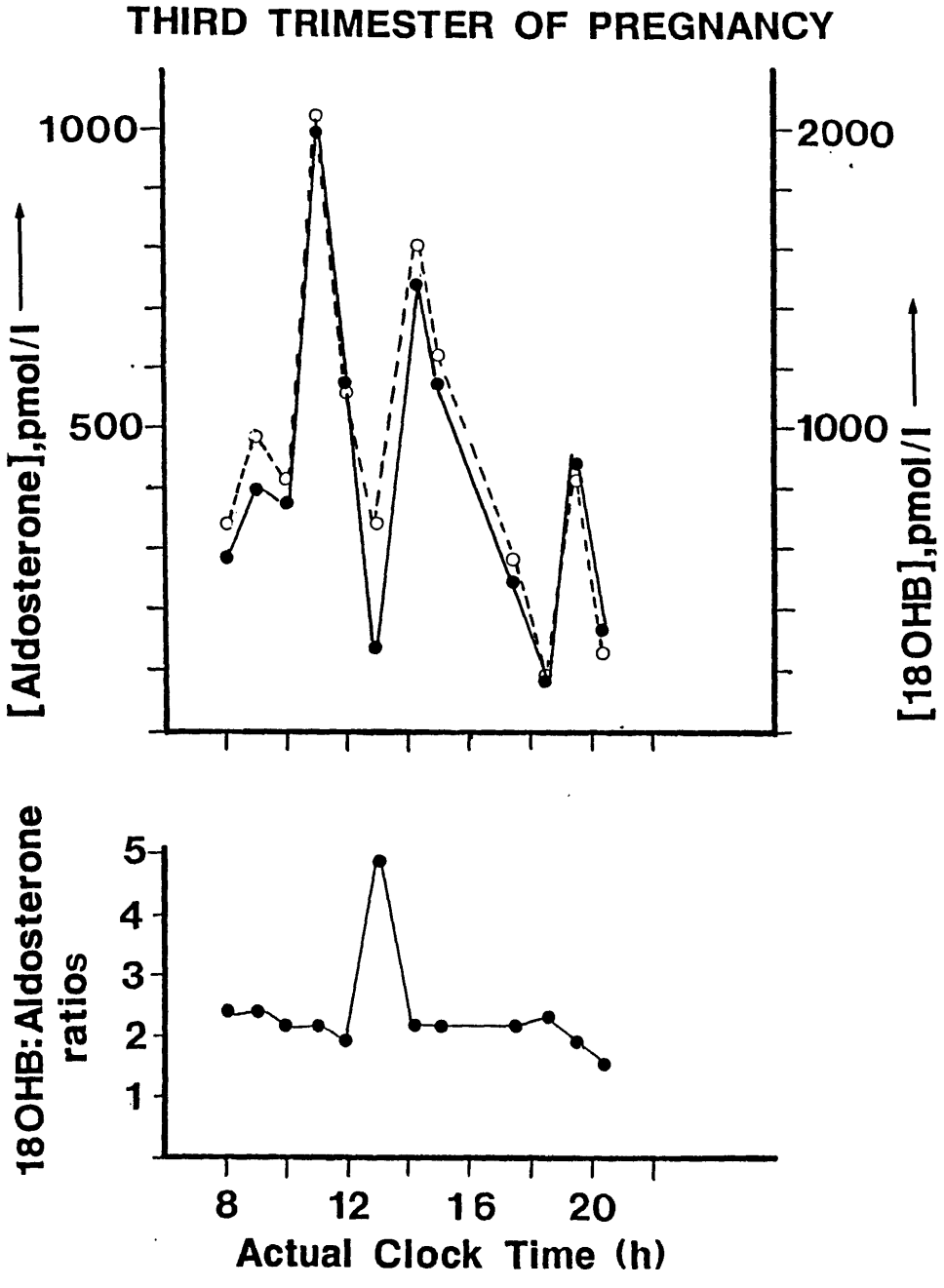


Figure 48

Diurnal salivary 18OHB and aldosterones profiles, and 18OHB:aldosterone ratios during the third trimester of a normal pregnancy. Symbols: Bold circles for aldosterone; open circles for aldosterone.

Chapter 4

General Discussion

The principal aim of the work for this thesis was to develop a sensitive, accurate and specific method, robust enough for routine use, to measure 18OHB especially in saliva. This has been accomplished and the method adequately validated using saliva and plasma samples from healthy subjects obtained under physiological conditions and after corticotrophin administration. In this way the usefulness of plasma and salivary 18OHB determination for monitoring changes in adrenal secretory activity under physiological conditions and following corticotrophin stimulation was also assessed. In addition, saliva samples from subjects with primary and secondary hyperaldosteronism (pregnancy and ileostomy) were used in the validation.

The method described in this thesis involved a thin-layer chromatographic step (TLC) with tritiated 18OHB incorporated as a procedural loss marker. This is the first report which describes the separation of 18OHB in saliva and plasma using thin-layer chromatography. No material assayed by a reliable reference method was available. However the values obtained for plasma 18OHB concentration in normal subjects were broadly similar to those reported by previously investigators (Appendix IIIa). This, along with my own validation, encourages me to believe that my data are reasonably reliable.

Two aspects relating to the methodology will first be mentioned. A simple and novel procedure has been discovered for

preparing the 3-monoxime of corticosteroids having two carbonyl functional groups. Carboxymethoxylamine-hemihydrochloride ($(CMO)_2$-HCl) is the only compound available commercially for the formation of a steroid-carboxy-methoxyloxime (CMO) (Borek and Clark 1936). It had been assumed that this salt needed to be activated by the removal of hydrochloride with a basic solvent such as pyridine (Abraham 1975) or by the addition of a basic salt such as sodium acetate (Erlanger 1957, 1959). The known lability of 18OHB (Schmidlin & Wettstein 1960; Kondo et al., 1965; Martin et al., 1975; Aragones et al., 1978; Usa et al., 1979; Cozza et al., 1985) suggested that extremely mild reacting conditions should be used. Although Belkien et al. (1980) apparently successfully used a short reaction time under acid condition it was felt better to avoid this possible complication. Having explored various possibilities with model compounds (cortisol, aldosterone and corticosterone) it was found that many corticosteroids react rapidly with $(CMO)_2$-HCl in pure methanol solution to yield a monoxime without the formation of any dioxime. However, care is required in the case of 18OHB to avoid formation of a polar artifact during the process of concentrating the solution of pure 18OHB-3-CMO. The 18OHB-3-CMO that was used for the preparation of the iodinated ligand for use as tracer in the RIA was stabilised by the addition of 3 molar equivalents of NaOH. This material was homogenous on TLC and its nuclear magnetic resonance spectrum was

consistent with the formulation that it is a monoxime of 18OHB.

Of the immunoassays previously developed using commercially available [1,2-^{3}H]18OHB as the ligand, the best have a sensitivity of 3 pg/tube. The use of [^{125}I]radioligand has improved the sensitivity to ca. 1 pg/tube. Although this is not a remarkable improvement, the ease of using an [^{125}I]radioligand compared to liquid scintillation counting is a further advantage. It is more cost effective and has high through-put. Moreover, the stability of the [^{125}I]radioligand prepared has been improved to enable the ligand to be usable for more than 6 months without the need for re-purification. This is attributed to the use of the stabilised 18OHB-3-CMO derivative as its sodium salt for preparing the radioligand and to a modified TLC system used for the purification of the radioligand. The omission of the 1 % acetic acid present in the original solvent system of Nars and Hunter (1973) did not affect the resolution of the radioligand on the chromatogram but it has improved the stability of the radioligand, which was usable for 6 months instead of 3 originally.

No antibodies raised to 18OHB are sufficiently specific for measuring 18OHB directly, even in saliva, except that of Witzgall et al. (1981). However, the titre of their antibodies was low. Similarly 18OHB-3-CMO conjugated to an homologous protein was found to yield more specific antibodies but their low titres made them of no practical value.

The TLC method developed in this thesis for the purification of the saliva or plasma samples is convenient, rapid and has negligible blank detectable by RIA. The TLC plates purchased from a commercial source require no pre-washing and the solvents used for the TLC need no pre-treatment before use. Paper chromatography (PC) has been used by previous authors as a mean of purifying plasma samples prior to RIA. An inconvenience of PC is the requirement to boil the paper in methanol to reduce the blank inherent in PC (Martin et al., 1975). With LH-20 sephadex column chromatography, fractions collected were in the region of 20 ml (Watanabe et al., 1984) for one ml of plasma chromatographed. These fractions have to be evaporated prior to RIA. In my method, the TLC plate was eluted with assay buffer and hence no evaporation of organic solvent is necessary.

The TLC assay was validated for its inter- and intra- batch precision using a saliva pool with a concentration of 45.5 $\pm$ 5.6 pmol/l (mean $\pm$ SD, n=30) was 12.6 % and 8.0 % respectively. The inter-batch precision for plasma samples had a CV of 12% using a plasma pool with a concentration of 400 $\pm$ 48 pmol/l (mean $\pm$ SD, n=30). The recovery of 18OHB added to three saliva pools and a plasma pool at varying doses was accurate and linear up to 800 pmol/l tested. The TLC system used was specific for measuring 18OHB. No other immunoreactive material was measurable when the 18OHB obtained with this TLC system was re-chromatographed using a

second TLC sytem. Secondly the parallelism of the TLC eluate to the standard curve also indicates the specificity of the TLC method established.

Zager *et al*. (1986) have drawn attention to the *in vitro* redistribution of 18OHB between plasma and red blood cells (RBC) that takes place when blood cools after withdrawal. It is normal to centrifuge blood either at ambient temperature (22 - 25^{o}C) or at 4^{o}C. At these sub-physiological temperatures the binding of steroids to plasma protein is greater than at 37^{o}C. Consequently as blood cools there is 'stripping' of 18OHB from the RBC into the plasma. The concentration of 18OHB measured in plasma is therefore in excess of the true physiological (*in vivo*) value. This is of little practical significance provided that blood samples are always processed in a similar fashion and that this *in vitro* handling is taken into account when comparing data from different centres.

However, it does have an influence upon the saliva : plasma ratio because salivary 18OHB derives from plasma 18OHB at physiological temperature. Matched saliva and plasma samples were collected from twenty healthy ambulatory subjects at random time. The results showed that only 50% of the variance of saliva 18OHB concentration was due to the variance of plasma free 18OHB concentration ($r=0.71$). The *in vitro* distribution of 18OHB between RBC and plasma was also dependent on the plasma cortisol

concentration which varies episodically throughout the day. The higher the cortisol concentration the smaller is the discrepancy between plasma obtained at 37°C and ambient temperature (Zager et al., 1986). The lowering of temperature probably will not affect the percentage plasma free 18OHB measured by equilibrium dialysis because firstly there is excess of binding proteins such as albumin and corticosteroid binding globulin to bind the steroids stripped from RBC. Secondly, the equilibrium dialysis was performed at physiological temperature which could restore the equilibrium which exists between the free and protein bound steroid. The artefactually raised plasma 18OHB concentration will lower the real saliva 18OHB : plasma free 18OHB ratio. In this case, the true saliva 18OHB : plasma free 18OHB ratio would have been higher than found in this thesis. It would therefore be inaccurate to regard measurement of salivary 18OHB as an indirect measure of plasma free 18OHB.

Since the effect of temperature has no effect on the redistribution of 18OHB in saliva because of the absence of binding proteins or RBC, measuring 18OHB in the saliva confers a further advantage. Thus measuring saliva 18OHB concentration in practice is useful in addition to the several advantages of collecting saliva such as being non-invasive, non-stressful and can be done without supervision and on an out-patient basis.

It was found that the 18OHB:aldosterone ratio in plasma (mean

± SD (n=20): 4.2 ± 2.7; range: 0.9 - 15.8) is different from that in saliva (mean ± SD: 2.7 ± 1.5; range: 0.8 - 6.1) although the variation is similar (CV= 64% in plasma versus 56% in saliva). The reason is obscure. Nevertheless, the plasma concentrations of 18OHB in these subjects have magnitude similar to some authors (Martin et al., 1975; Witzgall et al. 1981; Belkien et al., 1980; Watanabe et al., 1984; Sowers et al., 1983; Kem et al., 1985; Appendix IIIa) but higher than others (Wilson et al., 1976; Nagahama et al., 1983). The plasma concentration of 18OHB measured by other authors were from ambulatory subjects with blood taken at 1200h. The subjects in my study were ambulatory but blood was taken at random times. The reason for random blood sampling was because the investigation was done solely for the purpose of studying the relationship between saliva and plasma 18OHB concentration. The reasonable agreement between the results obtained in this thesis and other authors suggests that the method for measuring 18OHB is sufficiently accurate.

Although the method developed for measuring 18OHB in saliva did not have a high-throughput because of the requirement of purification by TLC, frequent diurnal saliva samples were collected by normal subjects and patients with primary hyperaldosteronism (PHA) under natural diurnal posture for physiological and pathological investigations. By measuring

salivary 18OHB concentration in subjects going about their daily activities, we obtain data that is truly normal for the subject being studied. On the other hand the lack of standardisation of some of the factors that are known, or suspected, to influence 18OHB secretion (such as posture, exercise, intake of food and salt etc.) makes it impossible to unravel the factors that contribute to the observed diurnal fluctuation. To do this would require standardisation of many of these variables. This would almost certainly require to be done in a metabolic ward with the possibility that such admission would itself lead to hormonal changes.

The information which one is likely to obtain by measuring the spontaneous changes of salivary 18OHB levels as described in this thesis is the magnitude of change throughout the day, its changes in relation to aldosterone and glucocorticoids as indices of the renin-angiotensin system and ACTH respectively, under physiological conditions.

The requirement of 3 ml of saliva from normal subjects limited the diurnal study to half hourly saliva sampling intervals. This protocol was used to assess the temporal aspects of 18OHB secretion in 3 healthy subjects and 3 patients with PHA. The handling of the diurnal profile data is briefly discussed here. No attempt was made to combine the data from these subjects

into meaningful averages. This is because the subjects did not wake up at the same time, share the same physical activities, have the same food and salt intake. These factors are believed to affect aldosterone secretion (Coghlan and Blair-West 1967; Few et al., 1987b) and hence possibly would affect 18OHB secretion. Further, individual fluctuations of the saliva 18OHB concentrations would be obscured by differences in phases, means, amplitudes or frequencies of secretion as shown for cortisol (Weitzman et al., 1966). Grouping of plasma 18OHB data from different subjects has been used by Sower et al. (1982 , 1983). The results showed that 18OHB secretion exhibits a circadian rhythm though inter-subject variation is high. Until more individual data are obtained, the manner in which these data should be summarised is uncertain. The best method at present for presenting the diurnal fluctuations of 18OHB of different subjects is to plot individual data separately on an arithmetic scale. Only when more data is available will it be possible to determine which statistical tests will be most useful for investigating the possible synchrony of 18OHB and aldosterone secretion.

The salivary 18OHB concentration measured need not necessarily reflect biosynthesis. This relationship could be investigated by measuring the plasma concentration and specific activity of the steroid after administration of the radioactive

steroid as obtained for cortisol by Hellman et al. (1970). However, radiochemical data obtained in this thesis showed that the metabolic clearance rate of 18OHB is similar to that of aldosterone (Few et al., 1987b). Coghlan and Blair-West (1967) have deduced that an increase in aldosterone level in excess of 100% under physiological condition suggests secretion of the steroid rather than a reduction in the rate of clearance such as might be affected by postural change. Therefore, this magnitude of change has been used for delineating increased salivary 18OHB concentration being due to secretion rather than postural change.

The varying saliva 18OHB:aldosterone ratio throughout the day in patients with PHA also make it impossible at this stage to consider the use of the ratio for screening of patients with PHA except in samples collected under a protocol standardised for time, posture, daily activities, salt intake and environmental stimuli (Kater et al., 1985; Biglieri et al., 1979). The results reported by these authors showed that plasma 18OHB:aldosterone ratio varied over a small range probably because of the standardised protocol used where the patients were studied in a metabolic ward.

Although the diurnal saliva 18OHB concentrations in normal subjects under physiological condition changed broadly in synchrony with saliva aldosterone concentration, the fluctuation

of the 18OHB:aldosterone ratio indicated dissociation of the two steroids. The dissociation is unlikely to be due to the metabolism of the two steroids being different (Few et al., 1987b). The half lives of the biexponential decay of [^{3}H]18OHB and [^{3}H]aldosterone in normal subjects were found to be similar. Both steroids also show extremely rapid initial clearance of the injected steroids. Moreover, the percentage free 18OHB and aldosterone in plasma is similar.

In addition, the percentage binding of 18OHB to albumin and corticosteroid binding globulin (CBG) is similar to that for aldosterone (Zager et al., 1986). In addition, the percentage plasma free 18OHB described in this thesis and aldosterone (Few et al., 1986b) obtained by equilibrium dialysis is the same. There are no data on the binding affinities of both steroids to albumin. However, the binding affinities to CBG · said to be different (Zager et al., 1986). The affinity constant of 18OHB was $2x10^6$ M^{-1}, which was intermediate between cortisol ($3x10^7 M^{-1}$) and aldosterone ($0.9x10^6$ M^{-1}). There was insufficient data in this report to test whether the difference in binding affinities of 18OHB and aldosterone is statistically significant. Hence, the evidence is in favour of the assumption that the metabolism of 18OHB and aldosterone is similar.

Diurnal fluctuations of 18OHB and aldosterone concentration

occur not only in normal subjects but also in patients with PHA. This demonstrates the secretion of 18OHB and aldosterone in patients with PHA is either intermittent or semi-autonomous (Vetter & Vetter, 1975 & 1978; Davies et al., 1979; Ganguly et al., 1973; Schambelan et al., 1976; Kem et al., 1976; Fraser et al., 1979). Both exogenous AII (Mason et al., 1976 & 1977; Belkien et al., 1980; Fraser et al., 1981; Beretta-Piccolli 1983; Sowers et al., 1983) and ACTH (Guthrie et al., 1981; Wilson et al., 1976; Martin et al., 1978; Tuck et al., 1981; Witzgall et al., 1981; Schoneshofer et al., 1981; Sowers et al., 1983, Beretta-Piccoli et al., 1983) have been demonstrated to influence the secretion of these steroids. The two patients with idiopathic hyperaldosteronism had as many peaks of aldosterone as 18OHB. However, in one subject with an aldosterone-producing adenoma (APA), there were five diurnal 18OHB peaks with no aldosterone peak. In contrast the aldosterone concentration declined biexponentially from 0630h onwards. In addition, there were more 18OHB peaks than glucocorticoid (GC = cortisol + cortisone) peaks. Out of the five GC peaks, three of them coincide with that of 18OHB. This finding suggests the influence of both AII (Fraser et al., 1979; Davies et al., 1979; Wisgerhof et al., 1981) and ACTH in patient with IHA whereas ACTH is the predominant factor which regulates the secretion of 18OHB in patients with APA (Wenting et

al., 1978; Wisgerhof et al., 1981). In the patient with APA, the saliva aldosterone concentrations after the early morning high concentration were normal whereas the saliva 18OHB concentration remained high. Therefore, if a blood sample had been taken only at 0830h, two hours after waking up, aldosterone concentration would appear to be normal. But 18OHB concentration remained abnormally high for a longer period of time throughout the day. Thus it is useful to have a measure of 18OHB concentration simultaneously with that of aldosterone.

Saliva 18OHB concentration is better correlated with saliva GC concentration than aldosterone with GC in both normal subjects and patients with PHA. 18OHB and aldosterone levels were always highly correlated. This again indicates that 18OHB and aldosterone secretions may be under the predominant influence of AII but 18OHB secretion is also under the regulatory control of ACTH. The stimulatory influence of AII and ACTH on aldosterone biosynthesis is believed to occur early in the biosynthetic pathway while AII has an additional role of stimulating the conversion of corticosterone to aldosterone (Fraser et al., 1978 & 1979).

In normal subjects ambulation leads to increases in the concentrations of aldosterone and 18OHB relative to the values measured while still recumbent. In contrast patients with APA have anomalous postural fall of both steroid concentrations on

ambulation following overnight recumbency (Biglieri et al., 1979). However, overnight saliva 18OHB concentration in normal subjects declined significantly. The aldosterone and 18OHB:aldosterone ratio did not change significantly, but saliva GC decreased in parallel with 18OHB. This suggests the influence of ACTH on 18OHB secretion. The four hours of ambulation is now found to be an inappropriate protocol for studying the effect of change of posture on 18OHB or aldosterone response. The period is perhaps too long and hence the influence cannot be due to ambulation alone. A postural change from overnight recumbency to standing upright without any ambulation could be a potent stimulus but this is impractical and causes discomfort to the subjects.

In conclusion, the most important finding in this thesis is that the 18OHB:aldosterone ratio cannot be regarded as a static parameter which previous investigators have found with single blood samples or samples collected several hours apart. Because my assay technique is easier to perform, and because of the ease of collecting saliva samples, I have been able to study the hour by hour fluctuations in the levels of these two hormones. This shows that the ratio is changing continously. Though small differences in the kinetics of clearance will make some contribution to this ratio I believe that most of it is due to the secretion of 18OHB and aldosterone not being completely synchronous. This suggests

that the secretion of the two compounds might be independently controlled which in turn suggests that 18OHB might have a physiological role independent of it possibly being a precursor of aldosterone. The methodology described in this thesis should enable us to accumulate data on the changes in 18OHB level more readily and so facilitate elucidation of its physiological significance.

Appendix I

Preparation of cortisol-3-CMO crystals

F in methanol (140 µmoles in 8 ml) + $(CMO)_2$-HCl in methanol (70 µmoles in 3 ml)

↓

stirring, 15', r.t. (23^0)

↓

added 0.1M Na_2CO_3 (10 ml)

↓

extract with Dichloromethane (50 ml x2)

↓

aqueous layer acidified with 0.1M HCl (10 ml)

↓

extract with ethyl acetate (50 ml x 3)

↓

water wash(50 ml x 3)

↓

dry with Na_2SO_4

↓

dryness in round bottom flask

↓

dissolved in ethyl acetate (3 ml)

↓

saturate the solution by removing excess ethyl acetate under nitrogen gas

↓

heat the solution in a water bath with a reflux arm attached

↓

add iso-octane dropwise until the solution became opalescent

↓

leave to crystallise at -20 C

↓

remove mother liquor

↓

wash crystal with ice cold ethyl acetate

↓

decant ethyl acetate

↓

dry crystal

↓

melting point determination

Results

Batch 1 m.p. = 195-196 C
Batch 2 m.p. = 199-201 (1st harvest)
m.p. = 196-198 (2nd harvest)

Concentration of steroids in plasma and saliva from various sources. + indicates values obtained from the book entitled "Hormones in blood Vol. 3 (1979) with page number quoted above in the reference column. Values were used as guide line for cross-reactivity study with antiserum raised against 18OHB using antiserum #5/12/6 and [^{125}I]radioligand.

Steroids	Plasma concentration (pmol/l) except * (nmol/l)		References
1. 18-OH-DOC	?random	578 - 4624	Mason and Fraser 1975
	0800h, supine	289 ± 188	Sulan & Sparano 1978
	Ambulant	272 ± 121	
	0800h (supine for 2h)	237 ± 112	Ojima & Kambegawa 1979
2. 18-OH-Prog	Urinary excretion: 0.034 ± 0.013 µg/24h n=17		Lewicka et al., 1986
3. 18-OH-F	random	870 - 3920	Corrie et al., 1985
4. Progesterone (Prog)	Follicular	* 1	Guerrero et al., 1976
	Luteal	* 16 - 112	(RIA method)
5. 17a-OH-Prog	Age: 0.5 yr - 16 yr		Hughes & Winter (1976) + pp218
6. Androstene-dione	Follicular	4896 ± 2276	+ pp380
	Mid-cycle	7586 ± 1551	
	Luteal	4138 ± 827	

7. Testosterone	Female:			
	Follicular		1173 ± 174	Vermeulen (1979)+ pp380
	Mid-cycle		1457 ± 243	
	Luteal		1319 ± 208	
	Male	*	22.6	Vermeulen (1979)+ 376
8. Oestradiol	Follicular		106	Baird & Guevara (1969)
	Midcycle		1167	+ pp 323
	Luteal		700	
9. Oestrone	Follicular		148	Baird & Guevara (1969)
	Midcyle		629	+ pp323
	Luteal		447	
10. Oestriol	Pregnancy (30 wk)	*	10 - 21	Cohen & Cohen (1974) + 326
11. Aldosterone	random		120 - 935	Few et al., 1986a
12. DOC	random	*	29 - 346	Arnold & James (1971)
13. Corticosterone (B)	0800 Male:	*	11.4 ± 6.6	Nabors et al., 1974
	Female:	*	18.9 ± 7.8	+ pp218
14. Cpd S (11-deoxycortisol)	random	*	4.9 ± 0.28	Newsome et al (1972) + pp217
15. Cortisone		*	10 - 30	Few & Cashmore, 1980
16. Cortisol (F)	0800h	*	138 - 552	Rolleri et al., 1976

17.	Saliva Cortisol	0900h	* 9.7 ± 4.0 (n=4)	Walker et al., 1978
18.	Saliva 17a-Prog	(0900h)	90 - 1500	Walker et al., 1979
19.	Saliva aldosterone	random	0 - 150	Few et al., 1986a

Appendix IIIa

Plasma 18OHB concentration in normal subjects:

Authors, year	Posture and Actual Time of blood sampling (h)	Plasma 18OHB mean ± SD (n) (pmol/l)	Salt intake
1. Martin et al. (1975)	Standing (0830)	621 ± 256 (17)	ad. libitum salt intake
	30 min recumbent (1000-1030)	273 ± 105 (6)	
(1978)	-	617 ± 274(SE)(15)	Low salt (Na=9meq/day)
		170 ± 81 (SE)(15)	High Na diet (Na=249 meq /day)
2. Wilson et al. (1976)	Recumbent overnight (0800)	416 ± 212 (9)	-
	Ambulant : (1200)	311 ± 168 (9)	
	(2300)	165 ± 66 (9)	

Appendix IIIa

Authors, year	Posture and Actual Time of blood sampling (h)	Plasma 18OHB mean ± SD (n) (pmol/l)	Salt intake
3. Belkien et al. (1980)	Ambulant (0800-0900)	768 ± 243 (16)*1 944 ± 119 (6)*2	Normal diet
	Upright (0900)	631 ± 298 (6)	
	1h recumbent (1000)	311 ± 61 (6)	
4. Ojima & Kambegawa (1979)	Before standing	311 ± 49 (30)	ad. libitum sodium intake
	2h standing	560 ± 108 (30)	
(1980)	After lying for 2h (0800)	342 ± 127 (10) *1 381 ± 113 (10) *2 284 ± 116 (20) *3	
5. Witzgall & Hassan-Ali (1981)	Ambulant (0800-1300)	1243 ± 82 (15) *4	Normal diet

Appendix IIIa

Authors,year	Posture and Actual Time of blood sampling (h)	Plasma 18OHB mean ± SD (n) (pmol/l)	Salt intake
6. Watanebe et al. (1984)	Supine but duration was not indicated	471 ± 248 (20)	Ad. libitum salt intake
7. Biglieri et al.(1979) *5	(0800) 4h upright (1200)	256 ± 55 (17) 621 ± 85 (17)	metabolically balanced Na & K (Na=112-122 meq/day; K= 58-82 meq/day)
8. Biglieri et al.(1982)	Recumbent overnight (0800)	511 ± 49.7 (20)	as 7
9.Bravo et al. (1983)*5	recumbent overnight	400 to 717 (20)	Normal dietary Na intake

Appendix IIIa

Authors, year	Posture and Actual Time of blood sampling (h)	Plasma 18OHB mean ± SD (n) (pmol/l)	Salt intake
10. Imaizumi et al. (1984) *6	recumbent overnight (0800)	590 ± 240 (14)	Ad. libitum Na intake
	2h recumbency (1000-1200)	420 ± 140 (6)	
11. Schoneshofer et al. (1985) *7	2h upright (0800 to 1000)	550 to 1680	Ad. libitum dietary intake
12. Kem et al. (1985)	Seated for 15 minutes. Blood taken with a cathether inserted into the antecubital vein	1795 ± 1436 (9) *8	Ad. libitum dietary intake

Appendix IIIa

Authors,year	Posture and Actual Time of blood sampling (h)	Plasma 18OHB mean ± SD (n) (pmol/l)	Salt intake
13.Kater et al. (1985)	recumbent overnight (0800)	643 ± 60 (15)	Overnight fast

*1 Female, follicular phase;

*2 Female, luteal phase;

*3 Male subjects;

*4 values obtained by estimation from graphical representation.

- means that conditions was not apparent.

*5 Antibodies for the assay from Martin et al. (1975);

*6 Antibodies for the assay from Ojima & Kambegawa (1979);

*7 Antibodies for the assay from Belkien et al. (1980);

*8, if the high level (5397 pmol/l) of an active female long distance runner is excluded, the value becomes (1334 + 480 pmol/l).

Appendix IIIb

Plasma 18OHB and aldosterone levels in patients with primary hyperaldosteronism and essential hypertension after overnight recumbency and 4 hours of ambulation where blood was sampled at 0800h and 1200h respectively. The authors are numbered as in Appendix IIIa:

Authors	CONC	Actual time of blood sampling:			
	(pmol/l)	0800h	1200h	0800h	1200h
6+.	18OHB	greater than normal range(4 out of 5 APA)			
			mean ± SD		
7.		APA (n=9)		IHA (n=14)	
	18OHB	4848 ± 99	2969 ± 524	668 ± 102	1353 ± 259
	Aldo	1716 ± 302	1322 ± 252	366 ± 33	902 ± 136
	18OHB: Aldosterone	2.9 ± 1.0	2.5 ± 0.3	1.9 ± 0.2	1.4 ± 0.2
	K^+	2.6 ± 0.17	2.9 ± 0.22	3.3 ± 0.09	3.5 ± 0.08
	PRC (ng/ml/h)	0.39 ± 0.20	0.37± 0.13	0.52 ± 0.08	1.1 ± 0.2
8.		APA (n=15)		IHA (n=10)	
	18OHB	3795 ± 613	-	784 ± 113	-
	Aldo	1458 ± 274	-	455 ± 42	-
9.	18OHB	greater than 1380 (n=29 out of 30)			
	Aldo	1244 ± 1016	-	1138 ± 399	-

Appendix IIIb

Authors	CONC	Actual time of blood sampling:			
	(pmol/l)	0800h	1200h	0800h	1200h
10.		APA (n=5)			
	18OHB	2620 to 5830	-	-	-
12*.		APA (n=23)		IHA (n=9)	
	18OHB	5911 ± 4226	-	2171 ± 558	-
	Aldo	1888 ± 1499	-	888 ± 494	-
	18OHB: Aldosterone	3.55 ± 1.30		3.30 ± 2.1	
13.		APA (n=31)		IHA (n=15)	
	18OHB	4505 ± 450	-	867 ± 80	-
	Aldo	1619 ± 166	-	422 ± 1.2	-
	18OHB: Aldosterone	2.85 ± 0.15		2.16± 0.19	

6+ Bilateral hyperplasia (n=1); 18OHB within normal range.

12* Essential hypertensives : 18OHB = 1759 ± 687 (n=10),
Aldo = 1011 ± 1072,
18OHB:Aldo ratio= 5.2 ± 2.5,
K^+ 4.19 ± 0.26 ; (the normal range reported by them is higher than values obtained by other authors).

\- Values not measured

References

Abraham GE (1969) Solid-phase radioimmunoassay of oestradiol-17ß. J Clin Endocrinol Metab 29: 866-870.

Abraham GE (1975) Radioimmmunoassay of steroids in biological fluids. J Steroid Biochem 6: 261-270.

Al-Dujaili EAS and Edwards CRW (1978) The development and application of a direct radioimmunoassay for plasma aldosterone using [^{125}I]-labelled ligand: comparison of three methods. J. Clinical Endocrinol. & metabolism 46: 105-113.

Aragones A, Gros EG, Lantos CP and Locascio GA (1978) Less polar forms and derivatives of 18-hydroxycorticosterone. J Steroid Biochem 9: 175-180.

Arnold ML and James VHT (1971) Determination of deoxycorticosterone in plasma. Steroids 18: 789-801.

Baird DT, Horton R, Longcope C and Tait JF (1969) Steroids dynamics under steady-state conditions. Recent Progress in Hormone Research 25: 611-656.

Bauknecht H, Vecsei P, Endres H and Hettenbach A (1982) Urinary free 18-hydroxycorticosterone, plasma aldosterone, and urinary aldosterone metabolites in normal pregnancy. Am J Obstet Gynecol 144: 28-34.

Belkien L, Schoneshoffer M and Oelkers W (1980) Development and characterisation of antisera to 18-hydroxycorticosterone and radioimmunoassay for serum 18-hydroxycorticoterone. Steroids 35: 427-437.

References

Biglieri EG and Schamebelan M (1979) The significance of elevated levels of plasma 18-hydroxycorticosterone in patients with primary aldosteronism. J Clin Endocrinol Metab. 49: 87-91.

Biglieri EG, Kater CE, Brust N, Chang B and Hirai (1982) The mineralocorticoid hormone pathways in hypertension with hyperaldosteronism. Clin and Exper Hyper -theory and Practice, A4 (9&10): 1677-1683.

Borek E and Clarke HT (1936) Carboxymethoxylamine. J Am Chem Soc 58: 2020-2021.

Bougas J, Flood C, Little B, Tait JF, Tait SAS and Underwood R (1964) Dynamic aspects of aldosterone metabolism. In: Aldosterone, (Baulieu EE and Robel P,eds), pp25-50. Backwell Scientific Publications, Oxford.

Bravo EL, Tarazi RC, Dustan HP, Fouad PM, Textor SC, Gifford RW and Vidt DG (1983) The changing clinical spectrum of primary aldosteronism. Am J Med 74: 641-651.

Caesar J, Shaldon S, Chiandussi L, Guevara L and Sherlock S (1961) The use of indocyanine green in the measurement of hepatic blood flow and as a test of hepatic function. Clin Sc 21: 43-57.

Cahn, Ingold and Prelog (1985) In: Advanced organic Chemistry, 3rd edition (March J ed) pp96-97. John Wiley & Sons New York Chichester Brisbane Toronto Singapore.

References

Cherrick GR, Stein SW, Leevy CM and Davidson CS (1960) Indocyanine green: observations on its physical properties, plasma decay and hepatic extraction. J Clin Invest 39: 592-600.

Coghlan JP and Blair-West JR (1967) Aldosterone. In: Hormones in blood, Volume 2, 2nd edn (Gray CH and Bacharach eds) pp 391-488. Academic press. London and New York.

Connolly TM, Vecsei, Haack D, Kohl KH, Abdelhamid and Ammenti A (1978) Aldosterone diagnosis in hypertension: Comparative evaluation of radioimmunoassays for urinary aldosterone and 18-hydroxycorticosterone. Klin. Wochenschr 56 (suppl. I): 173-181.

Corrie JET, Edwards CRW, Jones DB, Padfield and Budd PS (1985) Factors affecting the secretion of 18-hydroxycortisol, a novel steroid of relevance to Conn's syndrome. Clin Endocrinol 23: 579-586.

Cozza EN, Lantos CP and Burton G (1985) A highly lipophilic form of aldosterone. Isolation and characterisation of an aldosterone dimer. J Steroid Biochem 23: 511-516.

Damasco MC, Cenal FDJP and Lantos CP (1979) Acute effects of three natural corticosteroids on the acid-base and electrolyte composition of urine in adrenalectomised rats. Acta Physiol Latino Am 29: 305-314.

Nomogram of plasma volume using body mass and age (1970) In: Documenta Geigy, 7th edition (Diem K and Lenther C eds) pp556 Published by J.R. Geigy S.A., Basle, Switzerland.

Dunn JF, Nisula BC and Rodbard D (1981) Transport of steroid hormones: Binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 53: 58-68.

Ekins RP (1978) General principles of hormone assay. In: Hormone assays and their clinical application (Lorraine J and Bell ET eds) pp 1-72. Churcill Livingstone, London.

Erlanger BF, Borek F, Beiser SM and Lieberman S (1957) Preparation and characterisation of conjugates of bovine serum albumin with testosterone and cortisone. J Biol Chem 228: 713-727.

Erlanger BF, Borek F, Beiser SM and Lieberman S (1959) II Preparation and characterisation of conjugates of bovine serum albumin with progesterone, deoxycorticosterone and oestrone. J Biol Chem 234: 1090-1094.

Feldman H and Rodbard D (1971) Mathematical theory of radioimmunoassay. In: Principles of competitive protein-binding assays (Odell W and Daughaday W eds), pp158-203. J.B.Lippincott company, Philadelphia, and Toronto: Lippincott.

References

Ferriss JB, Brown JJ, Fraser R, Lever AF and Robertson JIS (1983) Primary aldosterone excess: Conn's syndrome and similar disorder. In: Handbook of Hypertension (Robertson JIS ed), pp132-161. Amsterdam : Elsevier.

Ferriss JB, Brown JJ, Fraser R, Lever AF and Robertson JIS (1981) Primary hyperaldosteronism. Clinics in Endocrinol Metab 10: 419-452.

Few JD and Cashmore GC (1980) Plasma cortisone in man: its determination, physiological variations, and its significance. Ann Clin Biochem 17: 227-232.

Few JD, Chaudry S and James VHT (1984) The direct determination of aldosterone in human saliva. J Steroid Biochem 21: 87-92.

Few JD, Paintin DB and James VHT (1986a) The relationship between plasma and saliva aldosterone during pregnancy. Br J Obstet Gynaecol 93: 928-932.

Few JD, Wallis PJW and James VHT (1986b) The relationship between salivary aldosterone and plasma free aldosterone concentrations. Clin Endocrinol 24: 119-126.

Few JD, Unwin RJ, Carmichael DJS and James VHT (1987a) Diurnal fluctuation in saliva aldosterone concentration. J Steroid Biochem 26: 265-271.

Few JD, Lee CY and Imms F (1987b) Kinetics of the clearance of aldosterone: comparison with cortisol and 18OHB. J Endocrinol 112 (supplement): Abstract 245.

Fraser R and Lantos CP (1978) 18-hydroxycorticosterone: A review J Steroid Biochem 9: 273-286.

Fraser R, Brown JJ, Lever, Mason PA and Robertson (1979) Control of aldosterone secretion. Clin Sc 56: 389-399.

Fraser R, Beretta-Piccoli C, Brown JJ, Cumming AMM, Lever AF, Mason PS, Morton JJ and Robertson JIS (1981) Response of aldosterone and 18-hydroxycorticosterone to angiotensin II in normal subjects and patients with essential hypertension, Conn's syndrome, and nontumorous hyperaldosteronism Hypertension (Supp. I) 3: I-87 - I-92.

Fraser R, Beretta-Piccoli, Brown JJ, Lever AF, Morton JJ and Robertson (1982) Control of plasma corticosteroid concentrations in patients with essential hypertension, Conn's syndrome and non-tumorous (idiopathic) hyperaldosteronism. In: Endocrinology of hypertension, Proceedings of the Serono Symposia Vol 50 pp 29-40 (Mantero F, Biglieri EG and Edwards CRW eds). Academic Press London New York Paris San Diego San Francisco Sao Paulo Sydney Tokyo Toronto.

Ganguly A, Melada GA, Luetscher JA and Dowdy AJ (1973) Control of plasma aldosterone in primary aldosteronism: Distinction between adenoma and hyperplasia. J Clin Endocrinol Metab 37: 765-775.

Guerrero R, Aso T, Brenner PF, Cekan Z, Landgren BM, Hagenfeldt K and Diczfalusy E (1976) Studies on the pattern of circulating steroids in the normal menstrual cycle. Acta Endocrinol (Copenh) 81: 133- 149.

Guthrie GP (1981) Multiple plasma steroid responses to graded ACTH infusions in patients with primary aldosteronism. J Lab Clin Med 98: 364-373.

Hellman L, Nakada F, Curti J, Weitzman ED, Kraem J, Roffwang H, Ellman S, Fukushima DK and Gallagher TF (1970) Cortisol is secreted episodically by normal man. J Clin Endocrinol 30: 411-422.

Huston G, Martin VI, Al-Dujaili EA and Edwards CRW (1981) Evaluation of the mineralocorticoid activity of 18-hydroxycorticosterone. Clin Sc 61: 201-206.

Imaizumi N, Morimoto S, Kigoshi T, Uchid K, Hosojima A and Yamamoto I (1984) Application of reversed-phase high performance liquid chromatography for radioimmunoassay of plasma 18-hydroxycorticosterone. J Chromatography 308: 295-300.

James VHT, Tunbridge D, Wilson GA, Hutton JD, Jacobs HS, Goodall AB, Murray MAF and Rippon AE (1978) Central control of steroid hormone secretion. J Steroid Biochem 9: 429-436.

References

Janoski AH, Shulman FC, Wright GE (1974) Selective 3-(o-carboxymethyl)oxime formation in steroidal 3,20-diones for hapten immunospecificity. Steroids 23: 49-64.

Kahnt FW and Neher R (1965) Uber die adremale steroid-Biosynthese in vitro. I Umwandling endogener und exogener vorstufer im Nebennieren-Homogenat des Rindes (German). Helv Clim Acta 48: 1457 - 1476.

Kater CE, Biglieri EG, Rost CR, Schambelan M, Hirai J, Chang BCF and Brust N (1985) The constant plasma 18OHB to aldosterone ratio. An expression of the efficacy of corticosterone methyloxidase type II activity disorders with variable aldosterone production. J Clin Endocrinol Metab 60: 225-228.

Katz FH and Shannon IL (1969) Parotid fluid cortisol and cortisone. J Clin Invest 48: 848-855.

Kem DC, Weinberger MH, Gomez-Sanchez C and Higgins Jr (1976) The role of ACTH in the episodic release of aldosterone in patients with idiopathic adrenal hyperplasia, hypertension, hyperaldosteronism. J Lab Clin Med 88 261-270.

Kem DC, Tang K, Hanson CS, Brown RD, Painton R, Weinberger MH and Hollifield HW (1985) The prediction of anatomical morphology of primary aldosteronism using serum 18-hydroxycorticosterone levels. J Clin Endocrinol Metab 60: 67-73.

Knupper R, Haupt M and Brewer H (1965) Formation of 15α-hydroxyoestradiol-17β and 18-oestrone by human adrenal tissue. J Endocrinol 33: 529-530.

Kojima I, Inano H and Tamaoki B-i (1982) The final step of aldosterone biosynthesis is catalysed by an NADPH-dependent and molecular oxygen-requiring enzyme. Biochem & Biophysical Res Comms 106: 617-624.

Kojima I, Ogata E, Inano H and Tamaoki B (1984) Oxygenation of 18-hydroxycorticosterone as the final reaction for aldosterone biosynthesis. Acta Endocrinologica 107: 395-400.

Kondo E, Mitsugi T and Tocile K (1965) Microbiological synthesis of aldosterone from corticosterone. J Am Chem Soc 87: 4655-4656.

Kowarski A, Katz H, Migeon CJ (1974) Plasma aldosterone concentration in normal subjects from infancy to adulthood. J Clin Endocrinol Metab 38: 489-491.

Lanthier A and Sandor T (1973) The effect of 18-hydroxycorticosterone on the salt-secreting gland of the duck (Anas platyrhynchos). Can J Physiol Pharmacol 51: 776-778.

Lee CY, Few JD and James VHT (1985) Measurement of 18-hydroxycorticosterone in saliva. J Endocrinol 107: Abstract 59.

Lee CY, Few JD and James VHT (1987) Comparison of the saliva and plasma concentration of 18-hydroxycorticosterone, aldosterone, cortisol in response to corticotrophin. J Endocrinol 112: Abstract 244.

Lewicka S, Bige K, Bauer H, Heinrich U, Abdelhamid S and Vecsei P (1986) 18-hydroxyprogesterone - New parameter in the 21-hydroxylase deficiency diagnosis J Steroid Biochem 25: (supplement 105) 5.

Martin LF, Mikulecky M, Blaschke TF, Waggoner JG, Vergalla J and Berk PD (1975) Differences between the plasma indocyanine green disappearance rates of normal men and women (39090) Proceedings of the Society for Experimental Biology and Medicine 150: 612-617.

Martin VI, Edwards CRW, Biglieri EG, Vinson GP and Bartter FC (1975) The development and application of a radioimmunoassay for 18-hydroxycorticosterone. Steroids 26: 591-604.

Marusic ET, White A and Aedo AR (1973) Oxidative reactions in the formation of an aldehyde group in the biosynthesis of aldosterone. Arch Biochem Biophys 157: 320-321.

Mason PA & Fraser R (1975) Estimation of aldosterone, 11-deoxycorticosterone, 18-hydroxydeoxycorticosterone, corticosterone, cortisol and 11-deoxycortisol in human plasma by gas-liquid chromatography with electron capture detection. J Endocrinol 64: 277-288.

References

Mason PA, Fraser R, Morton JJ, Semple PF and Wilson A (1976) The effect of AII infusion on plasma corticosteroid concentrations in normal man. J Steroid Biochem 7: 859-861n

Mason PA, Fraser R, Morton JJ, Semple PF and Wilson A (1977) The effect of sodium deprivation and of angiotensin II infusion on the peripheral plasma concentrations of 18-hydroxy-corticosterone, aldosterone and other corticosteroids in man J Steroid Biochem 8: 799-804.

Mckenzie JK and Clements JA (1974) Simplified radioimmunoassay for serum aldosterone utilizing increased antibody specificity. J Clin Endocrinol Metab 38: 622-627.

McVie R, Levine LS and New M (1979) The biologic significance of the aldosterone concentration in saliva. Paediatric Res 13 755-759.

Nars PW and Hunter WM (1973) A method for labelling oestradiol-17 with radioiodine for radioimmunoassays. Proc Soc Endocrinol 57: xlvii-xlviii.

Nagahama S, Fujimaki M, Kawabe H, Nakamura R, Saito I and Saruta T (1983) Effect of metoclopramide on the secretion of aldosterone and other adrenocortical steroids. Clin Endocrinol 18: 287-293.

Neher R (1979) Aldsoterone: Chemical aspects and related enzymology. J Endocrinol 81: 25p-35p.

Nicolis GL and Ulick S (1965) Role of 18-hydroxylation in the biosynthesis of aldosterone. Endocrinol **76**: 514-521.

Ojima M and Kambegawa A (1979) In vivo and in vitro studies on 18-hydroxy-11deoxycorticosterone and 18-hydroxycorticosterone in normal subjects and in those with various adrenocortical disorders. Folia endocrinol jap **55**: 994-1006.

Rolleri E, Zannino M, Orlandini S and Malvano R (1976) Direct radioimmunoassay of plasma cortisol. Clinica Chimica Acta **66**: 319-330.

Roy AK, Ramirez LC and Ulick S (1976) Structure and mechanism of formation of the two forms of 18-hydroxy-11-deoxycorticosterone. J Steroid Biochem **7**: 81-87.

Schambelan M, Norma Brust NL, Chang BCF, Slater KL & Biglieri EG (1976) Circadian rhythm and effect of posture on plasma aldosterone concentration in primary aldosteronism. J Clin Endocrinol Metab **43**: 115-131.

Schmidlin VJ und Wettstein A (1960) 125. Synthese Aldosteron-ahnlicher corticosteroids d,l-11-oxo-18hydroxy-cortexon. Helvetic Chimica Acta XLIII: 973-983.

Schmied U, Vetter W, Nussberger J and Siegenthaler (1975) Production and characterisation of antisera to 18-hydroxy-11-deoxycorticosterone. Steroids **26**: 478-487.

Schoneshofer M, Fenner A and Dulce HJ (1981) Assessment of eleven adrenal steroids from a single serum sample by combination of automatic high-performance liquid chromatography and radioimmunoassay. J Steroid Biochem 14: 377-386.

Sowers JR and Stern N (1982) Influence of sodium homeostasis and circadian rhythm on dopaminergeric modulation of 18OHB secretion in man. J Clin Endocrinol Metab 55: 1046-1051.

Sowers JR, Martin VI and Beck FWJ (1983) Effects of dietary sodium on circadian rhythm and physiological responses of 18-hydroxycorticosterone. Clin Sc 64: 295-301.

Sulan J, Sparano F, Genard P, Sciarra F, Cauwenberge HV and Conti C (1978) Episodic secretion of 18-hydroxy-11-deoxy corticosterone in normal and hypertensive men: relationship with cortisol and aldosterone secretion. In: The endocrine function of the human adrenal cortex (James VHT, Serio M, Gusti F and Martini L eds) pp 375-492. Academic Press London New York San Francisco.

Tait JF, Tait SAS, Little B and Laumas KR (1961) The disappearance of 7-H3-d-aldosterone in the plasma of normal subjects. J Clin Invest 40: 72-80.

Tait JF (1963) Review: The use of isotopic steroids for the measurement of production rates in vivo. J Clin Endocrinol Metab 23: 1285-1297.

Tuck ML, Sowers JR, Asp ND, Viosca SP, Berg G and Mayes DM (1981) Mineralocorticoids response to low dose adrenocorticotropin infusion. J Clin Endocrinol Metab 52: 440-446.

Ulick S and Lieberman S (1957) Evidence for the occurrence metabolite of aldosterone in urine. J Am Chem Soc 79: 6567-6568.

Ulick S and Vetter KK (1962) Identification of two C18 oxygenated corticosteroids isolated from human urine. J Biol Chem 237: 3364-3374.

Ulick S, Nicolis GL and Vetter KK (1964a) Relationship of 18-hydroxycorticosterone to aldosterone. In: Aldosterone (Baulieu EE and Robel P eds) pp3-17. Blackwell Scientific Publication. Oxford.

Ulick S, Gautier E, Vetter KK, Markello JR, Yaffe S and Lowe CU (1964b) An aldosterone biosynthetic defect in a salt-losing disorder J Clin Endocrinol Metab 24: 669.

Ulick S and Vetter KK (1965) Simultaneous measurement of secretory rates of aldosterone and 18-hydroxycorticosterone. J Clin Endocrinol Metab 25: 1015-1026.

Ulick S (1973) Aldosterone biosynthesis: Normal and alternate pathways in aldosterone biosynthesis. In: Proceedings of the Fourth International Congress of Endocrinology. Washington, D.C. June 18-24 1972 (Robert Scow ed) pp761-767. Excerpta Medica Amsterdam American Elsevier Publishing Company Inc. New York 1973.

Ulick S (1976) Diagnosis and nomenclature of the disorder of the terminal portion of the aldosterone biosynthesis pathway. J Clin Endocrinol Metab **43**: 92-96.

Usa T, Ganguly A and Weinberger MH (1979) M and L forms of 18-hydroxy-11-deoxycorticosterone and 18-hydroxycorticosterone: factors influencing conversion, stability and immunological chemistry. J Steroid Biochem **10**: 557-562.

Vaitukaitis J, Robbins JB, Nieschlag E and Ross GT (1971) A method for producing specific antisera with small doses of immunogen J Clin Endocrinol **33**: 988-991.

Vaitukaitis JL (1981) Production of antisera with small doses of immunogen: multiple intradermal injections. Methods in Enzymology **73**: 46-52.

Vaughan JR Jr., and Eichler JA (1953) The preparation of optically-active peptides using mixed carbonic-carboxylic acid anhydrides. J Am Chem Soc **75**: 5556-5560.

Vetter H and Vetter W (1975) Regulation of aldosterone secretion in primary aldosteronism. Horm Metab Res 7: 418-424.

Watanabe F, Ryoto T, Kobayashi Y (1984) Enzyme immunoassay for serum 18-hydroxycorticosterone and its clinical application. Steroids 43: 509-516.

Watanuki M, Tilley BE and Hall PF (1977) Purification and properties of cytochrome P-450 (11β and 18-hydroxylase) from bovine adrenocortical mitochondria. Biochim Biophys Acta 483: 236-247.

Weitzman ED, Schaumberg and Fishbein W (1966) Plasma 17-hydroxycorticosteroid levels during sleep in man. J Clin Endocrinol 26: 121-127.

Walker RF, Riad-Fahmy D and Read GF (1978) Adrenal staus assessed by direct radioimmunoassay of cortisol in whole saliva or parotid saliva. Clin Chem 24: 1460-1463.

Wenting GJ, Man In't Veld AJ, Derkx FH, Brummelen PV and Schalekamp MADH (1978) ACTH-dependent aldosterone excess due to adrenocortical adenoma: a variant of primary aldosteronism. J Clin Endocrinol Metab 46: 326-335

Wheeler HO, Cranson WI and Meltzer JI (1958) Hepatic uptake and biliary excretion of indocyanine green in the dog. Proceedings of the Society for Experimental Biology & Medicine 99: 11-14.

Wilson A, Mason PA and Fraser R (1976) Estimation of 18-hydroxycorticosterone concentration in human peripheral plasma by gas-liquid chromatography with electron capture detection. J Steroid Biochem 7: 611-613.

Wisgerhof M, Brown RD, Hogan MJ, Carpenter PC and Edis AJ (1981) The plasma aldosterone response to angiotensin II infusion in aldosterone-producing adenoma and idiopathic hyperaldosteronism. J Clin Endocrinol Metab 52: 195-198.

Witzgall A and Hassan-Ali S (1981) A simultaneous radioimmunoassay for aldosterone and its precursors:- Human plasma levels following the inhibition of converting enzyme, before and after blockade of prostaglandin biosynthesis. J Clin Chem Clin Biochem 19: 387-394.

Yanagibashi K, Haniu M, Shively JE, Shen WH and Hall P (1986) The synthesis of aldosterone by the adrenal gland. J Biol Chem 261: 3556-3562.

Zager PG, Frey HJ, Spalding CT, Wengs WJ and Brittenham MC (1986) Distribution of 18-hydroxycorticosterone between red blood cells and plasma. J Clin Endocrinol Metab 62: 84-89.

Appendum

Beretta-Piccoli C, Davies DL, Brown JJ, Ferriss B, Fraser R, Lasaridis A, Lever AF, Morton JJ, Robertson JIS, Semple PF and Watt R (1983) Relation of blood pressure with body and plasma electrolytes in Conn's syndrome. J Hypertension 1: 197-205.

Chu MD, Ulick S (1982) Isolation and identification of 18-hydroxycortisol from the urine of patients with primary aldosteronism. J Biol Chem 257: 2218-2224.

Davies DL, Beevers DG, Brown JJ, Cumming AMM, Fraser R, Lever AF, Mason PA, Morton PA, Robertson JIS, Titterington M and Tree M (1979) Aldosterone and its stimuli in normal and hypertensive man: are essential hypertension and primary hyperaldosteronism with tumour the same condition. J Endocrinol 81: 79p-91p.

Kirk DN, and Rajagopalan MS (1976) A synthesis of 11β,18, 21-trihydroxypregn-4-ene-3,20-dione (18-hydroxycorticosterone). J Chem Soc Chem Commun 2: 77-78.

Kirk DN and Slade CJ (1981) 18-substituted steroids. Part 8. An improved synthesis of 18-hydroxycorticosterone. J Chem Soc Perkin 1: 703-705.

Tori K, Tomita T, Itazaki H, Narisada M and Nagata W (1963) Nuclear magnetic resonance spectroscopic evidence for distorted conformations of the C rings in 8β, 11β- and 11β, 13β-bridged steroids. Chem Pharm Bull (Japan) 11: 956-959.