THE EFFECT OF ETHANOL ON CORTISOL METABOLISM IN MAN

A thesis presented in fulfilment of the requirements for the degree of Master of Science in Biochemistry at Massey University

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ABSTRACT

Methods were developed for the estimation of human plasma cortisol by radioimmunoassay and urinary 6β-hydroxycortisol (6βOHF) by colorimetry after separation by thin layer chromatography (TLC). In addition profiles of urinary neutral steroids were obtained by gas chromatographic separation of methoxime-trimethylsilyl derivatives from urine extracts on a glass capillary column. This approach was found to be more sensitive and reproducible than profile studies based on TLC separation and colorimetric estimation.

Pilot studies of the plasma cortisol levels of normal subjects showed a consistent rise in cortisol during alcohol loading under the conditions of the observations, but in hospital patients admitted with acute alcohol intoxication, variability in the experimental conditions masked any consistent changes. Large variations in method reproducibility as well as subject differences affected results from the measurement of 6βOHF and chloroform extractable 17-hydroxycorticosteroids in one normal and four alcoholic subjects, rendering apparent initial differences insignificant. The results suggest, but do not demonstrate, that alcohol ingestion may divert normal cortisol metabolism into a pathway leading to the production of 6βOHF.

Urinary steroid profiles obtained from two normal subjects, one normal subject under conditions of alcohol load and one alcoholic subject suggest that any effects of alcohol on cortisol metabolism are subtle and would require study of a large number of cases to define them.

This work has served to delineate the faults and potential of various approaches to the study of cortisol metabolism and the possible effects of alcohol thereon. It would seem that their application in carefully designed and well controlled experiments to a larger number of subjects is necessary to obtain the information desired.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

GENERAL

A.C.T.H.	adrenocorticotrophic hormone
BTZ	Blue Tetrazolium (chloride)
CBE	cholesterol n-butyl ether
TSIM	trimethylsilyl imidazole
TMS	trimethylsilyl
GC	gas chromatography
GLC	gas liquid chromatography
TLC	thin layer chromatography
PC	paper chromatography
RIA	radioimmunoassay
Kg	Kieselghur
Si gel	silica gel
17-KS	steroid with keto group at 17-carbon position
17-OHCS	steroid with hydroxyl group at 17-carbon position
EtOAc	ethyl acetate
EtOH	ethanol
MeOH	methanol
CH_2Cl_2, DCM	dichloromethane
C-19	steroid with no side chain at carbon 17
C-21	steroid with 2 carbon side chain at carbon 17

STEROIDS

Abbreviation	Trivial name	Systematic name
An	androsterone	5α-androstan-3α-ol-17-one
Et	etiocholanolone	5β-androstan-3α-ol-17-one
11-HAn	11-hydroxyandrosterone	5α-androstan-3α,11β-diol-17-one
11-HEt	11-hydroxyetio-cholanolone	5β-androstan-3α,11β-diol-17-one
11-KAn	11-ketoandrosterone	5α-androstan-3α-ol-11,17-dione
11-KEt	11-ketoetiocholanolone	5β-androstan-3α-ol-11,17-dione
DHEA	dehydroepiandrosterone	5-androsten-3αol-17-one
Pd	pregnanediol	5β-pregnan-3α,20α-diol
Pt	pregnanetriol	5β-pregnan-3α,17α,20α-triol
Atr	androstenetriol	5-androsten-3β,16α,17β-triol

E	cortisone	4-pregnen-17α,21-diol-3,11,20-trione
F	cortisol	4-pregnen-11β,17α,21-triol-3,20-dione
THE	tetrahydrocortisone	5β-pregnan-3α,17α,21-triol-11,20-dione
THF	tetrahydrocortisol	5β-pregnan-3α,11β,17α,21-tetrol-20-one
a-THF	allo-tetrahydrocortisol	5α-pregnan-3α,11β,17α,21-tetrol-20-one
αCo	αcortolone	5β-pregnan-3α,17α,20α,21-tetrol-11-one
βCo	βcortolone	5β-pregnan-3α,17α,20β,21-tetrol-11-one
αCor	αcortol	5β-pregnan-3α,11β,17α,20α,21-pentol
βCor	βcortol	5β-pregnan-3α,11β,17α,20β,21-pentol
6βOHE	6βhydroxycortisone	5-pregnen-6β,17α,21-triol-3,11,20-trione
6βOHF	6βhydroxycortisol	5-pregnen-6β,11β,17α,21-tetrol-3,20-dione

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CHAPTER I

GENERAL INTRODUCTION

The Effects of Alcohol on Human Endocrine Function

From reviews of this topic, such as that of Wright (1978), it may be concluded that the effects of ethyl alcohol on hormone secretion and metabolism are not large and dramatic, with the exception of its direct inhibition of the neurosecretion of the neurohypophyseal hormones vasopressin and oxytocin. However, as shown from alcohol consumption figures, the average adult in any western society has ethanol present in his bloodstream for several hours per day throughout life, and the tissues of many are never ethanol-free. Under these conditions, even minor disturbances of endocrine balance may become clinically significant and worthy of investigation.

The concentration of hormone to which a receptor tissue responds may be influenced by ethanol if this either (a) affects the rate of secretion of the hormone from the source tissue, or (b) modulates its rate of catabolism or metabolic activation, particularly if this occurs in the liver where ethanol is actively oxidized to acetaldehyde and acetate. Examples of both types of interaction have been reported.

Assessment of the Literature

In spite of the considerable volume of literature on the endocrine consequences of alcohol ingestion, its interpretation is complicated by a number of problems. Comparison of the changes involving acute administration of alcohol with those due to prolonged intake, such as occur in chronic alcoholism, and comparison of the response of habitual drinkers with the response of alcohol-naive subjects, has led to confusion and apparently conflicting results. In addition there has frequently been a failure to distinguish the endocrinological and metabolic effects of alcohol per se from those secondary to tissue (particularly liver) damage and a tendency to regard chronic alcoholics as a homogeneous group regardless of differences in drinking patterns, the type and quantity of liquor consumed, the history, nutritional status and the time interval between drinking and endocrine or

metabolic studies, all of which may profoundly affect the results obtained. Finally there is some difficulty in assessing data obtained before the introduction of modern hormone assays such as specific radioimmunoassays and of correlating results obtained from animal and human studies.

Hypothalamic-Pituitary-Gonadal Function

Hepatic cirrhosis in men is commonly associated with both hypogonadism and feminization. The similarities between the endocrine features of alcoholic and non-alcoholic cirrhosis initially suggested that it was the liver disease itself which was responsible for these changes. Recent evidence, however, suggests a possible direct effect of alcohol on testicular function. The changes described so far indicate that gonadal dysfunction may occur in the absence of overt liver disease but that in alcoholic cirrhosis, the cumulative effects of alcohol and hepatic dysfunction may produce more marked endocrine features. The subject is covered in some detail in the reviews by Adlercreutz (1974), van Thiel and Lester (1976) and Green (1977).

Secretion of Catecholamines

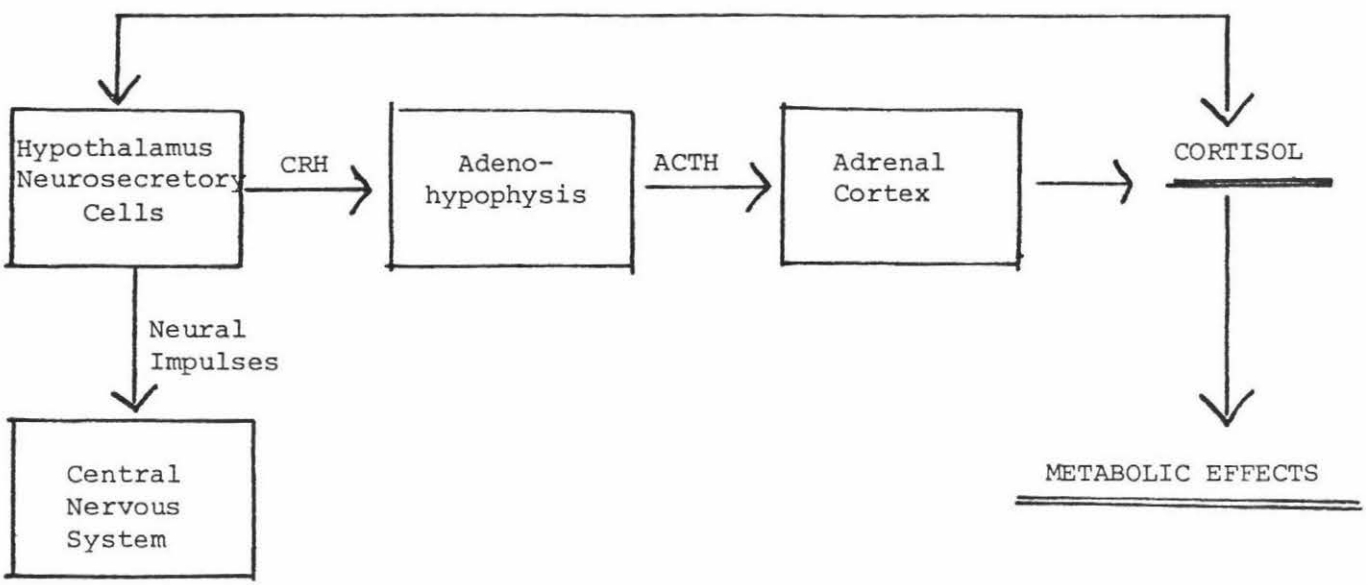
There is evidence from both human and animal studies that alcohol stimulates adrenal medullary secretion. Moderate doses of alcohol have been shown to produce a rise in both the plasma and urine catecholamines of normal human subjects (Perman, 1958; Anton, 1965), while similar effects have been observed in subjects with prolonged histories of drinking (Ogata et al, 1971).

Hypothalamic-Pituitary-Adrenocortical Axis

The effects of alcohol on endocrine function have been studied most extensively in relation to the hypothalamic-pituitary-adrenal (H.P.A.) axis and work in this field has been reviewed by Schenker (1970), Marks and Chakraborty (1973) and Wright (1978). The system and its regulation is shown schematically in Fig. 1i.

Figure 1i

Regulation of the Hypothalamic-Pituitary-Adrenal Cortex System



Short loops may exist between hypothalamus-pituitary and pituitary-adrenal, imposing further regulation.

Reproduced from Marks and Chakraborty (1973).

Cortisol is the major adrenocortical steroid hormone found in human blood. The circulating levels of cortisol have been shown to rise rapidly in response to trauma e.g. injury, surgery, burns etc. (as reviewed by Alberti and Johnston, 1977). Cortisol is the major anti-anabolic hormone: its ability to inhibit protein synthesis is thought to be responsible for its unique anti-allergic and anti-inflammatory effects. The secretion of cortisol is under the direct control of adrenocorticotrophic hormone (A.C.T.H.) produced by the adrenohypophysis (anterior pituitary) in response to the neuroendocrine releasing factor C.R.H. (corticotrophin releasing hormone). The release of C.R.H. is, in turn, determined by the action of external stimuli on the central nervous system as well as a circadian "clock".

A Review of the Literature on the Effects of Alcohol on Cortisol Release and Metabolism

Although H.P.A. function appears to be definitely disturbed in chronic alcoholics (Stokes, 1971) the literature reports are often contradictory. This appears to be due, in part, to the absence of suitable techniques for measuring the hormones involved, as well as the multiplicity of possible physiological, psychological and sociological contributions.

In man the effects appear to be dose related: while moderate to large doses may activate adrenocortical activity through higher regulatory centres (rather than by direct action on the adrenal or pituitary), lower doses are less predictable and it has been postulated that they may even decrease the activity of a previously aroused H.P.A. system via a sedative effect on the central nervous system.

Kissin et al. (1959) suggested that some of the observed abnormalities in the adrenocortical function of alcoholic subjects may be related to impaired liver function. A further investigation (Kissin et al., 1960) demonstrated increased urinary 17-OHCS and decreased plasma levels, accompanied by a marked diuresis, within two hours of a single dose of ethanol (1 g/Kg body weight) to alcoholic subjects. The similar effects of a water load seemed to indicate that the adrenocortical depletion may have been due to increased renal clearance, but a simultaneous water and

ethanol load produced a rise in plasma 17-OHCS with no appreciable change in urinary levels, suggesting an active stimulatory effect of ethanol on the adrenal cortex.

Perman (1961) however, failed to show a significant change in urinary 17-OHCS two to three hours after a 1 g/Kg dose of ethanol to non-alcoholic subjects; there were no corresponding plasma steroid measurements.

Margraf et al. (1967) found no significant difference in cortisol secretion rate or total excretion of 17-OHCS in alcoholic subjects as compared with non-alcoholic controls, although the distribution of the individual component steroids appeared to differ significantly from normal. In addition, 24 hour 17-ketosteroid excretion, response to A.C.T.H. and rate of metabolism of exogenous cortisol appeared to be lowered in alcoholics, while plasma corticosterone and its urinary metabolites were increased above normal levels, suggesting that alcohol affected steroid metabolism rather than adrenocortical function.

In reviewing the literature Schenker (1970) suggests that chronic alcoholics show a plasma 17-OHCS level significantly higher than that of partially rehabilitated alcoholics, which in turn, is higher than that of non-alcoholics. A marked rise in an alcoholic's plasma 17-OHCS is often associated with gastro-intestinal disturbance or withdrawal. After 12 hours without alcohol acutely withdrawn, chronic alcoholics showed a 9 am plasma cortisol significantly higher than normal, which fell following the ingestion of small amounts of alcohol (Merry and Marks, 1972). This compares with a distinct rise in plasma cortisol following infusion (Jenkins and Connolly, 1968) or ingestion (Merry and Marks, 1969; Bellet et al., 1970) of ethanol to/by normal subjects. These findings suggest that withdrawal represents a state of considerable stress to the alcoholic the symptoms of which may be relieved by alcohol. Alcohol ingestion by non-alcoholics, however, raises plasma cortisol levels probably by increasing pituitary-adrenocortical activity, since no such effects were noted in non-alcoholic patients with clinical adrenal insufficiency (Bellet et al, 1970).

The Aim of this Project

The goal of the present research was to elucidate some of the effects of ethanol consumption on adrenal corticosteroid release and metabolism in an endeavour to clear up some of the apparent inconsistencies in the literature. Initially, attempts were made to cover effects on both the plasma level of cortisol and its conversion to metabolites and to study both normal and alcoholic subjects. Both approaches required establishment of modern methods of analysis, which occupied most of the time available for this project.

CHAPTER 2

THE EFFECT OF ETHANOL ON PLASMA CORTISOL LEVELS IN THE HUMAN

INTRODUCTION

As outlined in Chapter 1 there is some controversy as to whether alcohol increases or decreases plasma levels of ACTH and cortisol, the predominant effect depending on the conditions and subjects of study. In an attempt to elucidate these effects, a radioimmunoassay (RIA) for plasma cortisol was established and validated to allow measurement of plasma cortisol in a variety of drinking situations.

A number of RIA methods for the estimation of cortisol are outlined in the literature including those of Abraham et al (1972); Ruder et al. (1972); Loriaux et al. (1973); West et al. (1973); Farmer and Pierce (1974); Foster and Dunn (1974). In this work the method of Ruder et al. was adapted for use. Pilot studies were made on five normal subjects during acute ethanol loading experiments under laboratory conditions, and on a larger heterogeneous group of subjects who were admitted to Palmerston North General Hospital suffering from alcohol intoxication.

One effect which confounds a study of plasma cortisol levels is the considerable diurnal variation present under normal conditions. Normal humans exhibit a peak of 120 ng/ml at about 7 am, with a subsequent decline to about 20 ng/ml in late evening (Kreiger, 1975). A large amount of data and covariance analysis methods are necessary to separate any effect of ethanol from the diurnal effect on plasma cortisol. Furthermore, some evidence also exists which suggests that ethanol metabolism in man (Reinberg et al., 1974; Sturtevant et al., 1975; 1976) and in mice (Goldstein and Kakhana, 1977) is also subject to circadian variations.

MATERIALS AND METHODS

MATERIALS

All chemicals, unless otherwise specified, were reagent grade or better, and supplied by May and Baker Ltd, British Drug Houses Ltd, or Sigma Chemical Co. Ltd.

Cortisol was obtained from Mann Research Laboratories, New York, U.S.A. A stock standard solution, containing 10 ng/ml, in ethanol,was stored at 4°C. A working standard of 1 ng/ml was prepared by freshly diluting the stock with ethanol.

(1,2,6,7 (n)-^{3}H)Cortisol was supplied in benzene:ethanol (9:1), by The Radiochemical Centre Ltd, Amersham, U.K. at a specific activity of 95 Ci/mmol (258 mCi/mg) and a radioactive concentration of 1 mCi/ml. 0.1 ml of this solution was diluted 1 in 250 (EtOH:H_2O, 1:24) to give a stock solution of approximately 4 µCi/ml, which was stored at 4°C. The working solution used in the RIA, was prepared by diluting the stock 1 in 10 with assay buffer, immediately before use. A 1 in 100 dilution of the stock in ethanol was stored at 4°C for use as a radioactive "spike" in estimating procedural losses during the extraction of plasma.

Anti-cortisol-3-BSA-serum, raised in rabbit, was kindly donated by Dr R. S. Fairclough, Ruakura Agricultural Research Centre, Hamilton, N.Z.. This was stored frozen as 1 ml aliquots in sealed glass ampoules at a 1 in 200 dilution in assay buffer. These were further diluted 1 in 10 with assay buffer immediately prior to use in the RIA.

The assay buffer was 0.01 M phosphate buffered saline, pH 7.3, containing 0.1% gelatin. To prepare this, 20 ml 0.5 M NaH_2PO_4 was added to 100 ml water, the pH adjusted to 7.3 by addition of 0.5 M Na_2HPO_4, 0.35 g thiomerosal and 28.6 g sodium chloride added before making up to 3.5 l. After checking the pH, 1 g gelatin was dissolved per litre.

Polyethylene glycol (PEG), 16.2% (w/v) solution of Carbowax 600 (Union Carbide or BDH) was added to assay tubes in such volumes as to give a final concentration of 12.5% in each tube.

A 3% solution of bovine gamma globulin (BGG) (Cohn Fraction II, Sigma Chemical Co.) in water was prepared daily.

The scintillation fluid was 9 g PPO (2,5-diphenyloxazole, BDH)

and 300 mg POPOP (1,4 bis(2-(5 phenyloxazolyl)benzene),Sigma) dissolved in a mixture of 2 litres toluene and 1 litre of Triton X-100 (Rhon and Haas).

Dichloromethane (DCM) and ethanol were redistilled twice before use, the latter following overnight treatment with m-phenylaminediamine.

For a plasma blank, a supply of wether plasma obtained from the Department of Physiology and Anatomy, Massey University was stripped of endogenous steroids (Torrey, 1971) by heating the plasma at 45°C for 2 min. with an equal volume of charcoal suspension (4 mg/ml in phosphate buffer, pH 7.8). It was then centrifuged and filtered twice through Whatman No.42 paper. The 50% stripped-plasma solution was stored frozen in small aliquots.

METHODS

A Radioimmunoassay for Human Plasma Cortisol

The plasma extraction process was adapted from that described by Ruder et al. (1972).

The standard curve

Best recoveries were obtained when standards extracted from 0.1 ml charcoal stripped wether plasma were employed. A series of standard tubes containing 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ng of unlabelled cortisol, following evaporation of the solvent,was equilibrated overnight at 4°C, each with a 0.1 ml aliquot of stripped wether plasma. The standards were extracted and assayed by the method described below for plasma samples.

Plasma samples

0.1 ml aliquots of "unknown" plasma samples were extracted into 4.0 ml DCM ("Zipette" automatic dispenser) by shaking, horizontally, for 10 min. (equipoise-type shaker, Analyte Pty, Aust.). After standing briefly, to separate the phases, 0.4 ml aliquots (Gilsen automatic pipette, 0.2-1.0 ml) of the organic phase were removed to assay tubes and evaporated to dryness in a water bath at 40°C, under a vigorous stream of air.

The radioimmunoassay

This was performed in batches of 24 tubes, which is the capacity of the centrifuge used in the assay.

To each tube of evaporated extract or extract plus standard was added:

1. 0.1 ml antiserum (AS) diluted 1:2,000 with assay buffer immediately before use.
2. 0.1 ml ^{3}H-cortisol (^{3}HF), diluted to approximately 0.4 µCi/ml with assay buffer before use.
3. 0.1 ml 3% bovine gamma globulin (BGG) made up freshly in distilled water.

The contents of each tube were mixed, briefly (vortex mixer), and allowed to stand covered for 1 hour at room temperature, followed by 2 hours at 4°C. These incubation conditions were shown to give the same results as an overnight incubation at 4° (Fig. 2i).

Free ^{3}HF was separated from that bound by the antiserum by the addition of 1.0 ml 16.2% PEG to precipitate protein. Tubes were allowed to stand 10 min. at 4°, then centrifuged at 2-4° for 10 min. at 7,700 g. The supernatant was aspirated off and discarded, and the precipitate redissolved in 1.0 ml distilled water with gentle warming. The solutions were decanted into scintillation vials, the tubes washed with two 5 ml aliquots of scintillation fluid which were added to the vials. Scintillation counting was performed in a Packard Tricarb liquid scintillation counter with automatic external standardization.

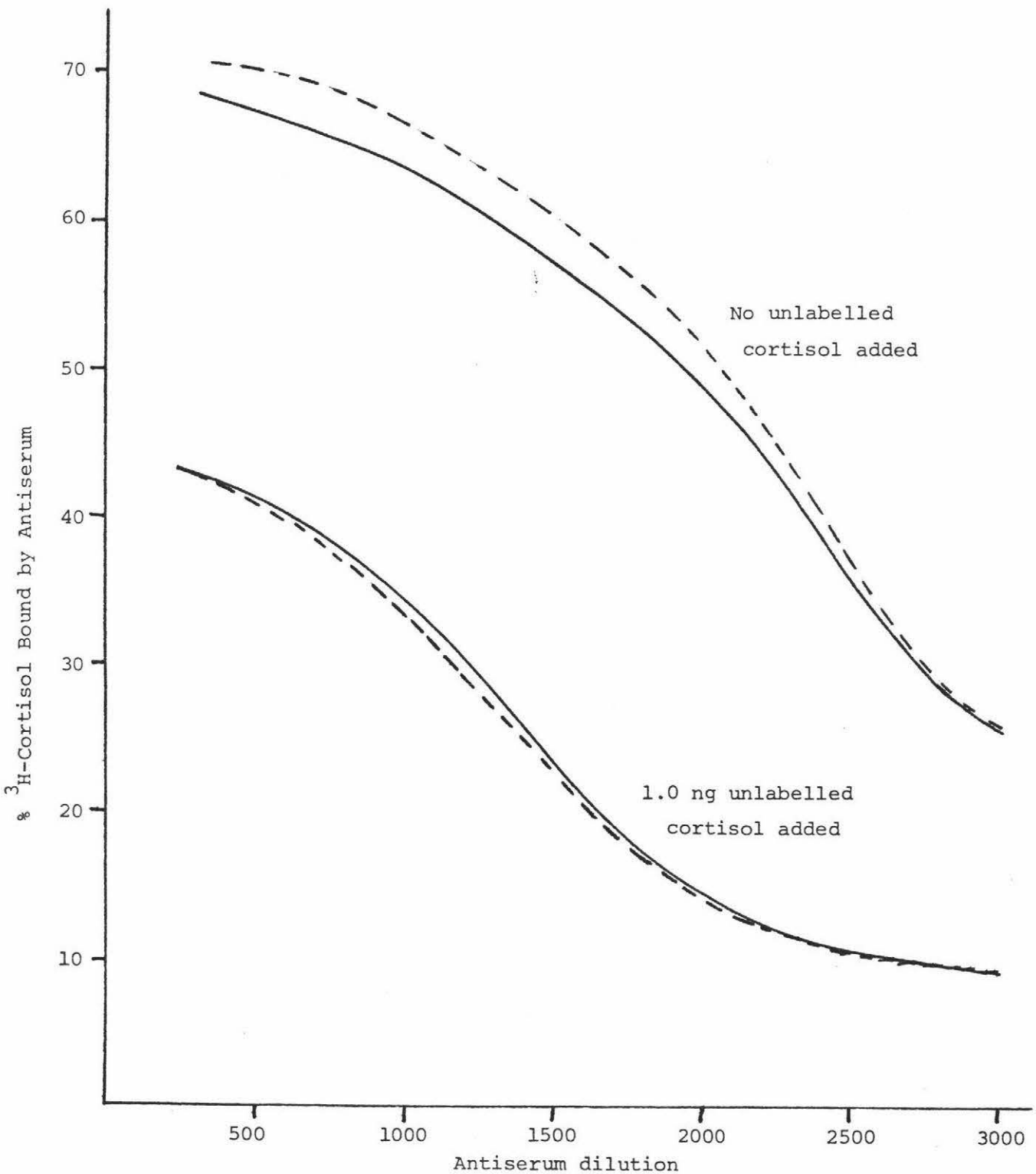
Background and total counts

Since the final counting solution was partially quenched and somewhat turbid, due to the presence of protein, the total added counts (TC) and background counts (BG) were determined as part of the assay.

Duplicate BG and quadruplicate TC tubes were set up containing 0.1 ml assay buffer, 0.1 ml diluted AS, and 0.1 ml BGG, and treated in the same way as the rest of the assay tubes, except that the TC precipitates were redissolved in 0.9 ml water (instead of 1.0 ml) and washed into scintillation vials containing 0.1 ml of the dilute ^{3}H cortisol used in the assay.

Figure 2i

Antiserum Binding Curves



------ Represents incubation for 18 hours at 4°C.

——— Represents incubation for 1 hour at room temperature followed by 2 hours at 4°C.

Correction for free labelled cortisol

The counts due to free ^{3}H cortisol, which had been incompletely separated from the bound hormone, were assumed to be uniform throughout a run and were determined by including two or more blank tubes containing 0.1 ml assay buffer, 0.1 ml ^{3}H cortisol and 0.1 ml BGG. The resulting counts were subtracted from the gross counts in all other assay tubes, excepting BG and TC.

Correction for non-specific binding by plasma (NSB)

All plasmas were found to bind the labelled cortisol to a small extent in the absence of antiserum, therefore "antiserum-blank" tubes were set up containing extracted male plasma with 0.1 ml assay buffer, 0.1 ml ^{3}H cortisol and 0.1 ml BGG. The final counts, less those due to free ^{3}H cortisol, indicated the degree to which cortisol was bound by the plasma.

Correction for procedural losses

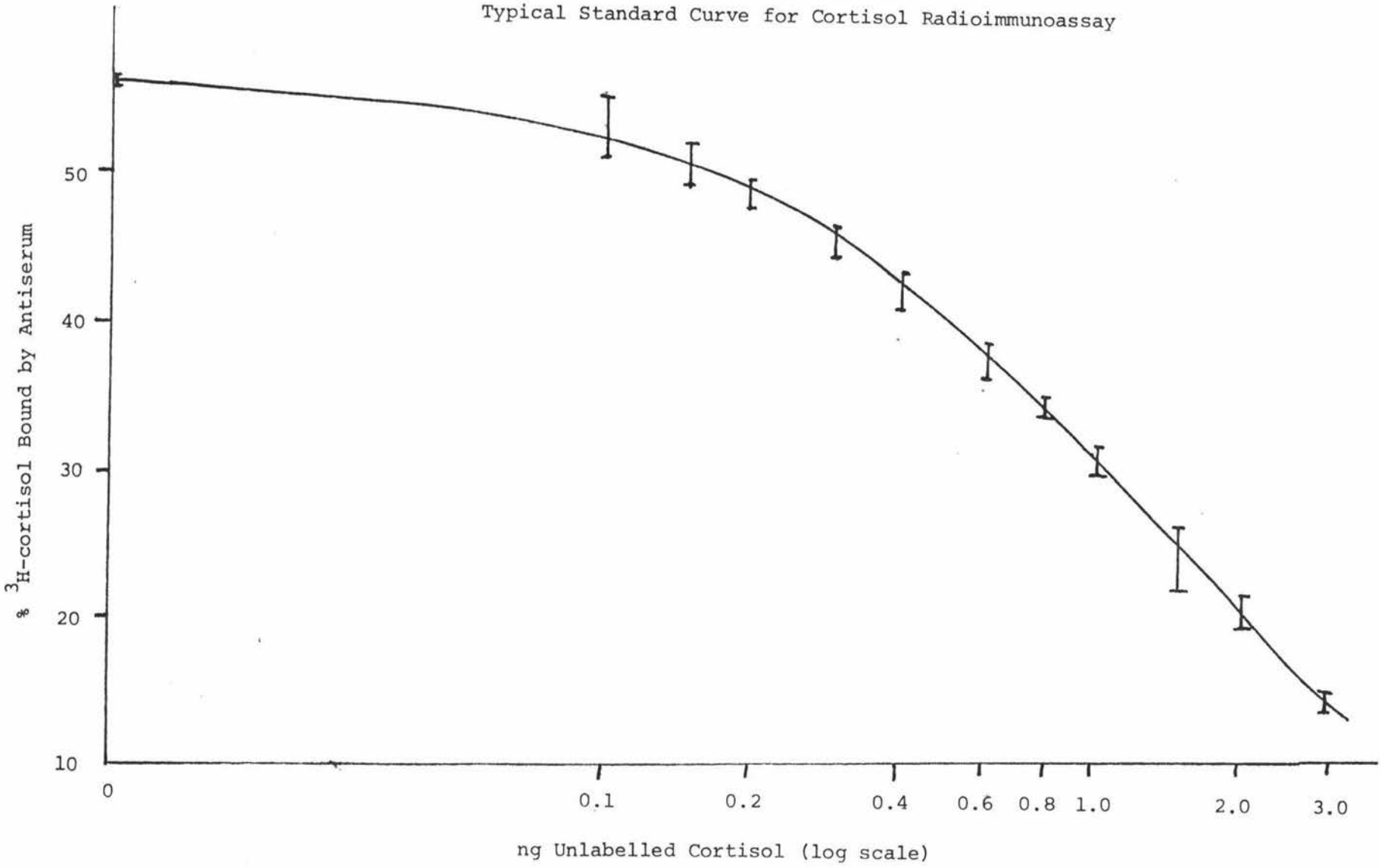
Extracted plasma samples and standards were corrected for procedural losses by the addition of 0.1 ml ^{3}H cortisol (approximately 0.4 Ci/ml) to all plasma containing tubes. Following extraction a fourth 0.4 ml aliquot of solvent phase was transferred from extraction tubes to scintillation vials, evaporated and counted together with vials containing 0.1 ml ^{3}H cortisol to assess total added counts. The recovery from the extraction procedure was determined as a percentage of total counts added (always better than 90%), and the final assay results corrected accordingly. It was also necessary in the final calculation to correct the total counts and those of background and free ^{3}H cortisol for the counts added in the recovery estimation.

Calculation of cortisol concentration in plasma

The following formula was used to calculate the percentage ^{3}H cortisol bound by antibody in each tube:

$$\% \ ^{3}H \text{ cortisol bound} = \frac{x-(a+b+c)}{y} \cdot \frac{100}{z} \cdot 100$$

Figure 2ii



Standards have 0.1 ml stripped wether plasma added, are extracted into 4.0 ml dichloromethane and 0.4 ml aliquots taken for assay.

where a = cpm BG

b = cpm free ^{3}H cortisol

c = cpm NSB

x = cpm sample or standard

y = cpm TC

and z = % recovery from extraction

The percentage bound was plotted against ng unlabelled cortisol per tube for each standard and the curve of best fit drawn through the points (semi-log paper).

The cortisol levels of the unknown plasma samples, whose binding percentages had been determined as above, were then found by direct interpolation of the standard curve, bearing in mind the one-tenth dilution of plasma in the assay, i.e. only one-tenth of the 0.1 ml of plasma initially extracted was assayed. A typical standard curve is shown in Fig. 2ii.

Antiserum dilution and incubation time

The percentage of ^{3}H cortisol bound by the antiserum was estimated at 6 dilutions of the AS in the absence of unlabelled cortisol and in the presence of 1.0 ng unlabelled cortisol. Results are shown in Fig. 2i. There appeared little difference between incubating the assay overnight at 4° and 1 hour at room temperature followed by 2 hours at 4°.

At a dilution of 1 in 2,000 the AS was shown to bind 47-53% of ^{3}H cortisol in the absence of cortisol and 12-15% in the presence of 1.0 ng cortisol.

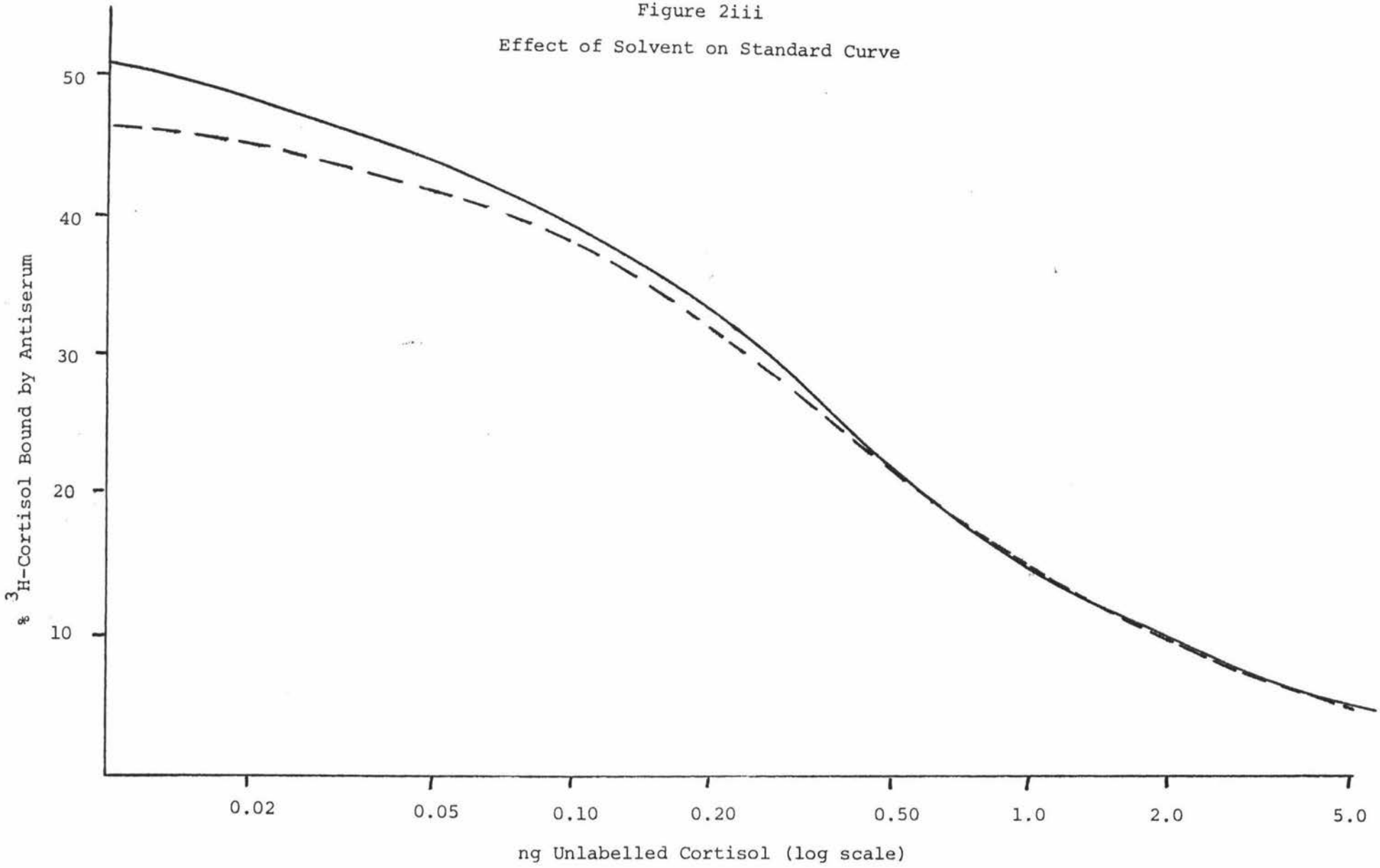
Extraction of standards

Before it was finally decided to add plasma and extract standards prior to assay, a number of standard curves were produced under varying conditions:

(a) Effect of dichloromethane residues: In an experiment designed to test the possibility that the assay might be affected by residues of solvents used to extract plasma samples, two identical series of unlabelled cortisol standards, in ethanol, were set up. 0.4 ml DCM was added to each tube of one curve; and all tubes were evaporated to dryness and subjected to identical RIA's. The curves so obtained are shown in Fig. 2iii.

Figure 2iii

Effect of Solvent on Standard Curve



——— Represents standard curve with no added solvent.

- - - - Represents standard curve with 0.4 ml dichloromethane added.

There appeared to be little difference in the linear regions of the two curves; however for cortisol concentrations less than 0.1 ng the slope of the curve to which DCM had been added was less and the percentage of ^{3}H cortisol bound in the absence of unlabelled cortisol, reduced by approximately 5%.

(b) Effect of extraction of plasma: Standards containing 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ng unlabelled cortisol were equilibrated with 0.1 ml wether plasma which had been stripped of endogenous steroids as previously described. The evaporated standards and plasma were equilibrated overnight at 4°C. Each was extracted into 4.0 ml DCM and three 0.4 ml aliquots removed from each. Losses during the extraction procedure were determined by addition of ^{3}H cortisol, as described for the routine assay. The response curve so obtained was compared with that of a set of standards to which no plasma had been added and which were assayed simultaneously (Fig. 2iv).

The curves were similar and at their points of widest variance showed a difference of about 5% binding. They intersected at 0.04 ng cortisol and it appeared that omission of the extraction step led to over-estimation of cortisol concentration at low values and under-estimation at high values. A sample of wether plasma extracted and assayed with the two standard curves showed an endogenous cortisol concentration of 21.5-28.5 ng/ml when calculated using the standard curve extracted from plasma, and 14.5-23.0 ng/ml by interpolation from the non-extracted curve.

At 0 ng cortisol the difference in binding between the two curves was around 2%, corresponding to 1.6 ng/ml cortisol on the non-extracted curve, which would not account for the discrepancy between the two methods in estimating the wether plasma sample. A non-specific binding of approximately 50 cpm by the plasma similarly fails to account for the difference.

(c) Effect of plasma proteins on assay: Two sets of standards, one without plasma and one equilibrated with 0.1 ml stripped plasma were extracted and compared with one to which plasma was added but not extracted:

Figure 2iv

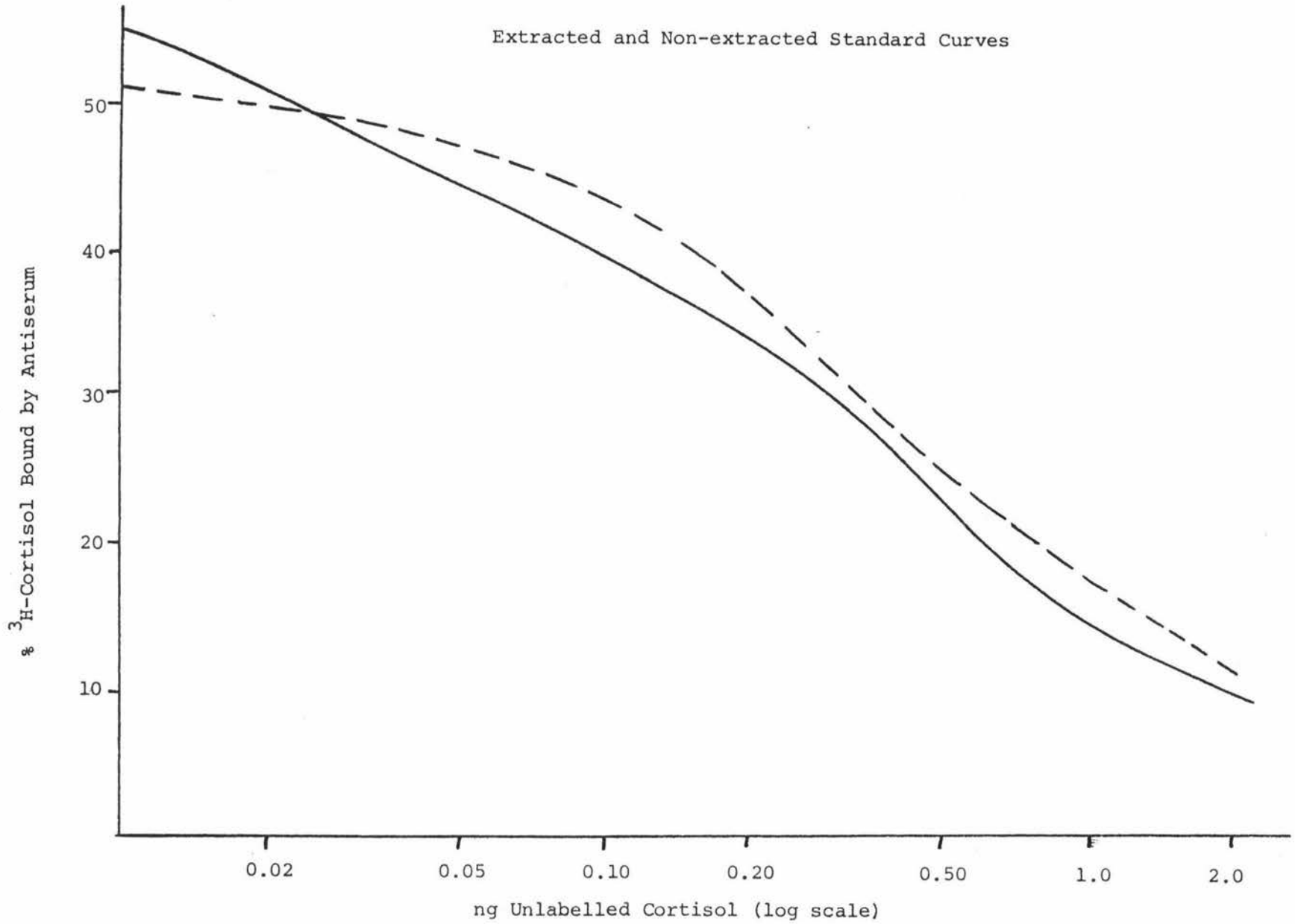
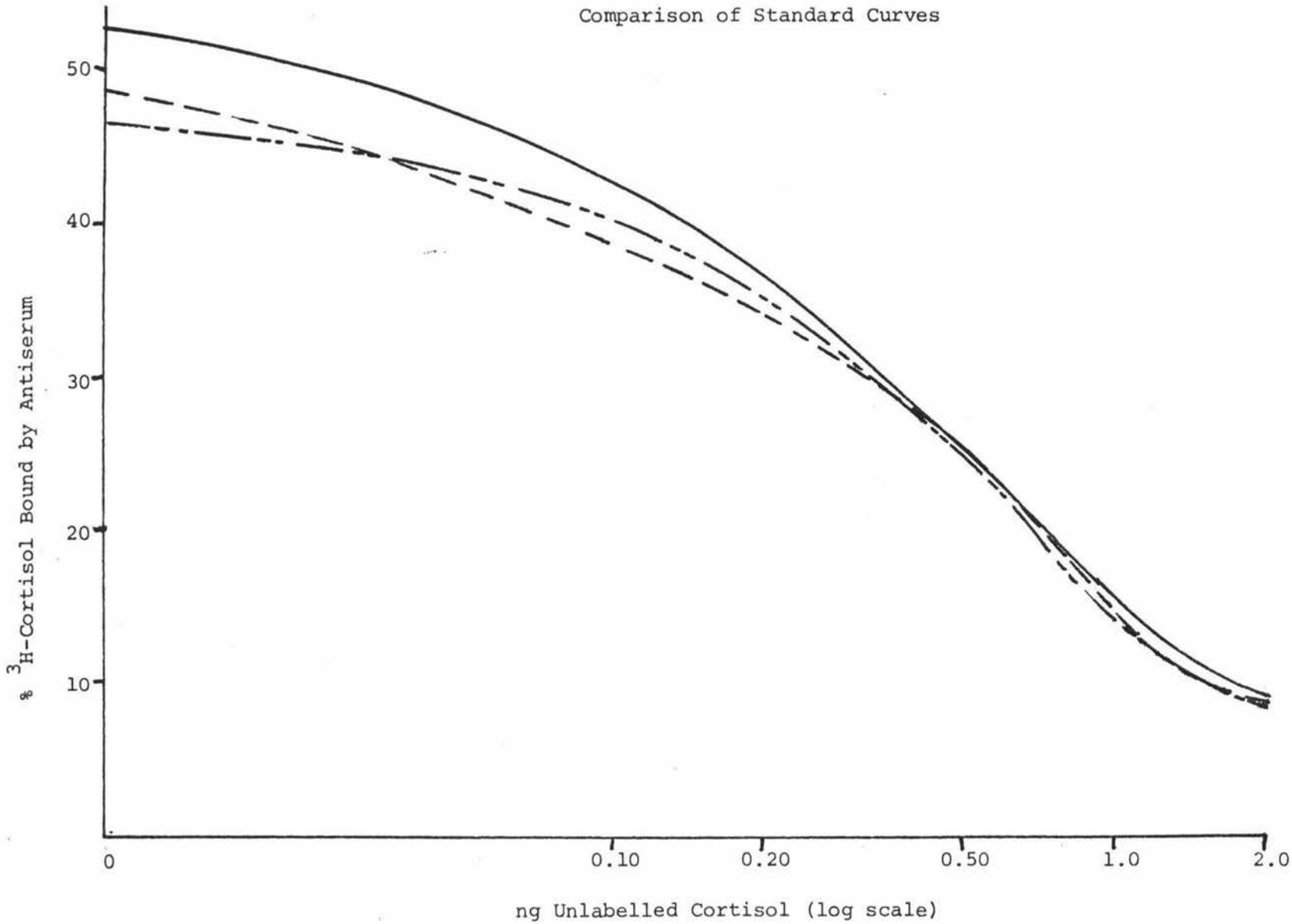

Extracted and Non-extracted Standard Curves
% ^{3}H-Cortisol Bound by Antiserum
50
40
30
20
10
0.02
0.05
0.10
0.20
0.50
1.0
2.0
ng Unlabelled Cortisol (log scale)
Represents non-extracted standards.
Represents standards extracted following addition of plasma.

Figure 2v

Comparison of Standard Curves



——— Represents standards with no added plasma.

- - - - - Represents standards with plasma added, but not extracted.

— -- — Represents standards with plasma added and extracted.

(i) contained 0, 0.1, 0.2, 0.5, 1.0 and 2.0 ng unlabelled cortisol.

(ii) contained 0, 1.0, 2.0, 5.0, 10.0 and 20.0 ng cortisol. Each standard was equilibrated with plasma, extracted and one-tenth aliquots taken for assay as usual.

(iii) contained 0, 0.1, 0.2, 0.5, 1.0 and 2.0 ng cortisol to each of which was added 0.1 ml of stripped wether plasma which had been diluted 1 in 10 with assay buffer to give a concentration identical with that in (ii) above.

0.1 ml assay buffer was added to all tubes in (i) and (ii), and the RIA performed as usual. The three curves so obtained are shown in Fig. 2v.

The extracted and non-extracted plasma standard curves differed by up to 1% in amounts of ^{3}H cortisol bound, well within experimental error. Plasma and non-plasma curves showed insignificant differences (±1%) between cortisol concentrations of 0.1-2.0 ng, 2% at 0.1 ng and 5% at 0 ng. Since 0.2-2.0 ng was considered close to the effective useful range of the curve, it was, at that stage, considered valid to use a standard curve without added plasma and not to extract (merely dilute 1 in 10 with buffer) unknown plasma samples. RIA's were carried out in a total volume of 0.4 ml (rather than 0.3) and the volume of added PEG increased to maintain a final concentration of 12.5%.

Later trials, however, showed that the most reliable "recovery" results were obtained when plasma was added to the standard curve and all plasma and plasma-standard samples extracted as outlined for the routine method: see "recovery of unlabelled steroid" in the following section.

Validation of Radioimmunoassay for Plasma Cortisol

The validation of this RIA is in accordance with the methods specified by the Journal of Endocrinology (J. Endocr. 63, 1-4, 1974).

Binding by plasma with no added antibody

Approximately 2% (i.e. 300 out of 15,000) of the total counts added to the plasma in the absence of antiserum appear to be bound by the plasma non-specifically.

Parallelism of standard curve and dilutions of plasma

A dilution curve which was both linear and parallel to the standard curve was obtained only when the "pool" plasma was diluted with stripped wether plasma, then extracted into DCM, and when the standard curve was equilibrated with stripped-plasma and similarly treated (Table 2i). Even then, this linearity was shown to extend over a relatively small part of the standard curve i.e. approx. 20-100 ng/ml of plasma.

The effect of dilution of a plasma pool containing 95.3 ng/ml of cortisol by 2, 4 and 8 is shown in Table 2i. The eight-fold dilution lies on that part of the curve which is no longer linear and approaches the lower sensitivity limits. The standard and plasma curves are shown in Fig. 2vi.

Table 2i

Comparison of Serial Dilutions of Plasma under Different Analytical Conditions

	Undil.	3/4	2/3	1/2	1/4	1/8
Experiment 1	180	115	107	84		
2	112			48	29	17
3	95			46	20	5.5

Concentrations are in ng/ml plasma, and are the means of three estimates.
All experiments used different plasma samples.
All dilutions were obtained by adding stripped wether plasma to the undiluted plasma pools before taking aliquots for assay.

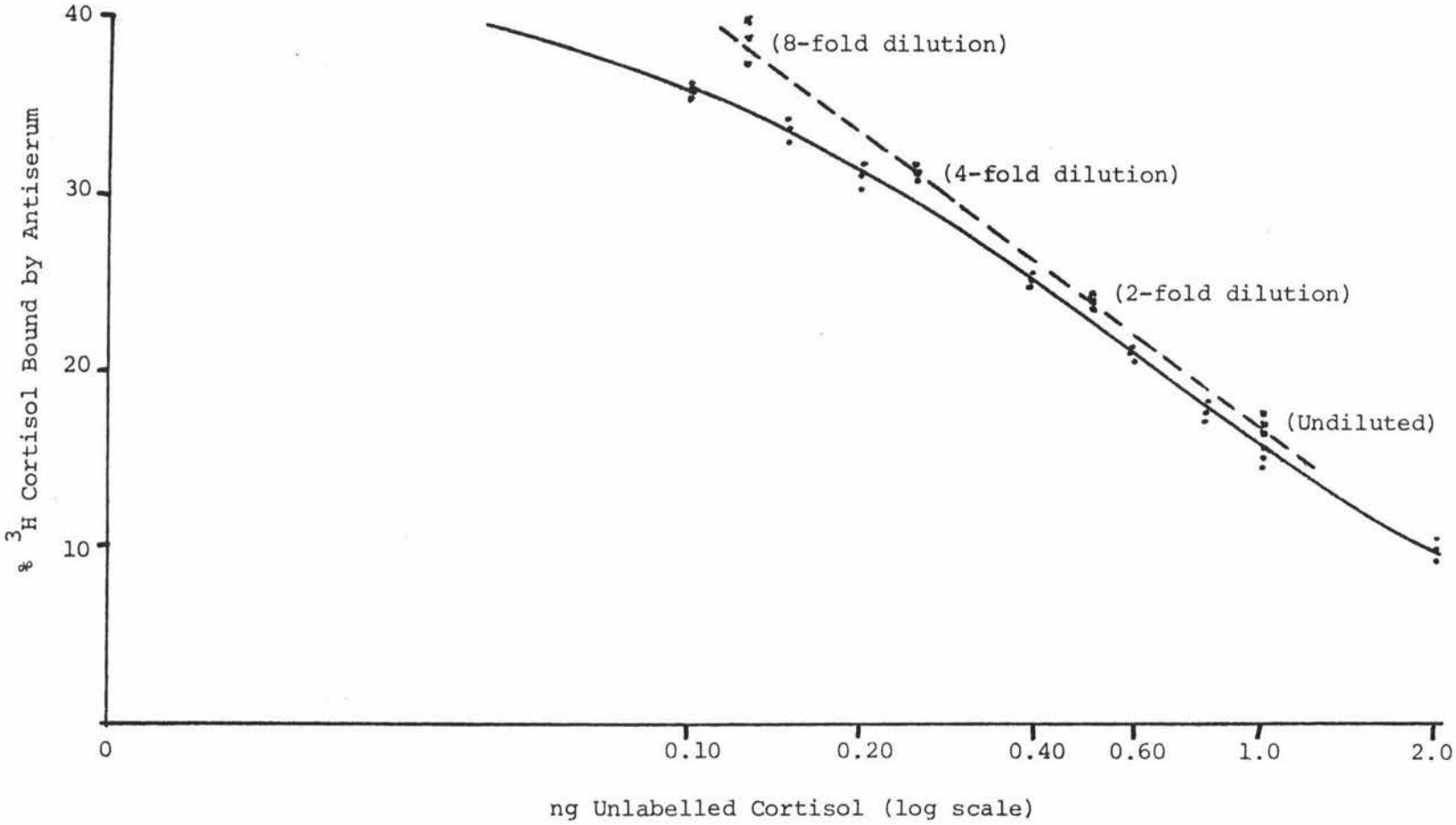
Experiment 1: No plasma added to standards; neither plasma samples nor standards extracted.

2: No plasma added to standards; plasma samples extracted, standards not extracted.

3: Plasma added to standards; plasma samples and standards extracted.

Figure 2vi

Parallelism of Standard Curve and Plasma Dilution Curve



——— Represents standard curve.

- - - - Represents plasma dilution curve.

Coefficients of variation

(a) Intra-assay variation: Two plasma pools containing high and low levels of cortisol were included in a number of assays, between three and nine estimations being performed in each run. Results, shown in Table 2ii, indicate intra-assay coefficients of variation ranging from 4.6% to 23.6%, with only one coefficient above 20%.

Table 2ii

Coefficients of Intra-assay Variation

A. LOW CORTISOL PLASMA POOL:

Assay	n	Mean (ng)	Standard Deviation (ng)	C.V.
1	9	0.269	0.026	9.8%
2	6	0.262	0.019	7.4%
3	6	0.262	0.020	7.8%
4	6	0.255	0.042	16.4%
*5	5	0.167	0.039	23.6%

B. HIGH CORTISOL PLASMA POOL (all pools different)

Assay	n	Mean (ng)	Standard Deviation (ng)	C.V.
1	8	1.76	0.21	12.0%
2	6	0.80	0.37	4.6%
3	3	1.12	0.12	10.7%
*4	7	0.96	0.09	9.2%

"n" is the number of separate estimates in each assay.
C.V., the coefficient of intra-assay variation is calculated as the standard deviation divided by the mean.
* these were assays in which both the standards and plasma samples were extracted prior to assay.

(b) Inter-assay variation: Coefficients of variation were compared over five successive assays of the low cortisol plasma pool. The mean of the intra-assay means was 0.243±0.43 (standard deviation), indicating a coefficient of inter-assay variation of 17.6% (where the coefficient of

variation between assays is calculated as the standard deviation of the means of each pool divided by the mean of the means over several assays). Table 2iii shows the comparison of variations between given points on the standard curve over four assays, where all coefficients are shown to be less than 5%, well within acceptable limits.

Table 2iii

Between Assay Variation: Comparison of Points on Standard Curves over Four Assays

ng Unlabelled Cortisol Added	Mean % ^{3}H Cortisol Bound ± Standard Deviation	Inter-assay C.V.*
0	54.0±1.3	2.4%
0.05	45.8±2.1	4.6
0.10	40.3±1.2	2.9
0.20	33.9±0.6	1.8
0.40	26.3±0.7	2.5
0.60	21.3±0.4	1.9
1.00	15.5±0.4	2.7
2.00	9.6±0.3	2.7

* C.V., the coefficient of inter-assay variation is calculated as the standard deviation of the means of each pool divided by the mean of the means over four assays

Recovery of unlabelled steroid from plasma

Varying amounts of unlabelled cortisol were added to aliquots of the low cortisol, pooled plasma and the recovery estimated by RIA. Recoveries closest to 100% were obtained when plasma samples were extracted prior to assay and the concentrations calculated by interpolation of a standard curve which had been extracted from stripped plasma. In particular, non-extracted plasmas tended to give recoveries well in excess of 100%. A tendency to over-estimate the concentrations of plasma samples fortified with cortisol when results were based on non-plasma standard curves, was also shown. A number of recovery experiments,

employing different assay conditions are shown in Table 2iv.

Table 2iv

Comparison of Experimental Conditions for Estimating Recovery of of Unlabelled Cortisol from Plasma

ng Cortisol Added	0.1	0.2	0.4	0.5	0.6	0.7	1.0
Experiment 1	126			110			
2	122	110	118		155		
3	100	113		93			98
4	95	110	112			129	114
5	85	101		95			

All results are % recovery of cortisol added to the low cortisol plasma pool (approx. 26 ng/ml), and are the means of two to three estimates.
Experiment 1 and 2: No plasma added to standards; plasma samples not extracted.
3 and 4: No plasma added to standards; plasma samples extracted.
5: Plasma added to standards; plasma samples extracted.

Sensitivity

The lowest concentration of cortisol which could be estimated in plasma, by this assay, was around 10 ng/ml; obtained by assaying one-tenth of an extract of 0.1 ml plasma. In routine assays, the lower sensitivity limit was taken as 20 ng/ml, and estimates lying below this level were reassayed using a larger volume of extract.

Specificity of anticortisol-3-BSA serum

The percent cross reaction of the AS with other steroids is shown in Table 2v. At the time of testing, samples of corticosterone and 11-deoxycortisol, which may have cross-reacted significantly, were not available. Of those steroids tested, only cortisone showed significant (9.6%) cross reaction. This was not, however, considered a problem for

the purposes of this assay, since the normal plasma concentration of cortisone has been estimated at around 10% that of cortisol.

Table 2v

Specificity of Antiserum: Cross Reaction with Other Steroids

Steroid	% Cross-reaction
Cortisone	9.6
Deoxy corticosterone	0.9
Tetrahydrocortisol	<0.5
17-hydroxyprogesterone	<0.1
Progesterone	<0.1
B-Methazone	<0.1
Aldosterone	<0.1
Testosterone	<0.1
Androstenedione	<0.1
Estradiol-17B	<0.1

% Cross-reaction defined as (a/b).100

where a is the mass of unlabelled cortisol required to displace 50% of the bound ^{3}H cortisol

and b is the mass of cross reacting steroid required to displace 50% of the bound ^{3}H cortisol.

EXPERIMENTAL SUBJECTS

Normals

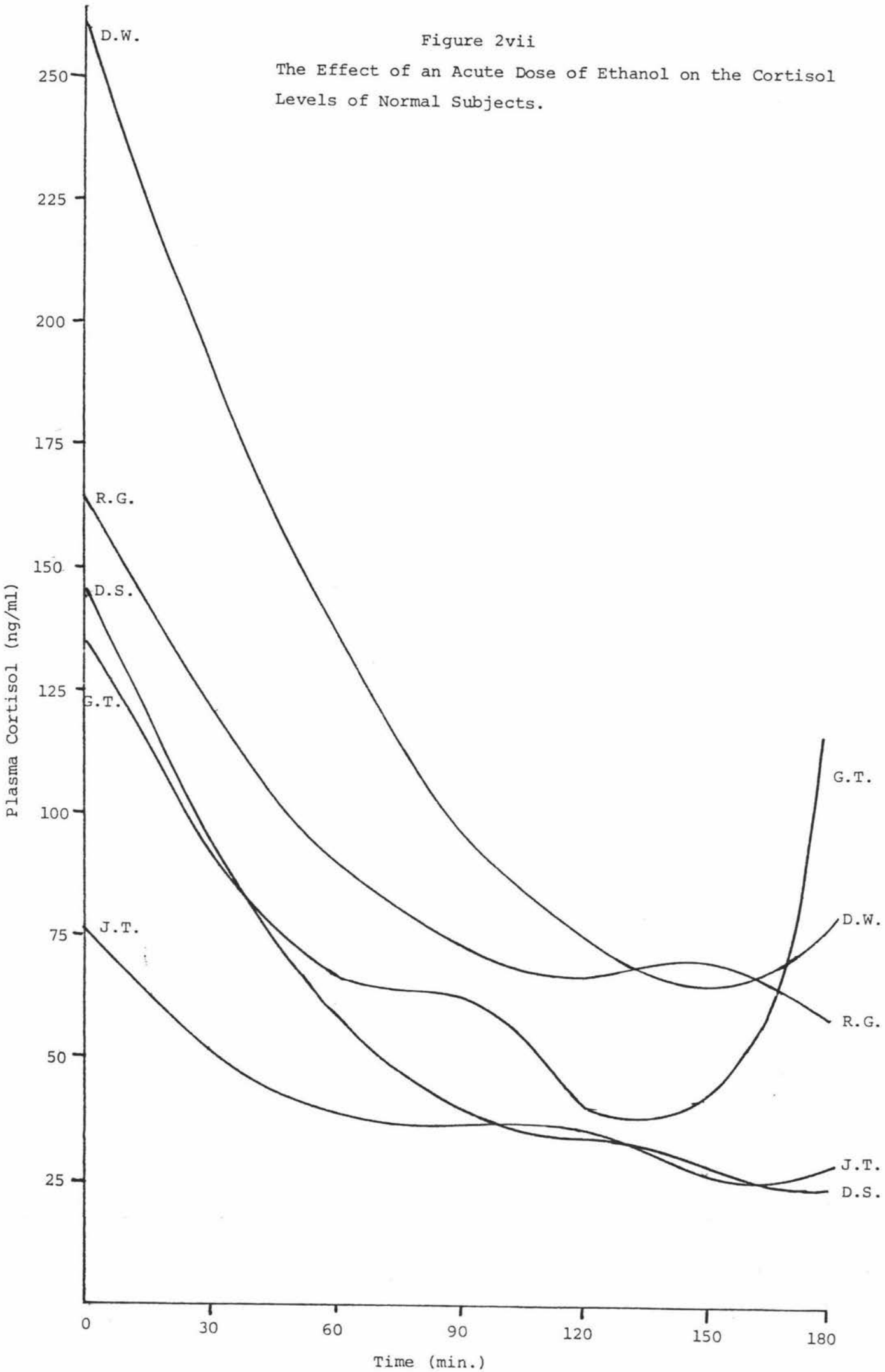
Plasma cortisol was measured in five normal subjects (including one female) who were part of a group studied to determine the effects of alcohol loading on a variety of blood metabolites (Couchman, 1974). These subjects were given an oral alcohol load of 0.5 g/kg body weight (as diluted vodka) at approximately 9.30 am in fasting condition. Blood alcohol levels were measured by K. G. Couchman, using gas chromatography of headspace gas equilibrated over a perchlorate extract of blood.

Alcoholics

Sixty-seven blood samples were obtained from Palmerston North General Hospital from eleven patients (two were studied twice) admitted in acute alcohol intoxication. Blood alcohol levels were measured by the hospital laboratory using a gas chromatographic method. These samples were a heterogeneous group taken in varying numbers and at various times from the subjects whose intake of alcohol was not known. All of the patients would be classed as heavy drinkers or chronic alcoholics.

Figure 2vii

The Effect of an Acute Dose of Ethanol on the Cortisol Levels of Normal Subjects.



RESULTS AND DISCUSSION

Normal Subjects

The data from the five normal volunteers is given in Table 2vi and graphed in Fig. 2vii. In all subjects the highest cortisol level was found before alcohol was taken and fell throughout the study, except for the last sample in three subjects where a rise may have been associated with hunger. Since all of these studies were made in the period 8.30 am - 12.30 pm where the diurnal cycle of cortisol is still in a sharply declining phase, it is highly likely that much of this consistent decrease is due to normal diurnal cycling. The possibility remains that alcohol modifies the shape of this decline, but further studies with non-loaded controls would be necessary to demonstrate this. This pilot study underlines the need to take diurnal cycling of plasma cortisol into account when studying the effects of alcohol.

Table 2vi

Effect of Acute Doses of Ethanol on the Cortisol Levels of Normal Subjects

Time:	0	15 min	30 min	60 min	90 min	120 min	150 min	180 min
J.T.	77	62	52	40	41	36	27	29
G.T.	133	115	92	68	65	41	42	116
R.G.	165	130	121	92	74	66	72	58
D.S.	145	122	85	61	41	37	27	26
D.W.*	260	208	174	142	90	77	63	78

* Female subject.

Alcoholic Subjects

The basic data from the 67 clinical samples is listed in Table 2vii. When all samples were combined the mean blood alcohol was 167±91 mg/100 ml (± standard deviation) and plasma cortisol was 163±80 µg/ml.

Table 2vii

Cortisol and Blood Alcohol Levels in Alcoholic Subjects

Patient No.1

Time	1900	2050	2200	2350	2400	0050	0100	0150	0200	0250
Cortisol	108	190	160	184	164	142	108	94	78	92
BAC	460	368	290	271	170	179	212	216	184	216

Patient No.2(a)*

Time	2150	2300	0100	0300
Cortisol	70	26	86	68
BAC	327	331	184	133

Patient No.2(b)*

Time	2250	0050	0250	0550
Cortisol	105	172	86	98
BAC	299	258	161	138

Patient No.3

Time	1925	2175	2350	0550
Cortisol	65	160	157	237
BAC	221	189	169	40

Patient No.4

Time	1650	1700	1800	1850	1900	1950	2000	2050	2100	2150	2200	2250
Cortisol	320	345	309	251	379	334	284	245	222	150	159	132
BAC	230	248	234	212	184	147	152	156	133	133	92	101

Patient No.5(a)*

Time	1600	1750	1900	2050	2150
Cortisol	166	150	127	148	101
BAC	277	234	161	132	74

Patient No.5(b)*

Time	2200	0050	0250	0450	0650
Cortisol	141	229	97	138	101
BAC	398	122	103	83	59

Patient No.6

Time	2350	0100	0300
Cortisol	70	49	214
BAC	151	64	54

Patient No.7

Time	1750	2050	0500
Cortisol	114	102	192
BAC	210	175	260

Continued...

Table 2vii continued

Patient No.8

Time	0200	0400	0650
Cortisol	120	178	145
BAC	144	84	92

Patient No.9

Time	1350	1600	1775	1950	2150	2350
Cortisol	246	217	205	133	172	138
BAC	210	148	144	82	58	23

Patient No.10

Time	1550	1800	1900	2225
Cortisol	310	235	168	196
BAC	154	94	68	44

Patient No.11

Time	2100	0100	0300	0500
Cortisol	41	83	74	288
BAC	188	122	57	19

Cortisol levels estimated in ng/ml on 67 samples derived from 11 patients over 13 admissions as shown.

Blood alcohol (BAC) levels estimated by hospital laboratory in mg/100 ml of blood.

* Patients nos 2 and 5 admitted twice.

A scatter diagram of plasma cortisol versus blood alcohol concentration for all samples showed little correlation between these variables. The coefficient of linear regression was -0.05.

In an attempt to remove the effect of the diurnal rhythm on the variance of the plasma cortisol data a multiple regression analysis was carried out using the BAR 3 statistical programme on an IBM 1620 computer. In order to simulate the diurnal rhythm, the data on sample collection time on a 24 hour clock (T) was introduced into the computation as a polynomial combining 1st, 2nd, 3rd and 4th powers of T.

As shown in Table 2viii, at no stage did correlation for the time-of-day variance lead to a significant correlation between plasma cortisol and blood alcohol, although the analysis suggested that any correlation was negative, i.e. high blood alcohol tended to be associated with low circulating cortisol levels.

As seen from Table 2ix, the blood alcohol level was significantly correlated with time of day in the 1st and 3rd order polynomial runs, which may be explained by a tendency for heavy drinkers to consume more alcohol at certain times of day or for hospitals to admit intoxicated patients more commonly at certain times.

Table 2viii

Correlation of Time-of-Day Variance with Plasma Cortisol and Blood Alcohol

Power of T in time-of-day polynomial (n)	Regression Coefficient of BAC on cortisol	Significance Students t	P
0	-0.055	-0.39	NS
1	-0.112	-0.80	NS
2	-0.153	-0.96	NS
3	-0.187	-1.21	NS
4	-0.235	-1.48	NS

Table 2ix

Correlation of Time-of-Day with Blood Alcohol Level

Power of T in time-of-day polynomial (n)	Regression coefficient of BAC on T^n	Significance	
		t	P
1	0.027	2.06	<0.05
2	0.072	0.88	N.S.
3	$9x10^{-8}$	-2.23	<0.05
4	0.000	1.30	N.S.

Notes: T: Time of sample collection in hours (24 hour clock)
BAC: Blood alcohol concentration
NS: Not significant

Conclusion

It may be concluded that many factors operate in heavy drinkers to affect their cortisol levels but the present survey does not allow us to say whether alcohol in the blood is one of these. A more controlled experiment designed to minimise other factors known to affect cortisol release, including all types of mental and physical stress would be necessary to test the effect of alcohol per se.

CHAPTER 3

EFFECT OF ETHANOL ON URINARY STEROID METABOLITE PROFILES

INTRODUCTION

Disturbance of Steroid Metabolic Pathways by Alcohol

There is fragmentary evidence in the literature to support the hypothesis that the usual pathways of steroid metabolism may be altered following the ingestion of significant quantities of alcohol. One example of this competition is reviewed in detail in Chapter 5.

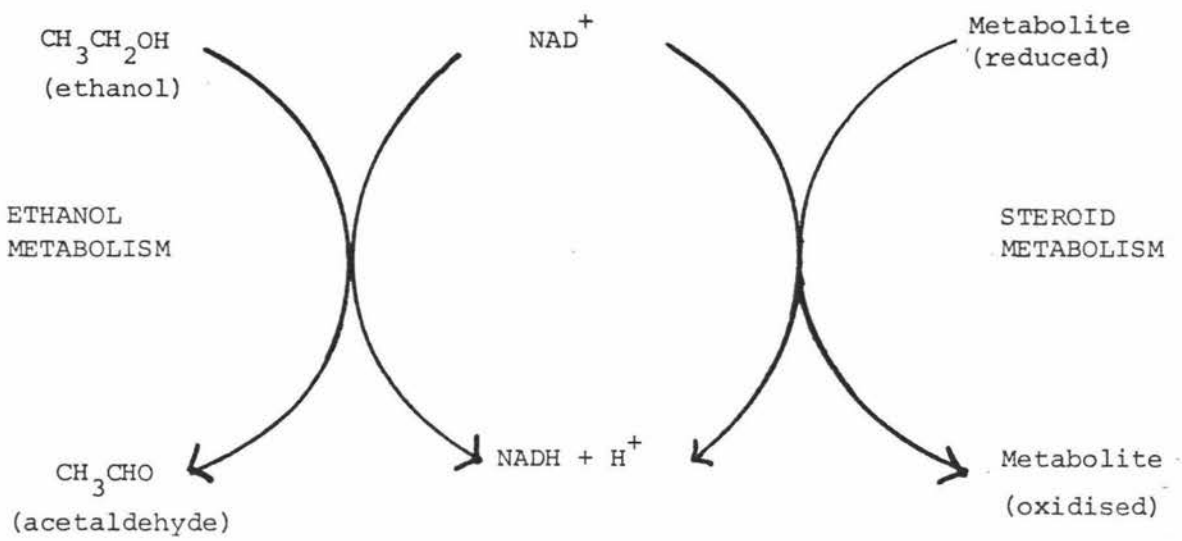
Isselbacher (1977) suggests that many clinical consequences of alcohol metabolism may be directly attributed to the generation of NADH and an increase in the $NADH/NAD^+$ ratio in hepatic cells, such a change could influence the reductive metabolism of steroids (Chronholm et al, 1971) (see Fig. 3i). Chronholm and Sjovall (1970) in fact showed a six- to ten-fold increase in the ratio of 17β-hydroxy steroids to 17 keto steroids following ingestion of sufficient alcohol to produce a level of 30-60 mg of ethanol/100 ml of blood. Furthermore, use of deuterium-labelled ethanol showed a rapid incorporation of the label into the D-ring of dehydroepiandrosterone-3-sulphate, suggesting NADH coupling of the metabolic pathways. The relative sizes of the hydroxy- and keto-steroid pools correlated directly with the level of ethanol suggesting that their ratio may be determined by the $NADH/NAD^+$ ratio in the liver cell cytoplasm.

The further influence of NADH and NADPH on the biosynthesis and metabolism of steroids is indicated (Chronholm et al., 1974) by the incorporation of deuterium from labelled ethanol into steroids, cholesterol and bile acids during NADH and NADPH dependent reductions.

In this section of the work it was intended to examine the immediate and long-term effects of simultaneous alcohol metabolism on the patterns of steroid excretion (particularly cortisol metabolites) with a view to showing possible differences generated in the relative levels of "oxidized" (keto-) to "reduced" (hydroxy-) pairs of metabolites by an increased cellular level of NADH and NADPH.

Figure 3i

Ethanol and Steroid Metabolism Related via $NAD^+/NADH$



Separation Techniques in Steroid Analysis

Until recently the isolation, identification and quantitation of biological metabolites such as steroids were carried out by milligram-level procedures based on such conventional methods as solvent extraction, chromatographic purification and crystallization using classical organic-chemical techniques. A major disadvantage of most of these was the requirement for comparatively large amounts of tissue, urine, blood etc. Although many of the electronic and optical methods developed since 1940 for quantitative analysis (e.g. spectrophotometric and fluorometric techniques) require only microgram quantities, they are relatively non-specific.

In the last two decades a large literature has appeared concerning the development of gas phase analytical procedures. Enjoying current popularity is the GC-MS-COM combination, involving gas chromatographic (GC) separation and estimation, mass spectroscopic (MS) identification and structural elucidation, with the results being analysed and collated by computer (COM). These methods are directly applicable to the estimation and study not only of single compounds, but of multi-component systems such as urinary profiles within the microgram and sub-microgram range.

Though elegant techniques, such as gas liquid chromatography (GLC) and high pressure liquid chromatography (HPLC) may now be unparalleled for biological steroid separations, the methodology is complicated and the capital cost of equipment high compared with the longer-established techniques of column, thin layer and paper chromatography. For many applications these latter methods are quite adequate, easier to use and far less costly.

All established methods in current use (particularly for cortico-steroids) are reviewed fully elsewhere (Heftmann, 1973; Makin, 1975; Sandberg and Slaunwhite, 1975).

Methods for the separation of corticosteroids by liquid column chromatography are well established and supports such as florisil (Eik-Nes et al, 1953), alumina (Loras and Migeon, 1966), celite (Larsen, 1968) and, more recently, Sephadex LH-20 (Setchell and Shackleton, 1973) have been used. Paper and thin layer chromatography have also found wide application for the separation and purification of corticosteroids.

(Paper: Bush, 1961; 1968; Hall et al., 1971; Daniilescu-Goldinberg and Giroud, 1974; Cawood et al., 1976. Thin layer: Butruk and Vaedtke, 1968; Duthie et al., 1969; Scandrett and Ros, 1976). Tables of systems suitable for both paper and thin layer chromatography of adrenal steroids are given by Smith and Hall (1974).

The TLC methods used in this chapter were based largely on those previously developed in this laboratory by Langford (1968).

MATERIALS AND METHODS

MATERIALS

Unless otherwise specified, all chemicals were reagent grade or better and supplied by May and Baker Ltd, British Drug Houses Ltd, or Sigma Chemical Co. Ltd.

All solvents were redistilled before use.

Kieselghur and Silica Gel GF-254 were supplied by E. Merck, Darmstadt, Germany.

β-Glucuronidase was supplied by Sigma Chemical Company, St Louis, Mo., U.S.A. as a crude extract from *Helix pomatia*, containing approximately 100,000 Fishman units of glucuronidase activity per ml. (1 Fishman unit defined as hydrolysing 1.0 µg phenolphthalein glucuronide per hour at pH 5.0 and 37°C); and 3690 µMolar units of arylsulphatase activity per ml, using nitrocatechol sulphate as substrate, pH 5.0; 37°C.

Cortisol, cortisone, THF, THE, allo-THF, α and β-cortol, α and β-cortolone, 11-hydroxyetiocholanolone, 11-ketoandrosterone and dehydroepiandrosterone were supplied by Mann Research Laboratories New York, U.S.A.

11-ketoetiocholanolone and 11-hydroxyandrosterone were from Sigma Chemical Co.; and 6β-hydroxycortisol was from Steraloids Inc., Pawling, New York.

Blue Tetrazolium was supplied by Mann Research Labs.

All water used was deionised and/or glass distilled.

METHODS

Collection of Urine Samples

This section of the work was performed on aliquots of a complete 24 hour urine collection from a normal female subject. The urine was collected in a plastic container, without preservative and stored at 4°C throughout collection. The volume was made up to 1 litre with water. 100 ml was processed immediately and the rest stored frozen as 100 ml aliquots in polythene containers.

Standard Hydrolytic Procedure

100 ml aliquots of urine were adjusted to pH 5.0±0.2 with a few drops of glacial acetic acid, covered and incubated for 32 hours in a gently shaking water bath at 37°C.

The pH of the hydrolysate was checked after 24 hours and adjusted if necessary. The activity of β-glucuronidase in the medium was also checked qualitatively after 24 and 32 hours incubation, as follows:

0.1 ml aliquots of hydrolysate were added to a test tube containing 0.25 ml of 0.002 M phenolphthalein glucuronide and 0.25 ml acetate buffer pH 5.0, and incubated at 37°C for 30 mins. The solution was adjusted to a pH≥8 by dropwise addition of 1 M NaOH. The presence of a pink, phenolphthalein colour in the alkaline solution indicated the continuing activity of the enzyme in the hydrolysis medium. A "blank" tube, containing 0.25 ml substrate and 0.35 ml buffer was routinely included.

The hydrolysate was stored overnight at 4°C if extraction did not take place immediately.

Solvent Extraction (adapted from Thrasher et al., 1969)

The urine hydrolysate (100 ml) was transferred to a 1 litre separatory funnel and extracted once with two volumes of dichloromethane by gentle swirling for 10 minutes. Complete separation of the phases was effected by centrifugation.

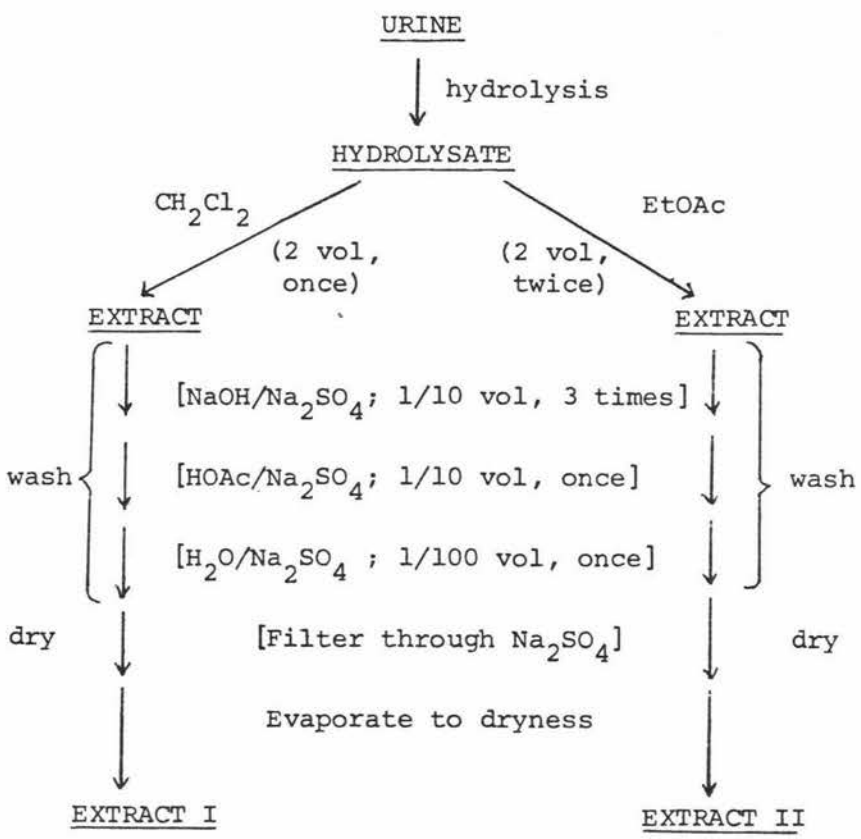
The organic phase was washed with one-tenth volume of 1 M NaOH, containing 15% Na_2SO_4 (w/v), until a clear wash was obtained (2-3 times), then washed once with one tenth volume 1 M acetic acid (containing 15% Na_2SO_4) and finally with one hundredth volume of aqueous 15% Na_2SO_4.

The extract was filtered through anhydrous Na_2SO_4 into a 1 litre round bottomed flask, and evaporated to dryness (Buchi "Rotavapor"). This residue is hereafter referred to as Extract I.

Approximately 15 g anhydrous Na_2SO_4 were added to the aqueous phase remaining after the first extraction. This was extracted twice with two volumes of ethyl acetate, the combined extracts washed and dried as above and similarly evaporated to dryness. This residue is hereafter referred to as Extract II.

Figure 3ii

Treatment of Urine Sample Prior to Fractionation of Steroids by TLC



Both extracts were transferred to conical test tubes: Extract I using aliquots of CH_2Cl_2:MeOH (9:1) and Extract II using EtOAc:MeOH (1:1). Each was evaporated to dryness under a stream of dry nitrogen and redissolved in 1.0 ml of the appropriate solvent (Fig. 3ii).

Thin Layer Chromatography

Thin layers of 0.25 mm thickness were prepared by spreading a slurry of 30 g of adsorbant (silica gel or Kieselghur) in 65 ml water on glass plates 20 x 20 cm and 20 x 10 cm using a Desaga TLC spreader (Heidelburg, Germany). The plates were left 10-15 min. to set and activated by heating at 110°C for 1-2 hours.

The plates were developed in glass tanks (22 x 8 x 23 cm) lined with Whatman 3 MM filter paper and closed with ground glass covers. Before use the solvent level was adjusted to approximately 1 cm, following equilibration of the tank with the solvent for several hours.

Continuous-elution chromatograms were developed in a "Shandon" continuous elution TLC tank fitted with a lid and solvent reservoir.

Partition Systems: The stationary phase was applied after the method of Langford (1968) by pre-running Kieselghur plates in solutions of ethylene glycol in acetone or methanol. The plates were dried in a stream of cool air, leaving a uniform impregnation of the thin layer and used immediately.

System "W": Kg//Glycol/CH_2Cl_2 (Butruk and Vaedtke, 1968) Kieselghur plates were pre-run in 10-15% solutions of ethylene glycol in acetone, the steroids and extracts applied at the origin and developed in dichloromethane saturated with ethylene glycol. The Rf values for the steroid standards used in this study are given in Table 3i. The system, without over-running, is effective in separating cortisone, cortisol (and THE) from some of their polar metabolites. Over-running for 1.5 hours completely separates cortisol and THE while the polar metabolites may separate to some extent. After 3 hours over-running the polar metabolites including the α- and β-cortols and cortolones, are separated completely.

Table 3i

Rf Values for Standard Steroids in Three TLC Systems

Steroid	System "W"	System "Y"	System "Z"
Cortisol	0.57	0.37	0.65
Cortisone	0.91	0.50	0.75
THF	0.20	0.21	0.55
THE	0.46		0.70
a-THF		0.23	0.57
α Cortol	0.06	0.07	0.34
β Cortol	0.07	0.07	0.34
α Cortolone	0.16	0.13	0.49
β Cortolone	0.17	0.10	0.45
11-HEt		0.53	0.79
11-HAn		0.57	0.81
11-KEt		0.60	0.82
11-KAn		0.63	0.81
6βOHF	0.05	0.23	0.63

System "W" : Kg//Glycol (10%)/CH_2Cl_2 (Approx. 30 min)
System "Y" : Si gel/$CHCl_3$/EtOH/H_2O (88:13:1) (Approx. 1 hr)
System "Z" : Si gel/CH_2Cl_2/MeOH (4:1) (Approx. 45 min)

All steroids were run as single "spots" and located by spraying with Lieberman-Burchard reagent, heating at 110°C for 10 min and viewing under long wavelength ultra-violet light.

System "X": Kg//Glycol/Benzene/Methylcyclohexane (1:1) (Langford, 1968). The Kieselghur plate was pre-run in a 30% solution of ethylene glycol in methanol, and developed with benzene:methylcyclohexane (1:1). The system is effective in separating the C-19 17-ketosteroid metabolites of cortisol: 11-hydroxyetiocholanolone, 11-hydroxyandrosterone, 11-ketoetiocholanolone, 11-ketoandrosterone and dehydroepianodrosterone.

Adsorption Systems: Silica gel GF-254 was used throughout this work because of its fluorescent indicator properties.

System "Y": Si gel/$CHCl_3$:EtOH:H_2O (88:13:1) (Bailey, 1966). This system was used to separate cortisone, cortisol and their polar metabolites. Rf values are shown in Table 3i.

System "Z": Si gel/CH_2Cl_2/MeOH (4:1) (Thrasher et al., 1969, adapted, see Chapter 5). The system gives separations similar to system "Y", but is able to separate 6β-hydroxycortisol from cortols and other polar metabolites.

Detection of steroids on TLC plates

Detection of the -4ene-3one group: Silica gel GF-254 (Merck) contains zinc silicate as a fluorescent indicator. Steroids with the -4ene-3one group appear as dark purple spots on a bright, yellow-green background, when viewed under short wavelength ultra-violet light. This property was used extensively in this work, since it enabled steroids to be detected without their destruction or chemical modification by spray reagents.

Spray Reagents: Specific and non-specific spray reagents for the detection of steroids, have been well documented in the literature. Those used in this work are described below. The reagents were applied using a "Quick-fit" chromatography sprayer and compressed air.

(1) Non-specific: Lieberman-Burchard reagent (Anthony and Beher, 1964).

2 ml acetic anhydride and 2 ml concentrated H_2SO_4 were added to 18 ml absolute ethanol, with cooling. After spraying, TLC plates were

heated at 110°C for 10 mins. This time was found to produce optimum colour development without charring (Langford, 1968). Viewing under long wavelength ultra-violet light revealed steroids as spots of varying colours which, in some cases, may be used as a tentative identification.

(2) Specific: (a) Blue Tetrazolium (Bush and Willoughby, 1957)

A 2% solution of blue tetrazolium in 3 M ethanolic KOH, was found to be stable for several months if stored below 0°C. In the cold the spray is specific for steroids with an α ketol side chain but is less specific if the plate is heated.

(b) Zimmerman reagent (Lisboa, 1964)

Equal parts of 2% ethanolic m-dinitrobenzene and 2.5 M methanolic KOH were mixed immediately before use. The spray is specific for 17-ketosteroids.

Extraction of Thin Layer Material

Following separation on thin layer plates, steroid material was eluted from unsprayed plates as follows: Kieselghur plates were heated at 45°C for 24 hours (Langford, 1968) to evaporate ethylene glycol before elution, while silica gel plates were eluted without prior treatment. Appropriate areas of solid support were carefully scraped off the glass plates with a razor blade, and transferred to 15 ml test tubes with the aid of a small funnel and a camel-hair brush.

Satisfactory elution of steroids (as determined by recovery of radioactive markers) was achieved by shaking the material ("Equipoise-type" shaker, Analite Pty, Australia) with 10 ml solvent for 15 min. The tubes were centrifuged, the solvent aspirated off, and the extraction repeated once more. The combined eluates (20 ml each) were evaporated to dryness and the steroids redissolved in a suitable volume of solvent.

Langford (1968) confirmed the inferior elution properties of EtOAc and CH_2Cl_2 when used alone to extract steroids from silica gel, and undiluted MeOH is known to destroy some C-21 corticosteroids. In this work CH_2Cl_2:MeOH (9:1) (Idler and Horne, 1968) was used for elution of

steroid material derived from "Extract I"; and EtOAc:MeOH (1:1) (Langford, 1968) for those derived from "Extract II".

Scintillation Counting

Recovery of some steroids i.e. cortisol and 6β-OH cortisol in the procedure was estimated by the addition of tritiated markers.

Purified extracts were eluted from thin layers as described above, and aliquots added to scintillation vials. After evaporation of the solvent, 10 ml of toluene scintillation fluid containing 0.4% P.P.O. (2,5,diphenyloxazole) and 0.04% P.O.P.O.P. (1,4,bis[2-(5 phenyloxazoyl)]-benzene were added. Vials were counted in a Packard Tricarb liquid scintillation counter.

Quantitative Estimation of Steroids

It was intended to quantitate purified steroids using the Porter-Silber colour reaction, described in Chapter 5.

RESULTS AND DISCUSSION

Attempts were made to separate cortisol, cortisone and some of their metabolites from aliquots of a 24 hour urine collection, using the TLC methods previously described. The steroids were identified by comparison of their Rf's with those of standard steroid markers, using the detection techniques detailed above. The abbreviations used for these steroids are given at the beginning of this thesis.

Samples were hydrolysed and extracted by the standard procedures described, to give two extracts (I and II) from each sample.

Initial separations were effected by applying bands of extract to Kieselghur plates impregnated with 10% ethylene glycol. A "Zaffaroni" type partition system was used because of the higher loading capacity compared with adsorption systems.

Steroid markers were generally applied at the edges of the plates so that, when necessary, the areas containing "samples" might be covered while the markers alone (and in some cases, small areas of extract) were sprayed.

Two typical separation experiments are described below and are shown, schematically in Figs 3iii and 3viii.

Experiment "A"

Attempts were made to separate and identify 15 steroids: E, F, THE, THF, a-THF, αCor, βCor, αCo, βCo, 11HEt, 11HAn, 11KEt, 11KAn, DHEA, 6βOHF.

A 24 hour urine sample was hydrolysed and extracted with CH_2Cl_2 (I) and EtOAc (II). Both extracts were applied as 8 cm bands to a Kieselghur/10% glycol plate, with a spot containing 5-10 µg cortisol (in EtOH) at each edge as a marker. An identical plate was spotted with the following steroid markers: F, E, 11HEt, THE, THF, αCor, αCo, DHEA, 6βOHF, and 1 cm bands of extracts I and II. The 2 plates were developed simultaneously in glycol-saturated CH_2Cl_2 (TLC system "W"), without over-running. After drying, the marker plate was treated with Lieberman-Burchard reagent and positions of individual steroids marked. Some indistinct bands of colour were evident where the urine extracts had been applied. The marker edges of the extract plate were sprayed

Figure 3iii

Schematic Representation of Experiment "A"; Fractionation of Steroids by TLC

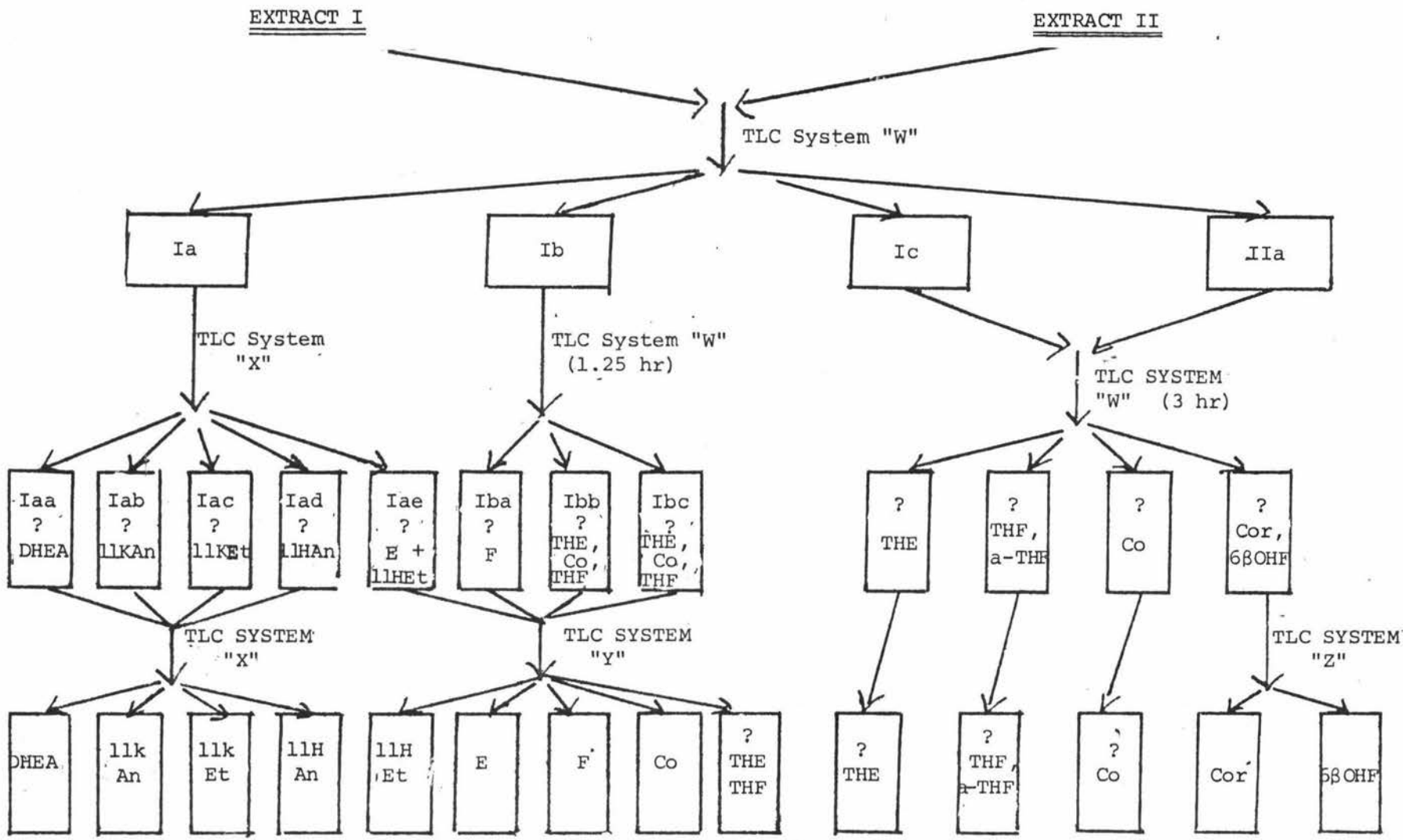
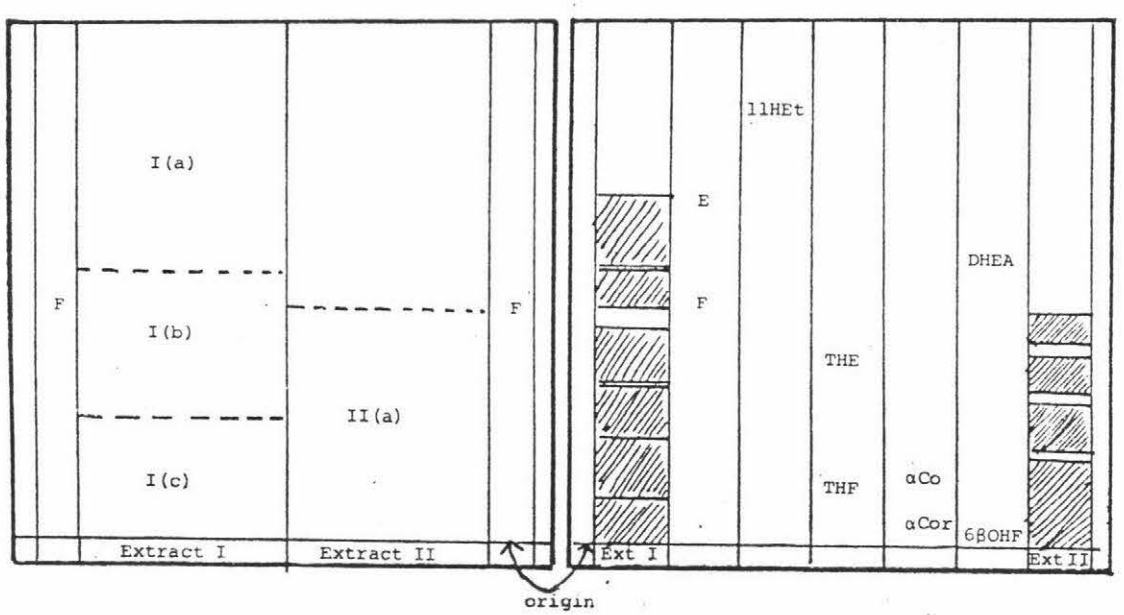


Figure 3iv

TLC EXPERIMENT "A"

Initial Separation of Extracts

in TLC System "W"



Edges sprayed with dichlorofluoroscein

Plate sprayed with Lieberman-Burchard Reagent

with dichlorofluoroscein to identify the cortisol markers and allow the plates to be aligned.

The extract plate was marked out into areas which, on the basis of standard markers would contain: cortisone and 17 ketosteroids (Ia), DHEA, cortisol and THE (Ib), THE, THF, and the more polar metabolites (Ic). Extract II showed no bands "above" cortisol and little obvious separation. These areas and marker positions are shown schematically in Fig. 3iv. The areas Ia, Ib, Ic and IIa were eluted separately.

Area Ia was rechromatographed in system "X". Kg/glycol/benzene/methylcyclohexane, as a short band, together with each of the 17-ketosteroid and cortisone markers. After development, the 17KS markers and a 1 cm band of extract were treated with Zimmerman reagent, while the cortisone marker and a further 1 cm band of extract were sprayed with Blue Tetrazolium. Positions of the steroids are shown in Fig. 3v.

Both E and 11HEt remained at the origin, however all other markers showed clean separation. No coloration was observed in those bands of the extract which were sprayed. Unsprayed areas corresponding to DHEA (Iaa), 11KAn, (Iab), 11KEt (Iac) and 11HAn (Iad) were removed, eluted and rechromatographed as small spots in the same system. After spraying the entire plate with Zimmerman reagent, single spots corresponding to the appropriate markers, were observed from each eluate.

The origin area, Iae, thought to contain E and 11HEt, was eluted and chromatographed in TLC system "Y" (Si gel/$CHCl_3$/EtOH/H_2O) for 1.5 hours, which effected a satisfactory separation (Fig. 3vi).

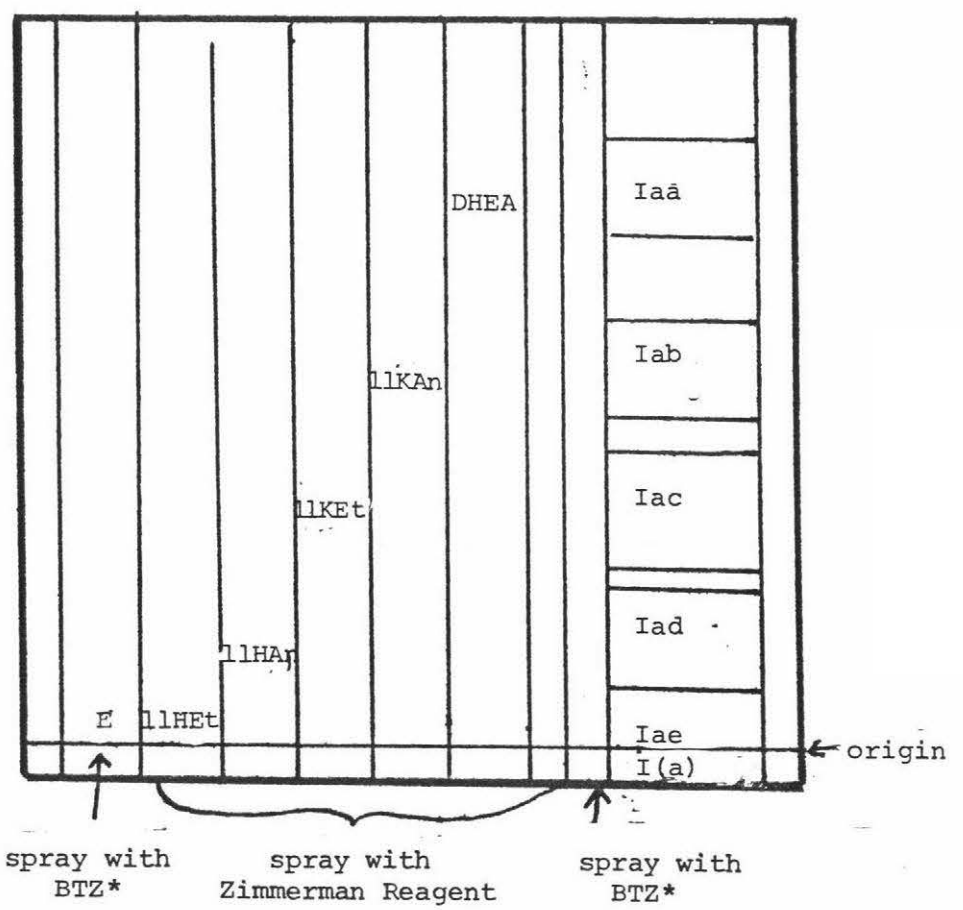
Eluate Ib was further separated by over-running (total time 1.25 hours) in TLC system "W", with markers of F, DHEA, THE, THF, a-THF and Co. The plate was sprayed, divided up and eluted as shown in Fig. 3vii to give eluates Iba (?F, DHEA); Ibb (?THE); Ibc (?THF, a-THF, Co). These were run as spots in the TLC system "Y" for 1.5 hours. The entire plate was sprayed with Lieberman-Burchard reagent, heated and the spots marked under long wavelength ultra-violet light. Though some separations were indistinct, probably due to very small amounts of the steroids remaining in the eluates, it was observed that eluate Iba contained only cortisol. Ibb and Ibc showed some possible contamination by 17-ketosteroids, but the possible presence of cortolone was indicated in Ibc (Fig. 3vi).

Figure 3v

TLC EXPERIMENT "A"

Separation of Eluate I(a) [Cortisone and C-19,17-ketosteroids]

in TLC System "X"



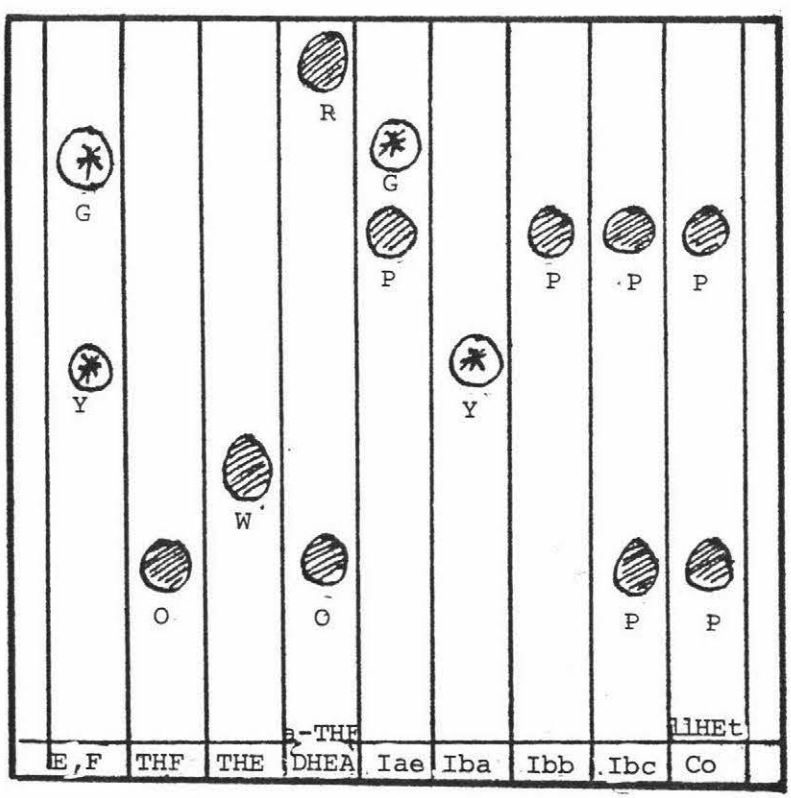
*BTZ: Blue tetrazolium reagent.

Figure 3vi

TLC EXPERIMENT "A"

Separation of Eluates Iae, Iba, Ibb and Ibc

in TLC System "Y" (1.5 hr)



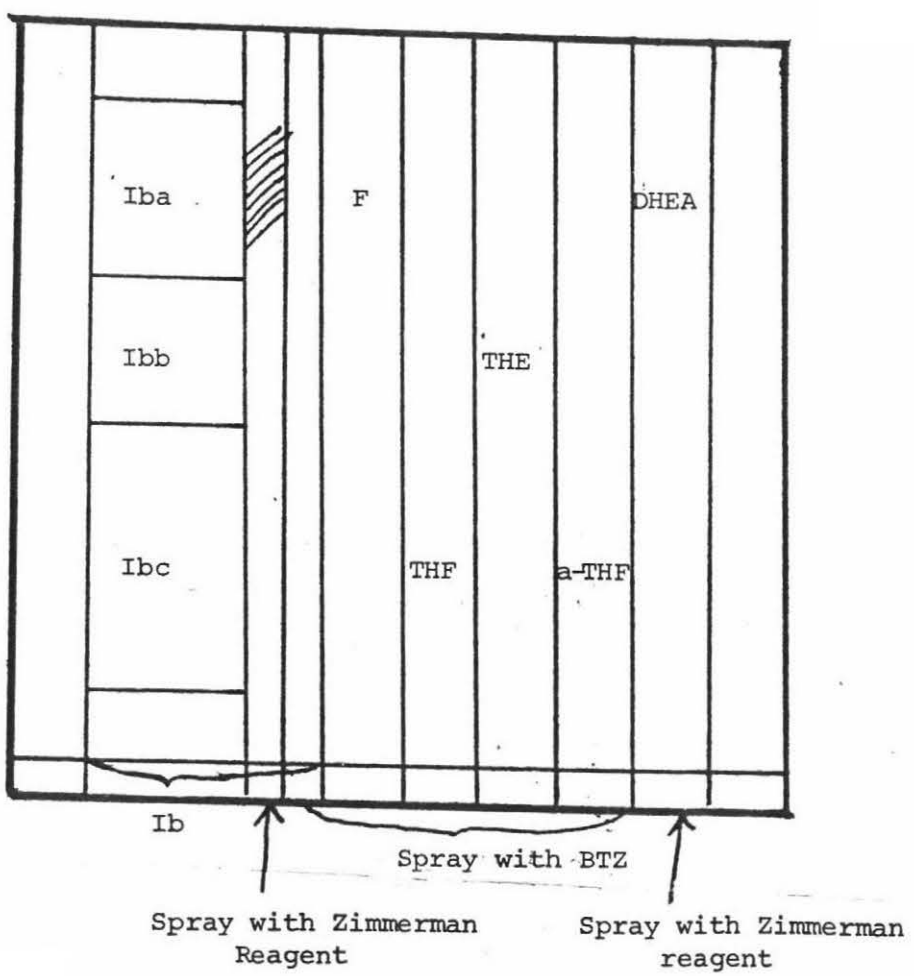
*denotes spots which fluoresced under short wavelength u.v. light, prior to spraying with Lieberman Burchard Reagent, i.e. steroids containing the 4-ene, 3-one grouping. Colours of spots are denoted by letters: G = grey; Y = yellow; O = orange; W = white and P = purple.

Figure 3vii

TLC EXPERIMENT "A"

Separation of Eluate I(b) in

TLC System "W" (1.25 hr)



Eluates Ic and IIa (Fig. 3iv) containing the very polar corticosteroids were applied to a Kieselghur plate (10% glycol) as two short (1.5 cm) bands and developed in TLC system "W" for approximately 3 hours. Although satisfactory separation of marker steroids was obtained, neither extract showed significant resolution of its steroid components.

Conclusions

The dichloromethane extract I may be readily separated into three areas by solvent system "W". Ia contains the 17-ketosteroids and cortisone, which may be further separated by system "X" to yield DHEA, 11KAn, 11KEt, 11HAn and (11HEt+E). The latter fraction, which remains at the origin, may be completely resolved by the system "Y".

Fraction Ib contains F, THE and possibly THF and Co. These are partially separated by over-running in system "W" (1.25 hours) and are further separated in the system "Y".

On the basis of results obtained using marker compounds, extract fractions Ic and IIa should be resolvable into their constituent, polar steroids: THE, THF, a-THF, cortolones and (cortols+6βOHF) by system "W", over-run for at least 3 hours. The last fraction containing cortol and 6βOHF could, theoretically be completely separated by TLC system "Z".

The procedure is shown schematically in Fig. 3iii.

Experiment "B"

Since the C-19 ketosteroids may be separated relatively simply, further work concentrated on the resolution of seven C-21 corticosteroids. In this type of experiment, hydrolysed urine samples were spiked with labelled and unlabelled free steroids to aid detection of particular compounds present and to give some indication of recoveries.

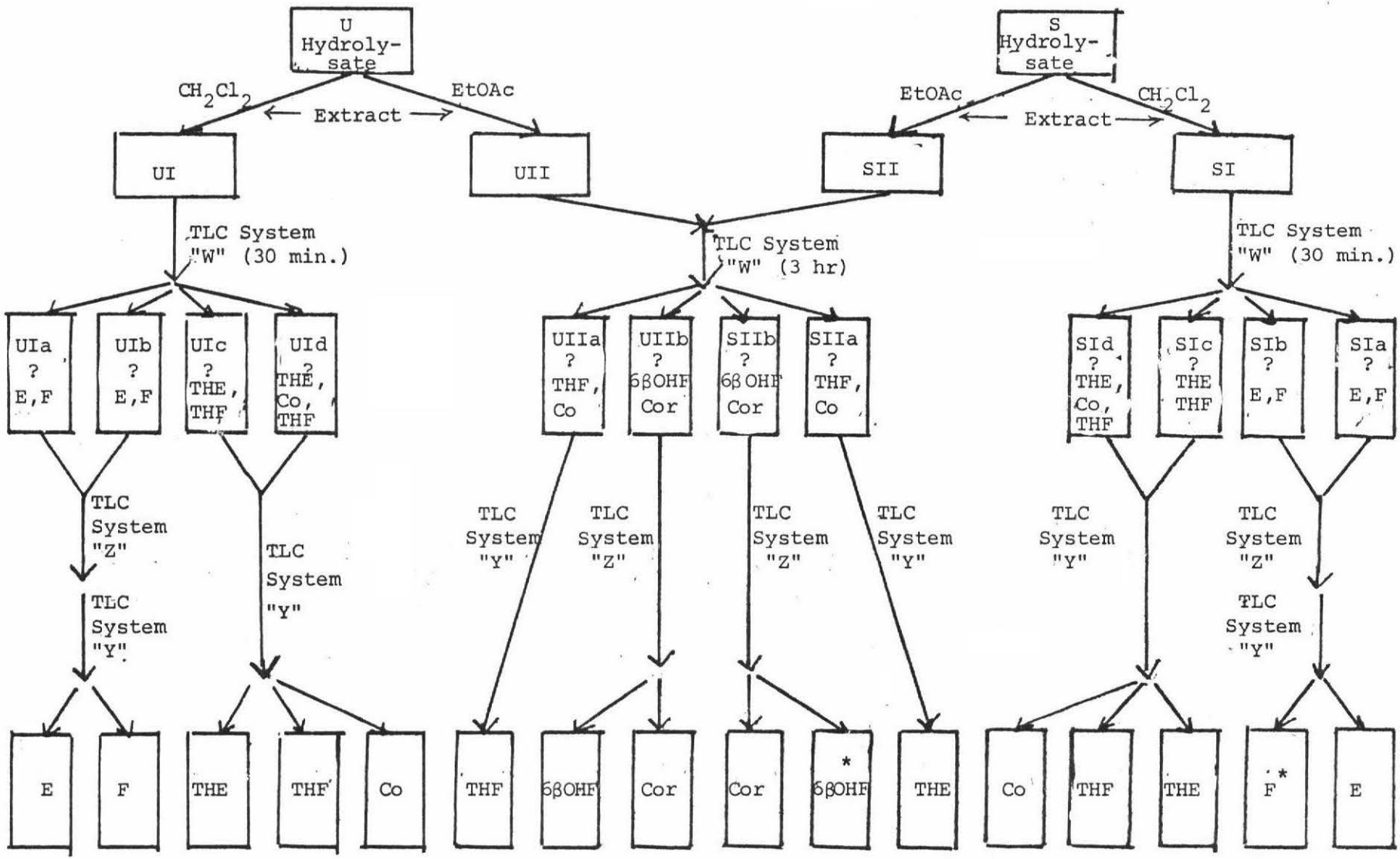
The procedures used in a typical recovery experiment are outlined schematically in Fig. 3viii. Details of TLC and eluted fractions are also shown in Figs 3ix-3xi.

One-tenth of a 24 hour urine collection was hydrolysed and divided into two 50 ml fractions: "U" and "S". "S" was spiked with approximately 15 µg F, 30 µg E, 20 µg 6βOHF, 250 µg THF, 150 µg THE, 10 µg αCor, and 50 µg αCo. These quantities were calculated as being of the same order

Figure 3viii

Schematic Representation of Experiment "B"; Fractionation of Steroids by TLC

(*Denotes tritiated steroid markers: cortisol and 6βOH Cortisol)



as the amounts in one-twentieth of a 24 hour urine collection from the normal values cited by Sandberg and Slaunwhite (1975) and Makin (1975). The steroids, dissolved in MeOH, were evaporated in a 100 ml Erlynmeyer flask and incubated with 50 ml of urine hydrolysate overnight at 4°C. Approximately 80,000 cpm of tritiated 6βOHF and 70,000 cpm tritiated cortisol were also added. The remaining portion "U" of the hydrolysate was used as a recovery control.

Both fractions were extracted with CH_2Cl_2 as previously described to give extracts UI and SI, and with EtOAc to give extracts UII and SII. The extracts UI and SI were chromatographed in TLC system "W" for 30 min, with E, F, THE and THF as markers. The markers and edges of the sample bands were sprayed with Blue Tetrazolium reagent. Areas corresponding to E or F and THF were found in UI, and separate areas corresponding to each of E, F, THE and THF were shown by SI (Fig. 3ix). The areas designated UIa, UIb, UIc, UId, SIa, SIb, SIc, SId in Fig. 3ix were removed from the plate and eluted.

UIa, UIb, SIa and SIb, thought to contain E and F, were further fractionated in system "Z", followed by system "Y". Complete separation of the individual steroids was shown by viewing the fluorescent plates under ultra-violet light. Elution and scintillation counting of all spots from the spiked extracts indicated a clean separation of cortisol from the other steroids, in particular cortisone, although "recovery" of ^{3}H-cortisol was only of the order of 10%.

Further purification of fractions UIc, UId, SIc and SId in the system "Y" showed that all fractions contained both THE and THF, and that UId and SId also contained cortolone. The complete separation of the three steroids was effected by this system (Fig. 3x).

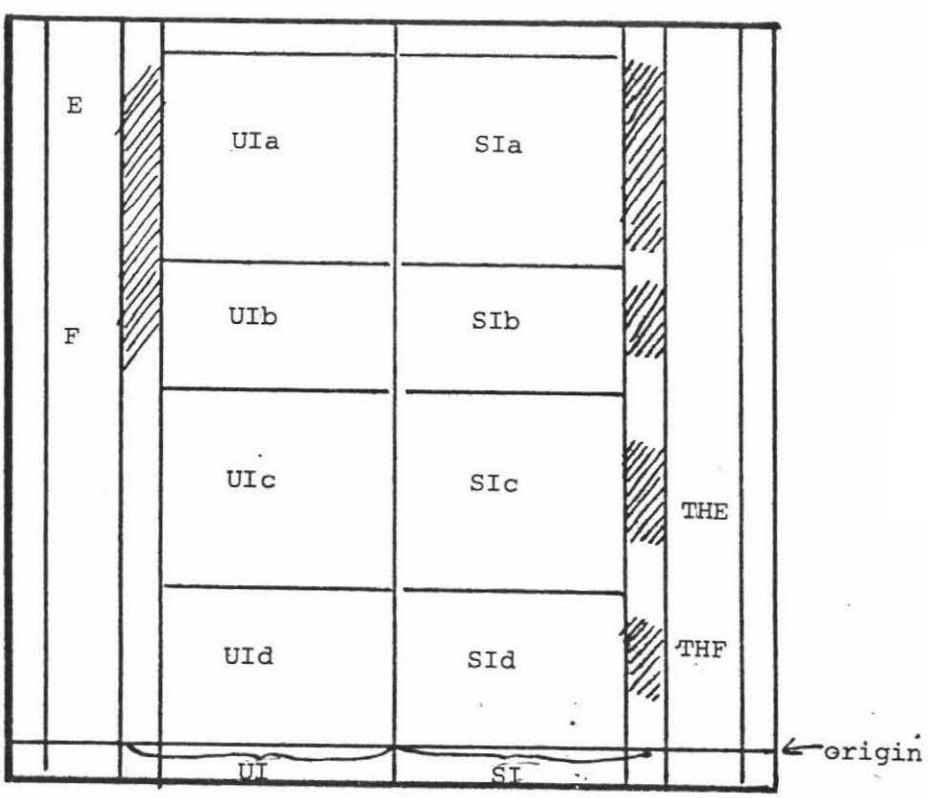
The EtOAc extracts UII and SII were chromatographed for 3 hours in TLC system "W", with THF and 6βOHF as markers. (THF had previously been shown to have an Rf similar to cortolone and 6βOHF to cortol, in this system). Fig. 3xi illustrates the separation of the extracts into groups containing (THF+Co) and (6βOHF+Cor) as detected by spraying markers and plate edges with Blue Tetrazolium.

The eluates UIIa and SIIa were shown to contain only THF, following chromatography in system "Y". Eluates UIIb and SIIb were separated into distinct fractions containing 6βOHF and cortol by system "Z". This was confirmed by the presence of a significant number of counts

Figure 3ix

TLC EXPERIMENT "B"

Initial Separation of CH_2Cl_2 Extracts in
TLC SYSTEM "W"



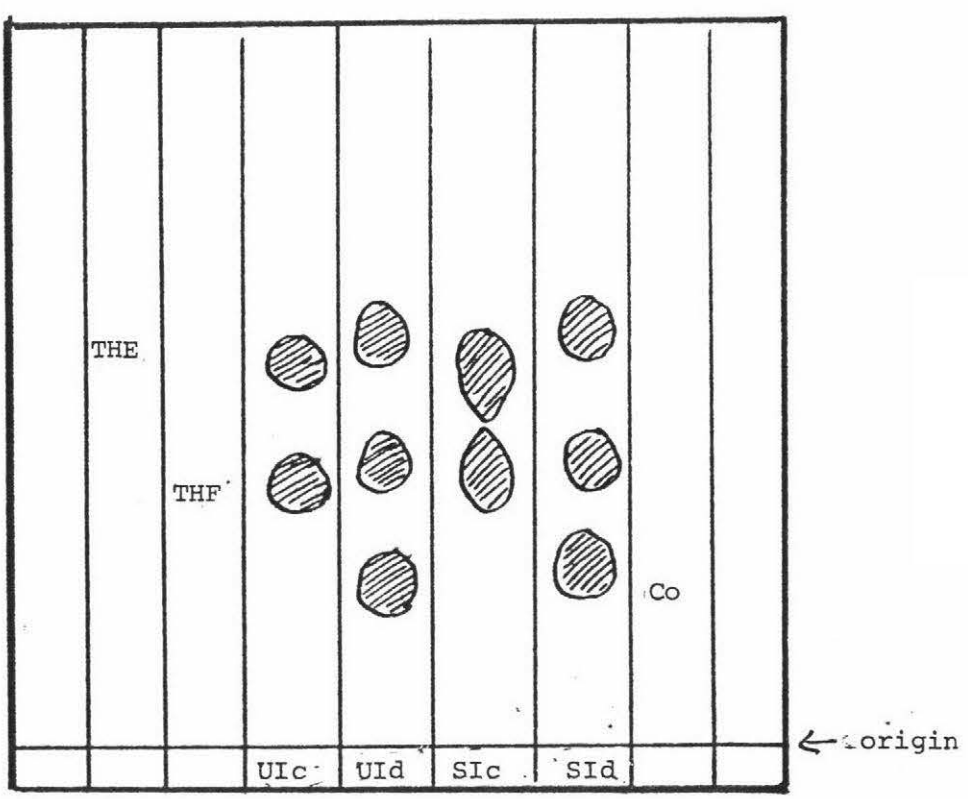
Markers and edges of extracts were sprayed with
Blue Tetrazolium

Figure 3x

TLC EXPERIMENT "B"

Separation of Eluates UIc, UId, SIc and SId

in TLC System "Y"



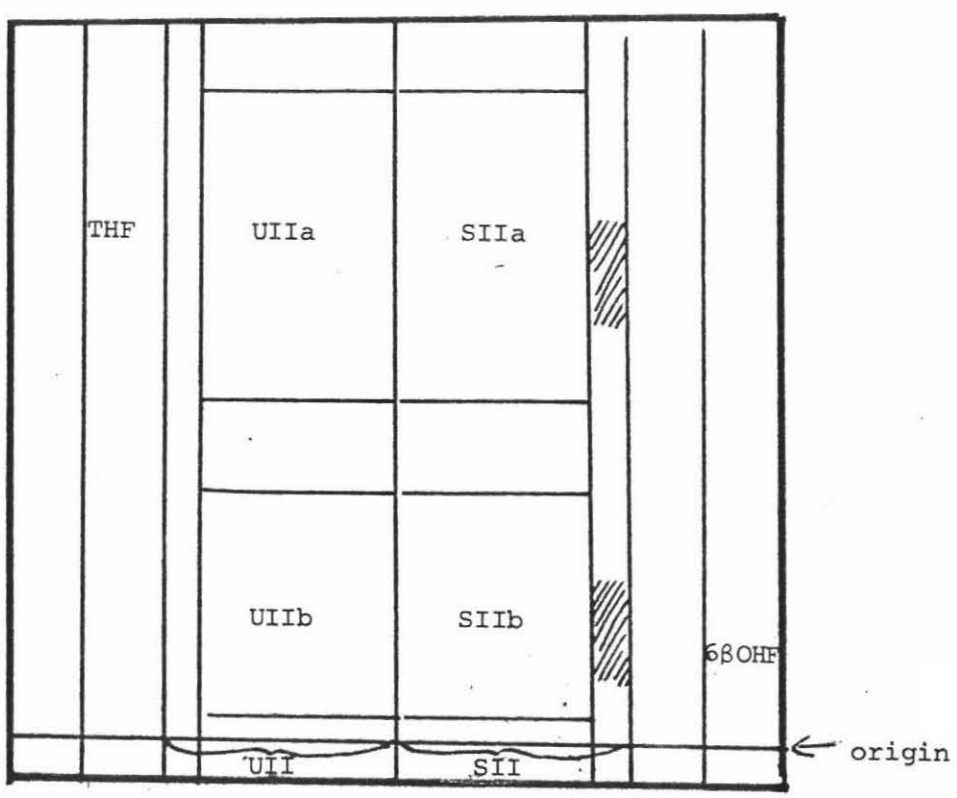
Steroids were visualised by spraying with Lieberman Burchard Reagent, heating and viewing under long wavelength u.v. light.

Figure 3xi

TLC EXPERIMENT "B"

Separation of EtOAc Extracts in

TLC System "W" (3 hr)



Steroids were located by spraying markers and extract edges with Blue Tetrazolium reagent.

in the supposed 6βOHF fraction, although these indicated a recovery of less than 10% of the counts added.

Conclusions

These experiments indicate that repeated TLC will separate the major corticosteroid metabolites found in urine reasonably cleanly. Because of the large number of steps required recoveries are necessarily low, and in many cases the final purified steroids are present in quantities too small for detection by sprays or for quantitation by colorimetric procedures such as the Porter Silber reaction. Quantitation was also hampered by inadequate information on recovery and purity of the final products without, for example, the addition of all labelled steroids to check for constant specific activity and recovery.

The procedures developed here are not only cumbersome and time consuming, but the preliminary work shows that they are subject to a number of cumulative errors and losses. Recoveries of tritiated markers did not exceed 10%, so that little significance could be attached to any quantitative estimates based on these separation methods.

It was thought unwise to waste time refining a system which would, at best, be relatively crude and extremely time consuming. Approximately one week's work was necessary to hydrolyse, extract and resolve a urinary sample. Efforts were, therefore, turned to adapting and developing a high resolution gas chromatography method, using a glass capillary column such as that developed in Horning's laboratory. Such a system held the advantage of having a potential resolving power in excess of any single TLC stage, with simultaneous quantitation of components.

The existence of a standard gas chromatograph in the laboratory, and the availability of commercially-produced surface coated open tubular columns, facilitated the development of such a procedure at this stage.

CHAPTER 4

APPLICATION OF CAPILLARY COLUMN GAS LIQUID CHROMATOGRAPHY TO URINARY STEROID PROFILING

INTRODUCTION

A Review of Recent Advances in Capillary Column Gas Chromatography of Steroids

For more than a decade now, biologically important steroids have been successfully estimated and at least partially resolved by the conventional methods of packed column gas chromatography, as reviewed by Polvani et al., (1967) and Eik-Nes and Horning (1968). Since 1970 attempts have been made to improve resolution by use of open tubular glass capillary columns. Initial work encountered problems in the preparation and pretreatment of the glass and the deposition and stability of the liquid phases, as well as requiring the modification of the injection and detection systems of conventional gas chromatographs. The development of the modern glass open tubular capillary system and its comparison with packed columns is reviewed by Ettre and March (1974).

The most commonly used methods for the pretreatment and deactivation of the glass are etching, silanisation, coating with interlayers of polymers and surface active agents; and the carbonisation of the wall (Rutten and Luyten, 1972; Alexander and Rutten, 1974). The thermal instability of liquid phases, shown by Merle d'Augibne (1971) was overcome by German and Horning (1973) with the introduction of an irregular surface into the film of liquid phase, using fine particles of silanised silicic acid suspended in the film. Prior to coating, absorptive sites on the glass are reduced by silanisation, and film uniformity is increased by the addition of benzyltriphenylphosphonium chloride as wetting agent during the coating process.

Coating of the glass is usually by one of two generally accepted methods (Merle d'Aubigne, 1971): the "dynamic" process, in which the phase, dissolved in solvent, flows through the column continuously to give an even deposition dependent on flow rate; or the "static" process, where the column is filled with phase in its solvent, one end sealed and

the solvent evaporated under vacuum (Rutten and Luyten, 1972).

The two step dynamic-evaporative procedure, developed in Horning's laboratory (German and Horning, 1973; German et al., 1973; Van Hout et al., 1974; Horning et al., 1974), has proved very successful in the preparation of columns to separate urinary steroids. One of the column's advantages lies in its ability to accept and resolve large samples of complex mixtures, containing components present in widely varying amounts. Their columns, ranging from 30 to 60 metres in length and with internal diameters around 0.3 mm show 50-150,000 "theoretical plates" (Pfaffenberger and Horning, 1977) compared with conventional packed columns 6-12 ft in length, using non-polar films on silanised diatomaceous earth supports, which give up to 6-7,000 theoretical plate efficiencies (German and Horning, 1973). Schomburg et al. (1974), have successfully used glass capillary columns up to 130 metres, with no treatment other than etching with dry HCl gas at high temperature.

The injection systems of commercially available gas chromatographs require modification when used with capillary columns. Some workers favour a solid injection system (Luyten and Rutten, 1974; Berthou et al., 1974; Shackleton and Honour, 1976); while others connect their columns to the gas chromatograph using an inlet stream splitter (German and Horning, 1972; 1973; Berthou et al., 1974; Schomburg et al., 1974). In each system, however, all components are of glass, particularly when used for steroid analysis.

German and Horning (1973) suggest that for steroid work it is preferable to inject samples in a large excess of solvent and claim to have devised a system where true gas phase splitting occurs by placing the splitter at the end of a short packed column, which also serves to protect the column from "solvent shock" and contamination by non-volatile material (German and Horning, 1972).

Modification of flame ionisation detectors is also necessary when used in conjunction with capillary columns, where carrier gas flows are around 2 ml/min compared with those used in packed column chromatography, where optimal flows are approximately 60 ml/min. Van Hout et al. (1974) and Berthou et al. (1974) describe a system where the capillary column effluent is mixed with an additional volume of heated "make-up" gas, to allow optimal performance of the detector with a minimum of "dead space".

Commercially-produced support coated open tubular (S.C.O.T.) and wall coated (W.C.O.T.) capillary columns are now being produced (e.g. by S.G.E. Pty. Ltd, Australia) with theoretical plate efficiencies around 50,000. These allow separations superior to those obtained with packed columns without the necessity for developing coating and deactivation procedures in each laboratory.

The current status of capillary column chromatography is amply reviewed by Schomburg et al. (1976), including the development of the method, progress in production, connection, sampling, injection, detection and advanced applications.

Preparation of Samples for GLC

Before the biological steroids present in urine, blood and tissue extracts may be analysed by gas chromatography certain purification procedures are required. The extent of such preliminary purification varies from one laboratory to another and depends largely on the compromise which must be made between the specificity of the measurement on one hand and the recovery of steroid and time of analysis on the other. The high resolution of capillary GLC gives inherent specificity and minimises sample purification requirements.

(a) Hydrolysis

Urinary steroids, except for a few very polar ones, are excreted as conjugates particularly of glucuronic and sulphuric acids. Since acid hydrolysis may result in structural changes to the individual steroids (Horning and Horning, 1970), enzymatic hydrolysis with relatively crude extracts of glucuronidase, sulphatase and combinations of the two are most common (Horning and Horning, 1970; Luyten and Rutten, 1974; Pfaffenberger and Horning, 1975; 1977; Setchell and Shackleton, 1975; Setchell et al., 1975; Setchell et al., 1976; Adlercreutz and Schauman, 1976).

In addition, solvolysis is often applied to completely free some androgens e.g. androsterone, from their sulphate conjugates (Janne et al., 1969; Shackleton and Honour, 1976; Pfaffenberger and Horning, 1977).

In general, the hydrolysis of conjugates represents the slowest and least efficient step in the entire analytical process (Shackleton and Honour, 1976).

(b) Extraction and purification

Steroids have been extracted from urine (and plasma) by several methods. Perhaps the earliest and still most popular, is by direct extraction with large volumes of solvents of suitable polarities. Of these ethyl acetate, dichloromethane and chloroform are most commonly used for the polar corticosteroids (Horning and Horning, 1970; Luyten and Rutten, 1974; Pfaffenberger and Horning, 1975; 1977). Stillwell et al (1973) describe a method for extracting polar steroids such as estriol and cortisol from urine and plasma into a small volume of solvent, following the addition of potassium or ammonium carbonate which acts essentially as a "salting-out" agent.

Many workers now combine or replace the bulk extraction procedure with chromatography on a non-specific neutral resin (such as "Amberlite XAD-2") eluting with a polar solvent (Luyten and Rutten, 1974; Axelson and Sjovall, 1974; Setchell and Shackleton, 1975; Setchell et al., 1975; Setchell et al., 1976; Shackleton and Honour, 1976).

Further purification, with the possibility of fractionating the steroids into groups, has been achieved by chromatography in miscible solvents on neutral or ion-exchange, lipophilic derivatives of "Sephadex" e.g. LH-20, "Lipidex" (which contains hydroxyl alkyl groups) and D.E.A.P. "Sephadex" (diethyl aminohydroxyl propyl) LH-20, alone or in combination (Axelson and Sjovall, 1974; Sjovall, 1975; Setchell et al., 1976; Shackleton and Honour, 1976). Often, solvent systems are chosen so that the steroids elute with the solvent front in a small volume while interfering compounds and steroids of widely differing polarities remain on the solumn.

(c) Derivative formation

Steroids containing hydroxyl and ketone groups, particularly members of the C-21 adrenocorticosteroid group with the 17α,21-diol-20one structure must be stabilised through derivative formation to prevent loss of the side chain by thermal decomposition during G.C. analysis. Chemical modification of reactive groups may also help to minimise their adsorption

to the glass and support material. A variety of methods are available, including the cyclic boronate, acetonide, and methylene deoxy procedures reviewed by Sakauchi and Horning (1971). By far the most popular however, would appear to be the formation of methoxime-trimethylsilyl ethers (Gardiner and Horning, 1966; Horning and Horning, 1970; Sakauchi and Horning, 1971; Vollmin, 1971; Thenot and Horning, 1972; Axelson and Sjovall, 1974; Pfaffenberger and Horning, 1975; Shackleton and Honour, 1976).

Demisch and Steib (1968) showed that the trimethylsilyl (T.M.S.) ether derivatives of testosterone and its metabolites gave improved stability and separation of isomers, shorter retention times, and required lower column temperatures than the parent compounds.

Reactive ketone groups are readily converted to enol-T.M.S. ethers, which are however readily hydrolysed and therefore are not always entirely satisfactory for analytical purposes (Sakauchi and Horning, 1971). It is therefore, preferable to deactivate reactive ketone groups prior to silylation. The O-methyloximes, described by Fales and Luukkainen (1965) are suitable for this purpose and are less prone to decomposition than the N,N-dimethyl hydrazones (Vanden Heuval and Horning, 1963).

Most workers use the now "classic" method of Thenot and Horning (1972) where methoxyamine hydrochloride is used to derivatise reactive ketone groups prior to trimethylsilylation. The highly hindered 11-ketone group does not form a methoxime, but neither does it show any significant enol-ether formation.

Silylation procedures vary, depending on the reactivity of the groups involved, and a number of silyl donors and reaction conditions are in use. Thenot and Horning showed that the least reactive of the adrenocorticosteroids, α cortol, could be persilylated in 2 hours at 100°C, when the powerful silyl donor trimethylsilyl imidazole was used in the presence of pyridine hydrochloride, a by-product of the methoxime reaction.

It has also been shown that the concentration of methoxyamine HCl used affects the ratio of syn- and anti- isomers produced, and problems may arise if its concentration is not kept constant (Pfaffenberger and Horning, 1975; Shackleton and Honour, 1976).

Devaux et al (1971) have demonstrated the successful use of benzyloxime ketone derivatives which improve the resolution of steroids

containing reactive ketones from those with non-reactive ones. It does however, result in longer retention times.

In systems involving liquid injection with stream splitting, the derivatives may be injected directly, without prior removal of the reagents. Solvents must however be removed when solid injection is employed. For this purpose passage of a hexane solution through a column of a lipophilic "Sephadex" derivative "Lipidex"-5000, removes reagents and any other contaminants which have escaped previous purification procedures (Sjovell, 1975).

Identification and Estimation of Steroids by GLC

For quantitation of steroids and correction for work-up losses, most workers routinely include at least one internal standard before derivatisation. For greatest precision it is advisable to include one standard which elutes before the steroids under investigation and one which elutes afterwards. 5α-androstane-3α,17α diol and cholesteryl butyl ether (respectively) are suitable for determination of androgens and adrenocorticosteroids (Shackleton and Honour, 1976).

A semi-quantitative estimate of steroid concentration may be based on comparison of the areas under steroid peaks with those of a suitable internal standard. However, equal amounts of different steroids often show differing G.C responses, so that separate "mass response factors" should be obtained experimentally for each steroid relative to a given standard. This variation has been attributed to differences in purity, efficiency of derivatization, detector response and column condition (Shackleton and Honour, 1976; Pfaffenberger and Horning, 1977).

In general the precision and accuracy of such a system decreases when a large number of compounds are estimated simultaneously, but in many cases the overall profiles are more informative than exact estimates of fewer individual compounds (Shackleton and Honour, 1976).

Specific steroid peaks are positively identified by combining G.C. with mass spectrometry, and though Pfaffenberger and Horning (1975) advise against identification solely on the basis of retention data, Luyten and Rutten (1974) suggest that such identification may be permissible for routine screening purposes.

A number of workers, in particular Horning (Pfaffenberger and Horning, 1975; 1977), have obtained and tabulated retention data relative to n-alkanes, which is known as the "methylene unit" and is characteristic of individual steroids, in a given chromatography system.

Using combinations of the above methods, workers have obtained profiles of human urinary steroids in normal and pathologic cases (Horning and Horning, 1970; 1971; Vollmin, 1971; German and Horning, 1973; German et al., 1973; Horning et al., 1974; Luyten and Rutten, 1974; Pfaffenberger and Horning, 1975; 1977; Shackleton and Honour, 1976). In general, profiles obtained from different laboratories show very close similarities though this approach is more used for identifying gross abnormalities in steroid metabolism than following subtle changes requiring exact measurements of concentration.

MATERIALS AND METHODS

MATERIALS

All chemicals, unless specified to the contrary, were of reagent grade or better, and were supplied by May and Baker Ltd, British Drug Houses Ltd or Sigma Chemical Co.

All solvents were redistilled before use and water was glass distilled and deionised by passage through an ion exchange resin.

β-Glucuronidase, supplied by Sigma, is described in Chapter 3.

Pyridine was either silylation grade (Pierce Chemical Co.), or analytical grade, obtained from Ajax Chemical Co. Ltd, Australia, and was stored over B.D.H. "molecular sieve".

Varaport-30, mesh size 100/120: Varian Aerograph, Walnut Creek, Calif., U.S.A.

Methyl silicone rubber gum SE-30: Applied Science Laboratories, Pa., U.S.A.

Trimethylsilyl imidazole (TSIM): Pierce Chemical Co., and Sigma, was stored, desiccated, at 4°C.

Methoxyamine hydrochloride was specially prepared by Dr D.R.K. Harding, Dept of Chemistry, Biochemistry and Biophysics, Massey University, N.Z., by the method of Hjeds (1965), and was stored desiccated.

Androsterone, DHEA, 11-KAn, 11-HEt, E, F, pregnandiol (Pd), THE, THF, a-THF, αCo, βCo, αCor, βCor were supplied by Mann Research Labs, Etiocholanolone, 11-KEt, 11-HAn, pregnantriol (Pt), androstenetriol (Atr), 6βOHE and Cholesterol n-butyl ether (CBE) were from Sigma Chemical Co. 6βOHF was supplied by Steraloids Inc., Pawling, N.Y., U.S.A.

METHODS

Gas Chromatography

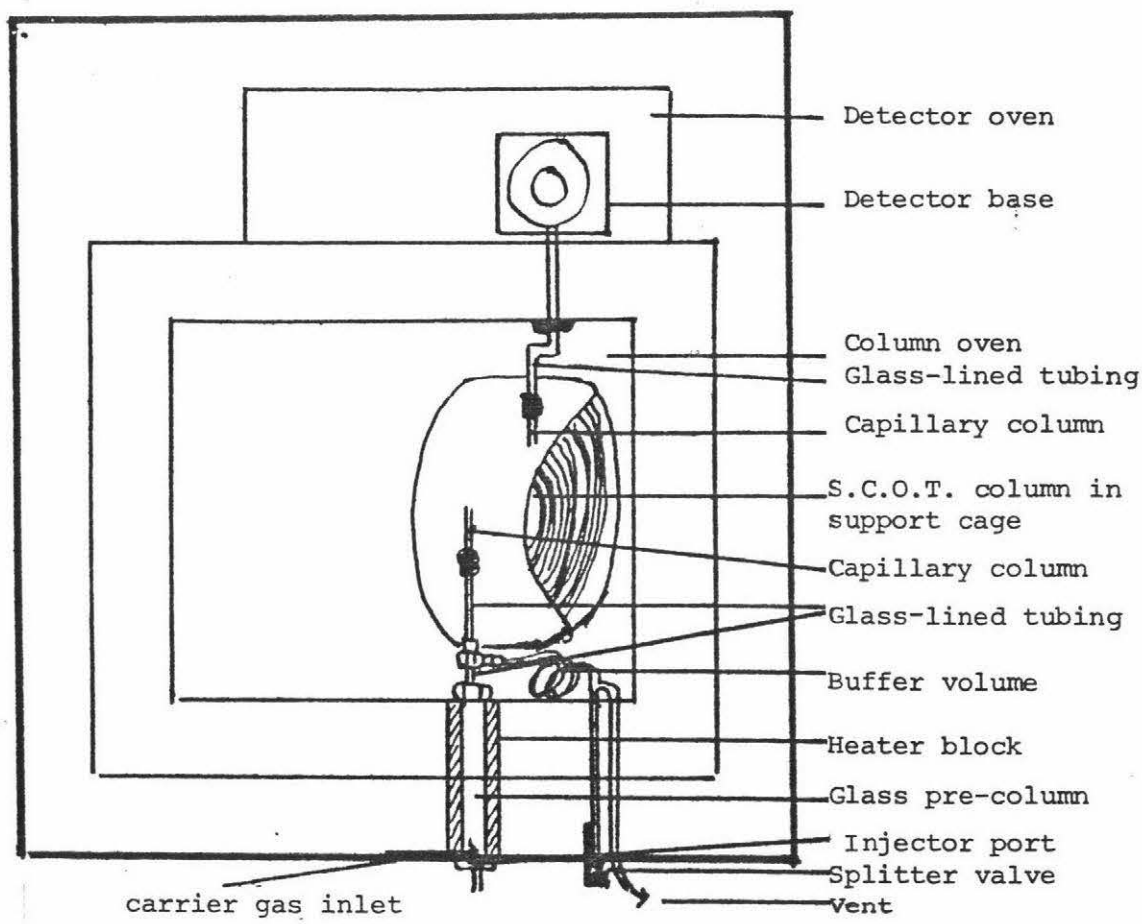
(a) Gas chromatograph:

A Varian 2700 series gas chromatograph was adapted to accept a glass "Support Coated Open Tubular" (S.C.O.T.), capillary column (GSD/SE-30/S; Scientific Glass Engineering Pty Ltd, Melbourne, Australia).

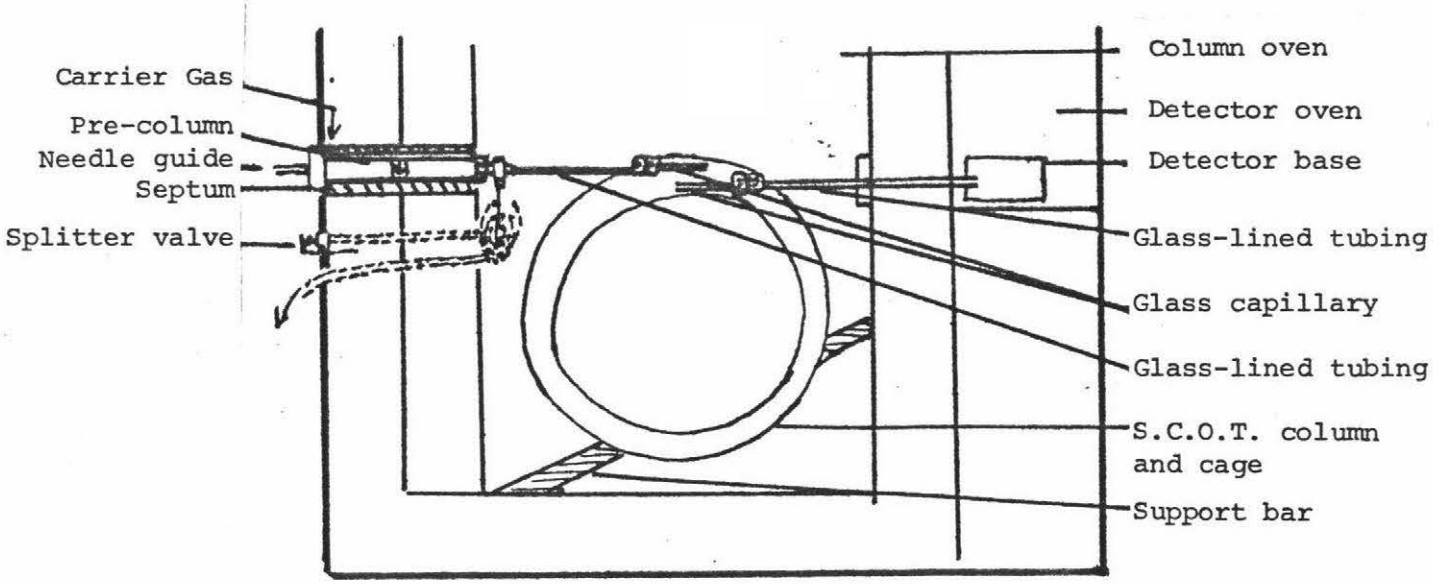
Figure 4i

Connection of S.C.O.T. Column to Gas Chromatograph

(a) Top view:



(b) Side view:



The column was of deactivated, neutral borosilicate glass, with a "Chromosorb" based support material, silanised, and coated with silicone SE-30, as specified in S.G.E. pamphlets CSA 1/74 and GSC 2/74.

The column supplied was 46 meters long with an internal diameter of 0.5 mm and showed an "ex-factory" efficiency corresponding to 46,200 theoretical plates.

The column was connected to the chromatograph as shown in Fig. 4i by means of stainless steel, glass-lined tubing, 1/16 inch O.D. and 0.5 mm I.D. using high temperature graphite ferrules, and was supported by a metal support cage (all supplied by S.G.E.). Glass-lined tubing was joined to the capillary by S.G.E. "zero dead volume" unions, with a reduced bore at one end, according to the instructions in S.G.E. pamphlet SGE 9, and reproduced in Fig. 4ii. Swagelok^{R} fittings were used throughout the system.

(b) Inlet splitter

To reduce the amount of sample passing through the column, the system was fitted with a S.G.E. inlet splitter system: GISS-4A (Fig. 4iii) which utilises the chromatograph's existing injector port connections. The system includes a 4 mm I.D. glass column, approximately 10 cm in length, connected to the splitter system as shown in Fig. 4iii.

This precolumn, together with the glass lined connecting tubing leading to the S.C.O.T. column, was silanised by soaking in a 5% (v/v) solution of dimethyldichlorosilane (Sigma) in toluene for approximately 1 hr (adapted from German and Horning, 1973). Glass cotton used in the column packing was similarly treated.

The precolumn was packed with a plug of 10% SE-30 on Varaport-30 approximately 1-2 cm in length. The packing material was prepared by coating Varaport-30 with a solution of SE-30 in chloroform according to the method of McNair and Bonelli (1967).

The injector port was fitted with a high temperature, low bleed, rubber septum (Supelco Pyrosep S-1, 6 mm) with adaptor and needle guide (also Supelco).

(c) Detector

The standard Varian flame ionisation detector was used without modification the S.C.O.T. column being connected directly to the

Figure 4ii

Connection of Glass-lined Stainless Steel Tubing to S.C.O.T. Column, using S.G.E. "Zero Dead Volume" Unions

(Reproduced from S.G.E. Pamphlet: SGE9)

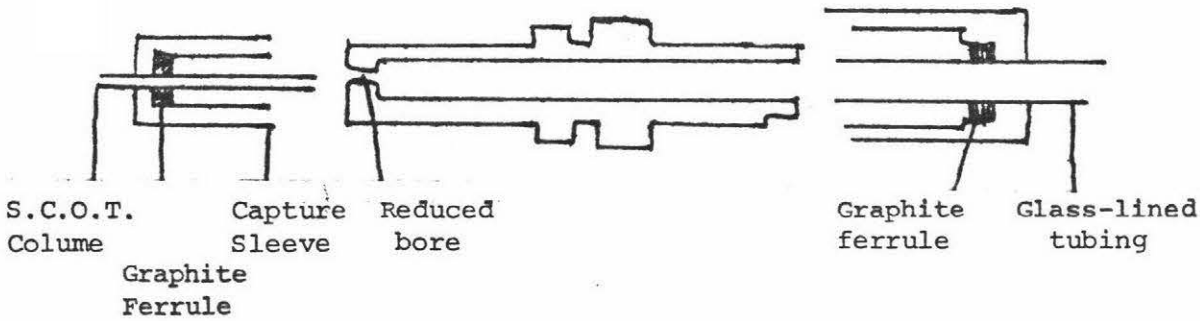
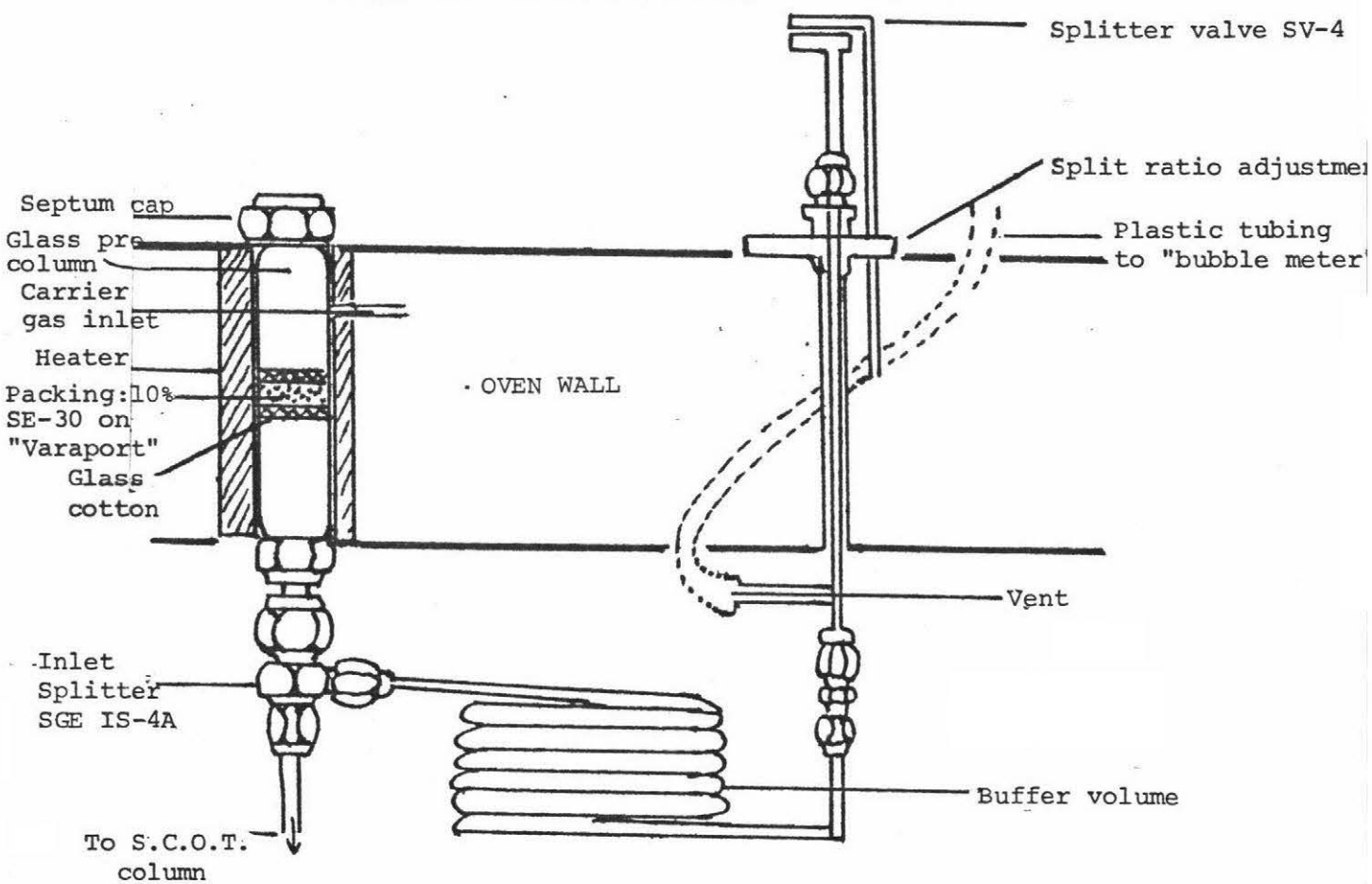


Figure 4iii

S.G.E. Inlet Splitter System: GISS-4A
(Reproduced from S.G.E. Pamphlet CSA1/74)



detector base with glass lined tubing as shown in Fig. 4i.

(d) Gas flows and regulation

The chromatograph was fitted with a Varian Pressure Gauge Flow Controller. A needle valve was fitted between the regulator and the column to allow fine regulation of the flow through the precolumn.

Carrier gas (nitrogen) flow rates were measured at the detector and splitter vents by bubble meters, the sum of the two being the assumed flow through the precolumn. In general, flow through the S.C.O.T. column was maintained between 2 and 2.5 ml/min and the splitter vent flow between 15 and 20 ml/min, giving a constant split-ratio of approximately 1:7-1:8, which was found not to vary over the temperature range used.

Unlike Horning's system, no make-up gas line to the detector was employed. Rather, a 40% mixture of hydrogen in nitrogen was supplied directly to the hydrogen inlet of the detector, at a rate of 75 ml/min, together with air at 300 ml/min. In the Varian F.I.D. detector the position of the hydrogen gas inlet is sufficiently close to the carrier gas inlet to allow the slow-moving carrier gas to mix with, and be swept through the detector by the hydrogen/nitrogen mixture, entering through the hydrogen inlet.

(e) Integrator

Emerging peaks were automatically integrated by a Varian Model 477 Integrator with automatic baseline correction.

(f) Recorder

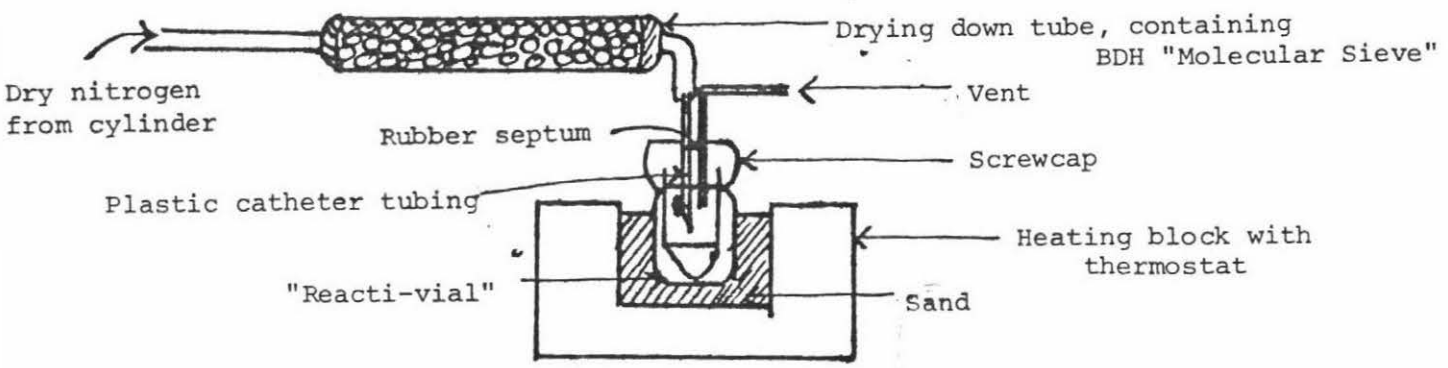
Peaks were recorded by a T.D.K. potentiometric recorder (Tohshin Electron) with 1 mV full scale sensitivity.

(g) Operating conditions

In general, chromatography conditions were maintained constant throughout the course of this work. The injector block temperature was 250°C; detector 300°C; and the initial oven temperature 200°C. The column oven was held at 200°C for 10-15 mins following injection, then temperature programmed at 1-2 degrees/min to a maximum of 300°C.

Figure 4iv

Apparatus for Drying Down Steroid Derivatives



Steroid Standards

Derivatisation of Pure Steroids: Methoxime-trimethylsilyl derivatives of a number of steroids were used as chromatography standards.

Derivatisation according to the method of Thenot and Horning (1972) was found to produce multiple peaks for any given steroid, probably due to formation of both syn- and anti-isomers (Shackleton and Honour, 1976). Single peaks were produced when the following derivatisation conditions were used.

500-100 µg of steroids in pyridine were evaporated in a 0.5 ml Kontes "reacti-vial", heated in a sand bath, maintained at 40°C, under a stream of dry nitrogen (Fig. 4iv). 5 mg methoxyamine HCl was added and the mixture redissolved in 50 µl pyridine with the aid of a teflon pestle. The vessel was fitted with a teflon lined screw cap and allowed to stand overnight (16-20 hrs) at room temperature. The solution was evaporated to dryness, as before, the residue redissolved in 50 µl TSIM and heated for 2 hrs at 100-110°C. Aliquots (2-5 µl) were taken directly for injection into the gas chromatograph.

Retention Times: These were calculated for 17 representative steroids, individually and in mixtures, following derivatisation, relative to the internal standard CBE and measured from the solvent front. Retention times were found to be reproducible within 0.01 units under constant conditions, and independent of the amount of steroid. The values obtained are shown in Table 4i. As found by Pfaffenberger and Horning (1977), β-cortolone and β-cortol coelute.

Although standard MO-TMS cortisone was detected by the system, other 3-oxo 4-ene compounds such as cortisol, 6βOHF and 6βOHE, were not.

Separation of Pure Steroid Mixtures: The method was found suitable for derivatising and resolving synthetic mixtures of most of the steroids under investigation. A typical "profile", so obtained, is shown in Fig. 4v.

Approximately physiological proportions (as determined from normal literature values - Makin, 1975) of pure steroid solutions, in pyridine, were evaporated in the same reacti-vial, under conditions identical with those used for single steroids; 100 µg CBE was added and mixed. The vial was sealed and left overnight at room temperature.

Table 4i

Retention Times of Steroid Standards

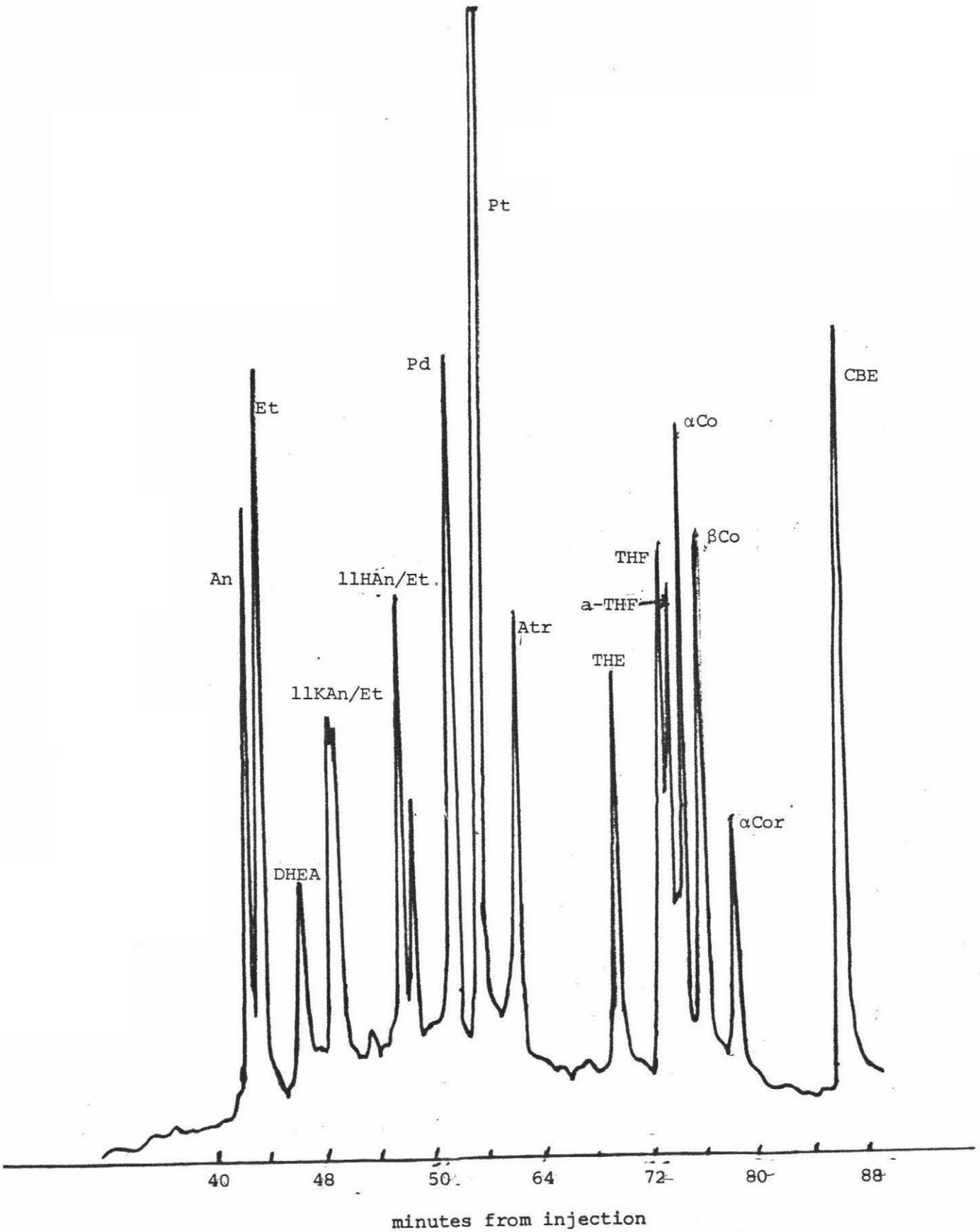
Steroid	Retention Time
Androsterone	0.41-0.42
Etiocholanolone	0.42-0.43
DHEA	0.46-0.47
11-KAn/Et	0.49-0.50
11-HAn/Et	0.56-0.57
Cortisone	0.57-0.58
Pregnandiol	0.61-0.62
Pregnantriol	0.63-0.65
Androstenetriol	0.67-0.68
THE	0.77-0.78
THF	0.82
a-THF	0.82-0.83
α Cortolone	0.84-0.85
βCo + βCor*	0.86-0.87
α Cortol	0.90
CBE	1.00

*Cortolone and cortol co-elute (Pfaffenberger and Horning, 1977)

Retention times were calculated relative to CBE and measured from the solvent front.

Figure 4v

GLC Profile of Synthetic Steroid Derivative Mixture



4 µl derivative in TSIM.
Initial oven temperature: 200°, held 12 min., then programmed at 1°/min
Electrometer range: 10^{-11} amp, attenuation: x16, chart: 15 cm/hr.

The solution was then evaporated to near dryness (it was often difficult to remove the last few microlitres of liquid under these conditions, but this was not found to interfere with silylation), and heated at 100-110° for 2 hrs with 100 µl TSIM.

4-5 µl aliquots were withdrawn and injected slowly into the chromatograph. A slow, even injection method was found necessary to minimise changes in gas pressure on top of the column. The oven temperature was maintained at 200°C for 12 min. following injection, and then raised by temperature programming at 1°/min.

The column, operated under these conditions, was unable to completely resolve the following steroid pairs: An and Et; 11-KAn and 11-KEt; 11-HAn and 11-HEt; THF and a-THF; also many chromatograms showed incomplete resolution of a-THF and αCo. As previously mentioned, βCo and βCor cannot be separated. The resolution was, however, considered sufficient for the studies intended.

Identification of the steroids was based on the retention data already obtained for single steroids, as well as comparison with published profiles. Although M.U. (methylene unit) values were not determined, the order of elution was found to be identical with that reported in the literature.

Reproducibility

The TMS derivatives of steroids are highly sensitive to traces of moisture (Pierce, 1968), and it was found necessary to minimise their contact with the atmosphere. During derivatisation, the vials were closed with screw-caps, lined with "neoprene" septa which had been covered with Teflon tape. Following the addition of silylating reagent, vials were completely sealed with Teflon tape and were not reopened at any stage. Aliquots for analysis were withdrawn directly into a Hamilton syringe (SGE, 5-10 µl) by injection through the septum, and the vials stored desiccated at 4°C. Even under these conditions, it was found that the reaction mixture separated into 2 phases and that initial steroid ratios changed within a week of derivatisation. Fresh standard mixtures were therefore prepared at least once a week.

Ratios of peak areas of individual steroids to that of the internal standard were found to be quite reproducible for consecutive

Table 4ii

Reproducibility of Steroid Peak Areas Over 3 Days
(Ratios of steroid peak areas to area of CBE peak)

Steroid	µg/100 µl	Day I	Day I	Day II	Day II	Day III	Mean
An	100	0.72	0.54	0.57	0.50	0.71	0.61
Et	100	0.83	0.70	0.75	0.64	1.03*	0.74
DHEA	50	0.24	0.23	0.31	0.25	0.24	0.25
11KAn/Et	50	0.50	0.50	0.58	0.41	0.78*	0.52
11HAn/Et	50	0.50	0.50	0.44	0.51	0.46	0.48
E	50	0.26	0.26	0.23	0.28	0.19*	0.26
Pd	100	0.75	0.78	0.72	0.70	0.84*	0.74
Pt	100	1.72	1.70	1.67	1.59	1.85	1.71
Atr	50	0.45	0.50	0.41	0.42	0.51	0.44
THE	100	0.55	0.53	0.48	0.48	0.53	0.49
THF	100	0.74	0.70	0.66	0.67	0.74	0.68
a-THF	50	0.59	0.56	0.53	0.54	0.58	0.55
αCo	50	0.84	0.79	0.77	0.71	0.87	0.78
βCo	50	0.68	0.55	0.68	0.60	0.76	0.66
αCor	25	0.37	0.33	0.23	0.26	0.37	0.31
CBE	100						

* Excluded from calculation of the mean.

Table 4iii

Linear Dilution of Standard Steroid Derivatives
(Ratios of peak areas of steroid/internal std)

Steroid	Undiluted**	Diluted 1:1	Diluted 1:3
An	0.61	0.30 (0.30)*	0.10 (0.15)*
Et	0.74	0.33 (0.36)	0.14 (0.18)
DHEA	0.25	0.06 (0.12)	0.01 (0.06)
11KAn/Et	0.52	0.20 (0.26)	0.05 (0.13)
11HAn/Et	0.48	0.24 (0.24)	0.06 (0.12)
E	0.26	0.08 (0.13)	0.02 (0.07)
Pd	0.74	0.34 (0.37)	0.14 (0.18)
Pt	1.71	0.79 (0.85)	0.40 (0.42)
Atr	0.44	0.19 (0.23)	0.08 (0.11)
THE	0.49	0.25 (0.25)	0.07 (0.12)
THF	0.68	0.35 (0.34)	0.15 (0.17)
a-THF	0.55	0.30 (0.28)	0.13 (0.14)
αCo	0.78	0.32 (0.39)	0.16 (0.19)
βCo	0.66	0.30 (0.33)	0.13 (0.16)
αCor	0.31	0.14 (0.15)	0.08 (0.08)

* Theoretical value, based on linear dilution of the undiluted, mean value.
**Mean values from Table 4ii.
Derivatised mixture of standard steroids, containing 100 µg CBE/100 µl, was diluted by addition of CBE (1 mg/ml, in pyridine).

injections on the day of silylation and for 1-2 days following, as shown in Table 4ii. In general it was found advisable for the analyses to be carried out immediately following derivatisation.

Standard Curves and Quantitation

A derivatised mixture of steroids containing 100 µg CBE/100 µl reaction mixture was diluted 1:1 and 1:3 with CBE in pyridine (1 mg/ml) to test the linearity of dilution.

Results showed that dilution of the reaction mixture, while maintaining a constant concentration of the internal standard, produces a fairly linear decrease in the ratios of peak areas of the individual steroids to that of the internal standard. This reflects only the response of the column and detector to varying amounts of steroid and in no way reflects the derivatisation reactions, which are assumed to go to completion. Typical results are shown in Table 4iii.

For most of the experiments intended, comparison of ratios of steroid to internal standard were sufficient for quantitation. However, the approximate linearity of the dilution curve allowed a semi-quantitative estimation of the amounts of individual steroids in an unknown solution. Thus, by taking the mean ratios shown in Table 4ii for the given amounts of steroid, relative to 100 µg CBE, the amount of a particular steroid could be calculated by simple proportion, for any 100 µl of solution to which 100 µg CBE had been added.

In the urinary profile experiments, addition of 100 µg CBE after hydrolysis was also able to compensate for any procedural losses prior to derivatisation.

Human Urinary Steroid Profiles

In this section of the work, methods were based extensively on those described by Pfaffenberger and Horning (1975; 1977).

Urine Collection

Urine samples were collected without preservative in polythene containers and stored at 4°C during collection and until processing. If hydrolysis could not be carried out within 72 hrs of collection the samples, or representative aliquots, were frozen.

Hydrolysis

A suitable aliquot of urine, usually corresponding to approximately 1/40 of a 24 hr urine collection, was added to an Erlynmeyer flask containing approximately 1 g sodium acetate trihydrate, and the pH adjusted to 5.0±0.2 with glacial acetic acid. 0.5 ml β-glucuronidase was added and the flask covered with "Parafilm" and aluminium foil, and incubated at 37°C in a shaking water bath.

After 24 hrs the pH of the hydrolysate was checked and adjusted if necessary. 100 µl aliquots were withdrawn and tested for β-glucuronidase activity, as described in Chapter 3 (it was not usually found necessary to add further enzyme), and the incubation continued for a further 24 hrs.

Extraction

100 µl of the internal standard CBE (1 mg/ml in pyridine) was added to 1.5 times the volume of urine hydrolysate of dichloromethane in a 1 litre separatory funnel. The urine hydrolysate was added and the mixture gently swirled for 10 min. The emulsion which was always found to form at the interface was broken by centrifugation for 10 min in 50 ml test tubes, using a bench centrifuge (Gallenkamp Junior). The organic phase was collected in a 1 litre, round bottomed flask, any solid or emulsified material being segregated with the urine phase.

The urine phase was returned to the funnel and similarly extracted with a further 1.5 volumes of CH_2Cl_2. The process was repeated once more with 1.5 volumes of ethyl acetate, and the combined organic phases evaporated to dryness (Buchi Rotavapor), the water bath temperature being kept below 40°C.

The residue was redissolved in 15 ml EtOAc and transferred to a 500 ml separatory funnel. The extract was washed three times with 10 ml aliquots of aqueous 5% $NaHCO_3$-10% NaCl, then three times with 10 ml saturated NaCl. The washed extract was dried over approximately 0.5 g anhydrous Na_2SO_4 in a 50 ml Erlynmeyer flask (approximately 30 min), filtered (Whatman 42 filter paper) into a conical test tube, and evaporated to less than 1 ml volume using a sand bath (40°C) and a stream of dry nitrogen. The residue was transferred quantitatively to a 0.5 ml reacti-vial and evaporated to dryness.

If derivatisation did not proceed immediately, the residues were stored as ethyl acetate solutions, at freezer temperature.

Derivatisation

This was carried out exactly as for the standard steroid mixtures. The methoxime was formed by overnight incubation of the dried extract with 10 mg methoxyamine HCl and 100 µl pyridine at room temperature and silylation carried out with 100 µl TSIM at 100-110°C for 2 hrs.

Aliquots were taken directly from the sealed vials for G.C. analysis.

RESULTS AND DISCUSSION

URINARY STEROID PROFILES

Normal Female

A 24 hr collection from a normal, premenopausal female, taking oral contraceptives was diluted to 1 litre with water. 1/40 of the collection was hydrolysed, extracted and derivatised according to the standard procedure.

The profile obtained is shown in Fig. 4vi(a); most of the usual steroids are present. Identification was based on retention times and on comparison of the chromatogram with that obtained when an aliquot containing the derivatised urine extract supplemented with a previously identified derivative of a standard steroid mixture (Fig. 4vi(b) and (c)) was injected.

The principle steroids identified were: An, Et, Pd, Pt, THE, THF, αCo, and βCo + βCor.

Normal Male

A profile was similarly obtained from a normal, adult male subject, using 1/40 of a 24 hr urine collection which had been diluted to 2 litres with water.

The steroid peaks were identified as outlined above for the female subject, and shown in Fig. 4vii(a), (b) and (c).

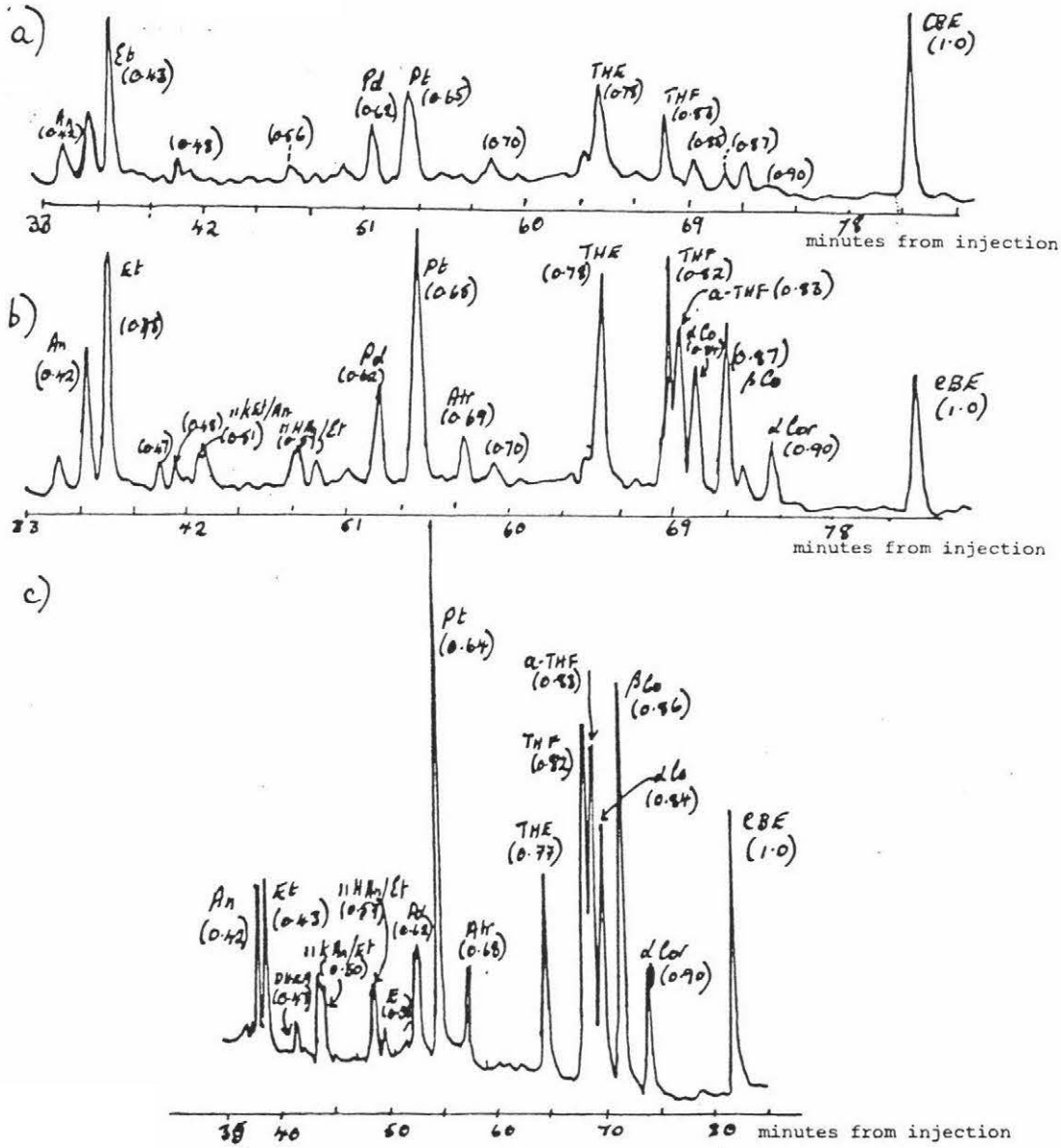
The principle steroids indicated were: An, Et, 11KAn/Et, 11HAn/Et, Pt, THE, THF, a-THF, αCo, βCo + βCor and αCor.

Alcohol Loading Experiment

Urinary steroid profiles were obtained from a normal, adult, 62 kg male on three consecutive days. The collections extended over the same 6 hr period on each day to minimise variations in steroid excretion due to diurnal rhythms. The urine was collected in plastic containers as three consecutive 2 hr samples each day. The fluid intake was maintained constant each day to minimise diuresis effects. Days I and III served as controls, but on day II the subject was maintained at an approximate blood alcohol level of 50 mg per 100 ml throughout the collection period.

Figure 4vi

GLC Steroid Profile from Normal Female

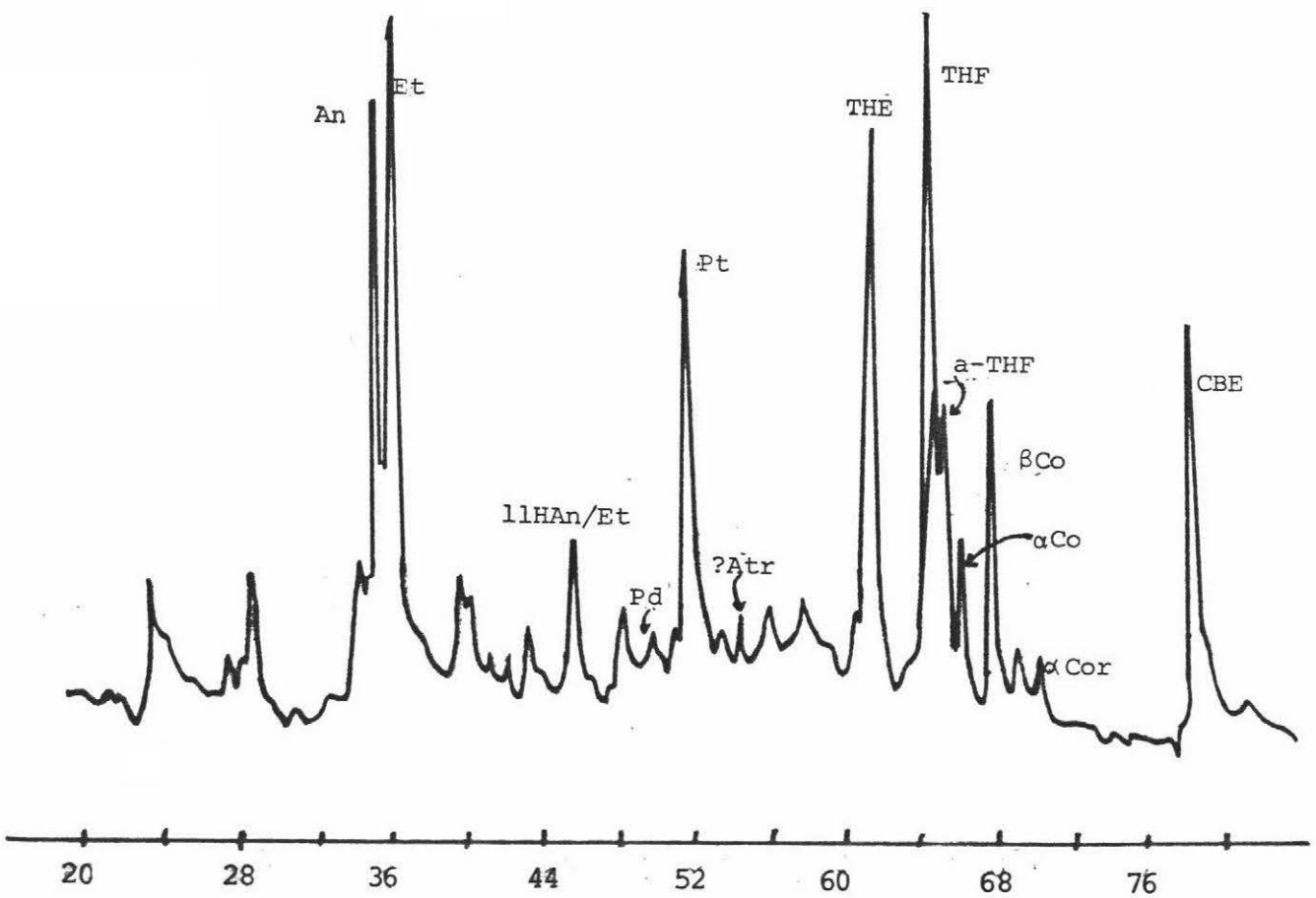


(a) 8 µl derivative of extract of 24 hr urine from normal female plus 2 µl CBE (1 mg/ml in pyridine).
(b) 6.5 µl urine extract (as above) plus 3 µl standard steroid mixture (below).
(c) 5 µl standard steroid mixture.

Oven temp. 200° for 12 min, then programmed at 1°/min.
Electrometer range: 10^{-11} amp; attenuation x32.
Relative retention times in parentheses.

Figure 4vii

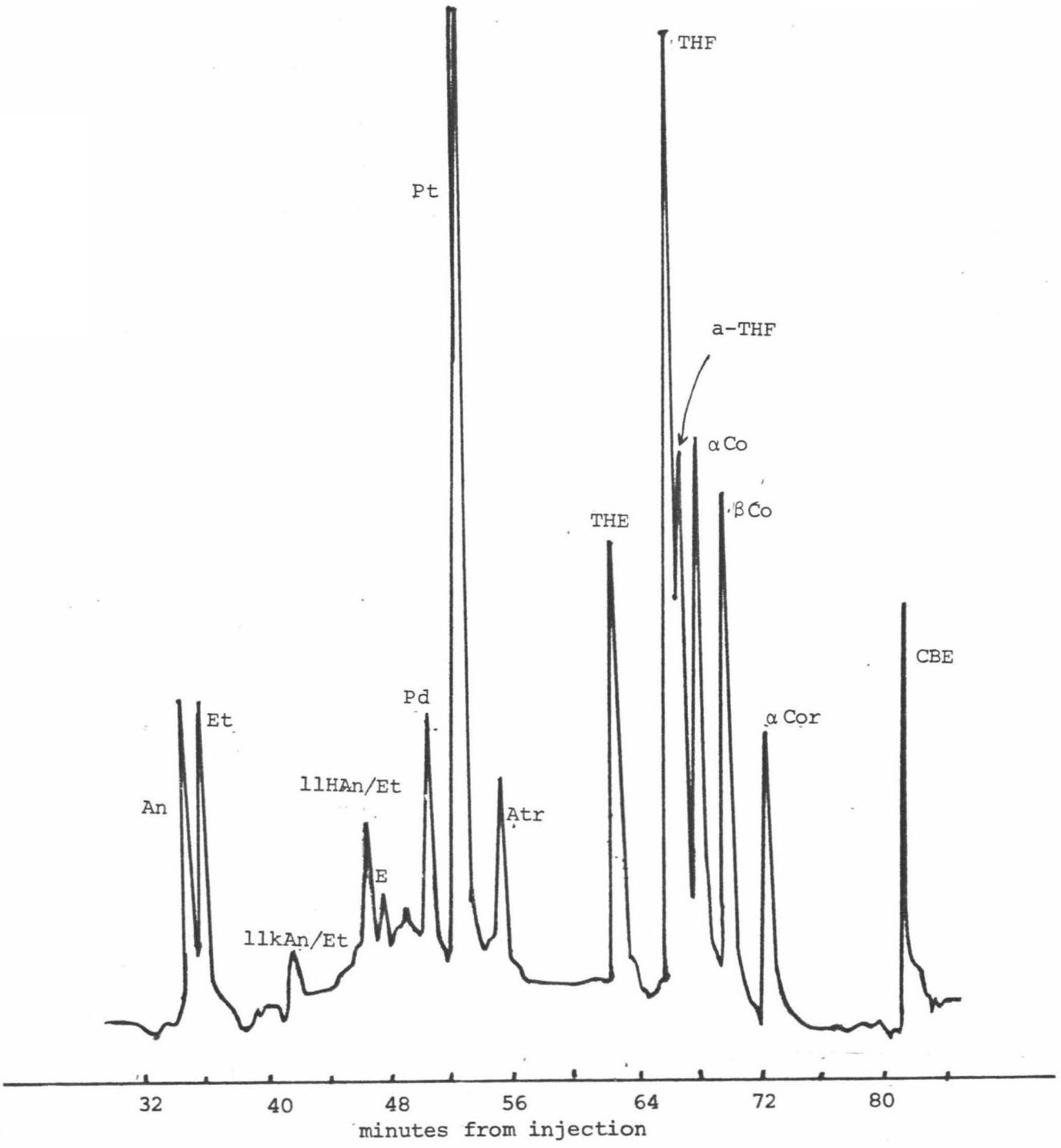
(a) GLC Profile of Steroids from 24 hr Urine of Normal Male



5 µl derivative.
Oven temp. 200° for 12 min. then programmed at 1°/min.
Range: 10^{-11} amps; attenuation x16; chart 15 cm/hr.

Figure 4vii

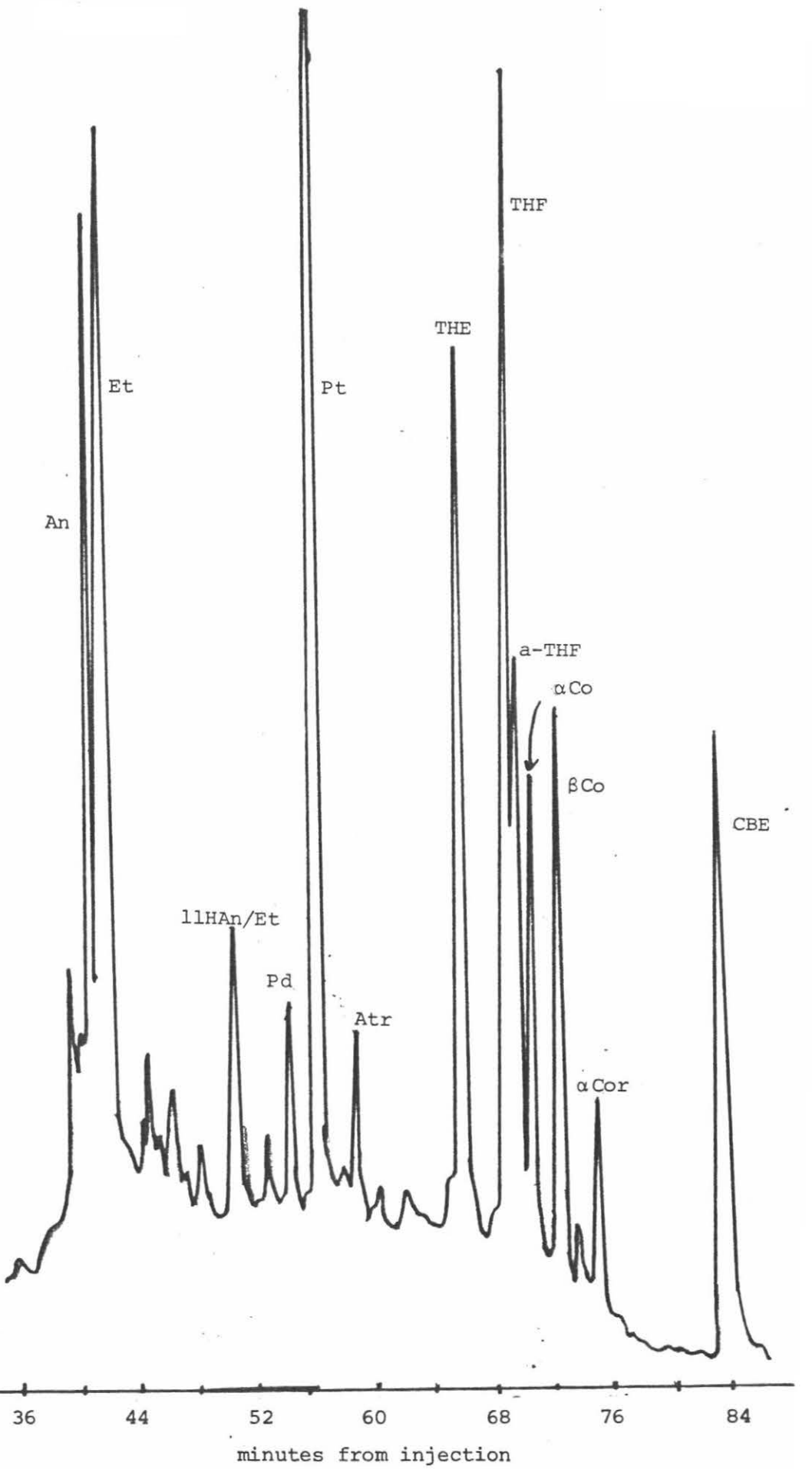
(b) Standard Steroid Mixture (5µl)



Chromatography conditions as for (a).

Figure 4vii

(c) Derivatised Urinary Extract from Normal Male (5 µl), Supplemented with Standard Steroids (2.5 µl)



... conditions as for (a) and (b).

The protocol used for control days I and III, together with a summary of the fluid balance, is given in Table 4iv. The protocol for day II and the observed fluid balance and breath alcohol levels are shown in Table 4v. Breath alcohol levels were obtained every half hour, immediately prior to drinking, using an "Alcolmeter" Model AE-D1, manufactured by Lion Laboratories, Cardiff, U.K.

Table 4iv

Protocol for Alcohol Loading Experiment
Control Days I and III

10 am — Empty bladder, drink 600 ml lemonade/lime mixture

10.30 am, and every following ½ hour — Drink a further 100 ml above mixture

URINE COLLECTIONS:		Day I	Day II
12 noon	Collect 2 hr urine from 10 am	Sample I(a)	Sample III(b)
	Total volume	230 ml	122 ml
	Aliquot for assay	75 ml	40 ml
2 pm	Collect 2 hr urine from noon	Sample I(b)	Sample III(b)
	Total volume	358 ml	136 ml
	Aliquot for assay	120 ml	45 ml
4 pm	Collect 2 hr urine from 2 pm	Sample I(c)	Sample III(c)
	Total volume	630 ml	125 ml
	Aliquot for assay	210 ml	40 ml

The amount of alcohol (gin) ingested was calculated to produce a blood alcohol level of approximately 50 mg%, based on body weight.

From each of the nine collections, approximately one-third was taken for assay, to correspond to 1/36 of a 24 hr collection. No dilutions were made prior to hydrolysis. Hydrolysis, extraction and derivatisation were performed as previously described. The chromatograms so obtained are shown in Figs 4viiia,b and c; 4ixa,b and c and 4xa,b and c. Ratios of steroid peak heights and areas, relative to CBE are shown in Tables 4vi, 4vii, 4viii and 4ix.

Table 4v

Alcohol Loading Experiment: Protocol and Fluid Balance for Day II, Alcohol Load

Time	Fluid Intake	Breath Alcohol (mg/100 ml)	Urine
10.00am	100 ml gin		
10.15	in 600 ml volume		
10.45	16 ml gin/100 ml	37	Sample II(a)
11.15	"	36	185 ml
11.30	-	44	60 ml for assay
11.45	15 ml gin/100 ml	42	
12.00 noon	-	48	
12.15pm	15 ml gin/100 ml	44	Sample II(b)
12.45	"	45	394 ml
1.15	"	47	130 ml for assay
1.45	"	48	
2.00	-		
2.15	15 ml gin/100 ml	50	
2.45	"	48	Sample II(c)
3.15	"	48	418 ml
3.45	"	52	140 ml for assay
4.00	-	44	
	total intake 1700 ml		total output 997 ml

Table 4vi

Alcohol Loading Experiment Ratios of Steroid Peak Heights/CBE

Steroid	Ia	IIa	IIIa	Ib	IIb	IIIb	Ic	IIc	IIIc
An	1.05	1.4	2.2	1.2	1.9	2.6	1.4	2.7	-
Et	0.81	1.4	2.4	0.98	1.9	1.8	1.4	3.6	-
DHEA	0.25	0.74	0.76	0.36	0.42	0.60	1.35	0.79	0.71
11KAn/Et	0.05	0.14	0.13	0.07	0.10	0.14	0.07	0.21	0.18
11HAn/Et	0.27	0.77	1.1	0.37	0.63	0.90	0.40	0.94	1.6
Pd	0.09	0.17	0.33	0.07	0.10	0.14	0.07	0.27	0.24
Pt	0.64	1.5	1.6	0.75	1.1	1.4	0.65	2.1	1.9
Atr	0.10	0.17	0.23	0.07	0.19	0.20	0.06	0.29	0.26
THE	0.64	1.8	2.4	0.56	1.6	4.2	0.79	2.3	
THF	0.88	2.0	2.7	0.69	1.4	3.4	0.91	2.7	
a-THF	0.38	0.97	1.2	0.33	0.71	1.0	0.33	1.1	3.1
αCo	0.18	0.57	0.50	0.16	0.42	1.1	0.26	0.83	1.4
αCor	0.10	0.17	0.23	0.10	0.17		0.09	0.17	0.34

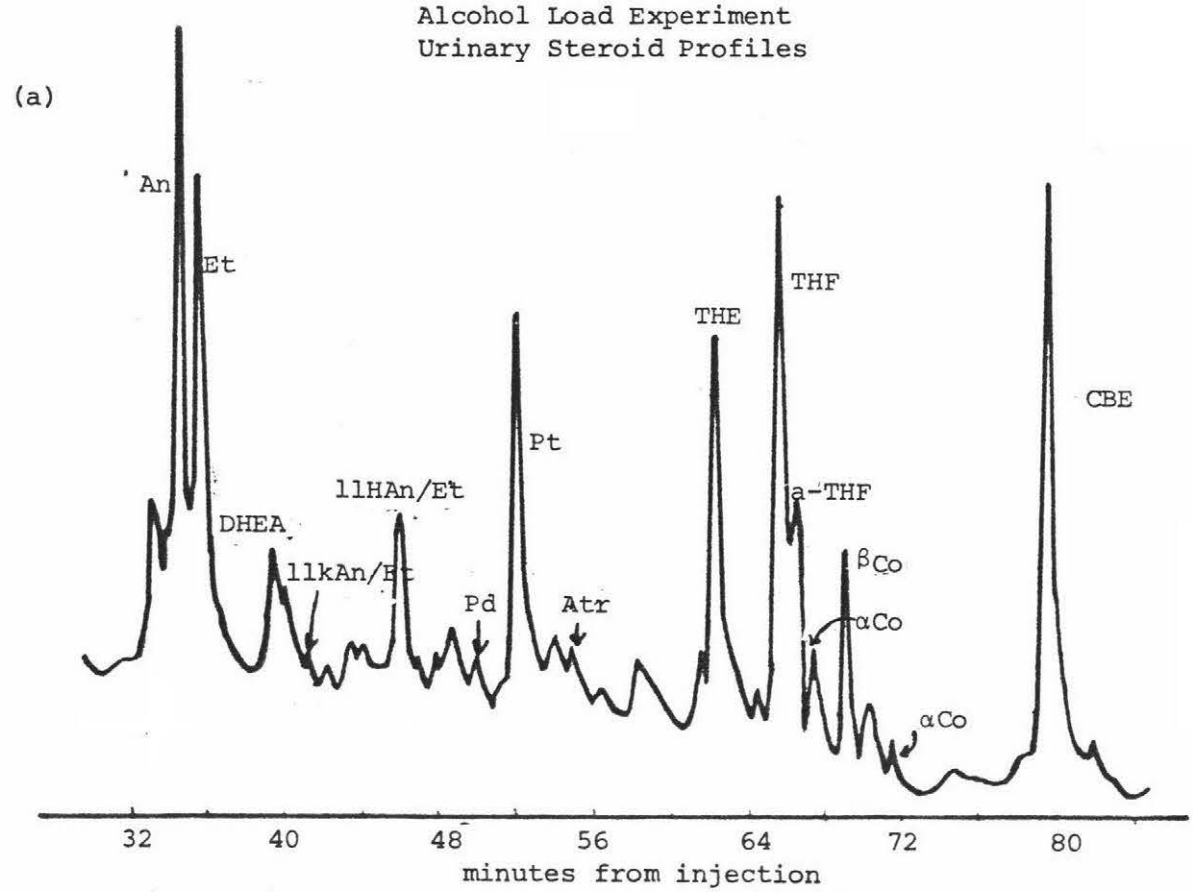
Table 4vii

Alcohol Loading Experiment Ratios of Steroid Peak Areas/CBE

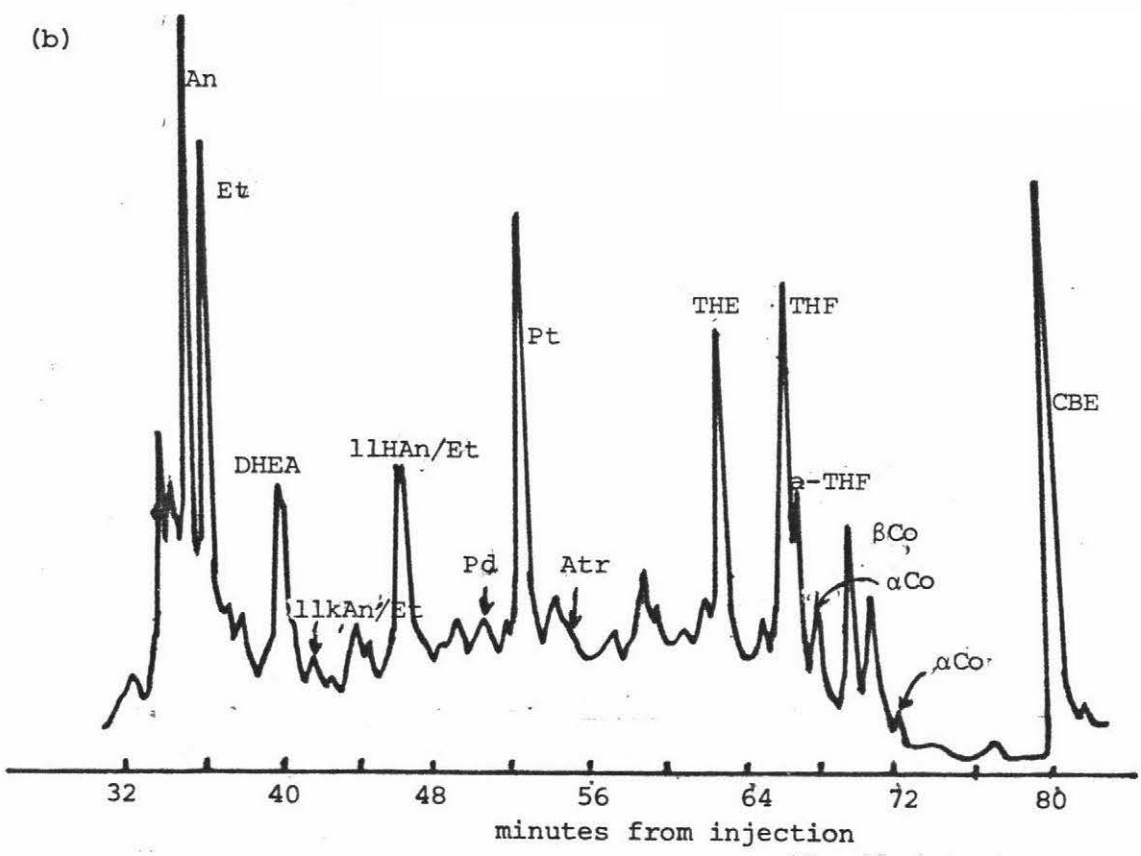
Steroid	Ia	IIa	IIIa	Ib	IIb	IIIb	Ic	IIc	IIIc
An	1.05	0.99	0.98	0.91	1.33		1.06	2.04	
Et	0.78	1.22	1.22	0.86	1.56		1.15	3.18	
DHEA	0.18	0.82	0.55	0.23	0.22	0.44	0.29	0.54	0.51
11HAn/Et	0.26	0.72	0.79	0.35	0.47	0.69	0.53	1.07	1.16
Pt	0.62	1.23	0.94	0.60	0.72	0.95	0.64	1.55	1.30
THE	0.77	1.53	1.80	0.43	1.32	3.30	0.68	1.85	6.7
THF	0.82	1.84	1.80	0.55	1.11	2.69	0.72	2.05	6.17
a-THF	0.19	0.54	0.50	0.14	0.36	0.67	0.12	0.58	1.71
αCo	0.08	0.33	0.15	0.03	0.22	0.57	0.12	0.40	0.83

Figure 4viii

Alcohol Load Experiment
Urinary Steroid Profiles

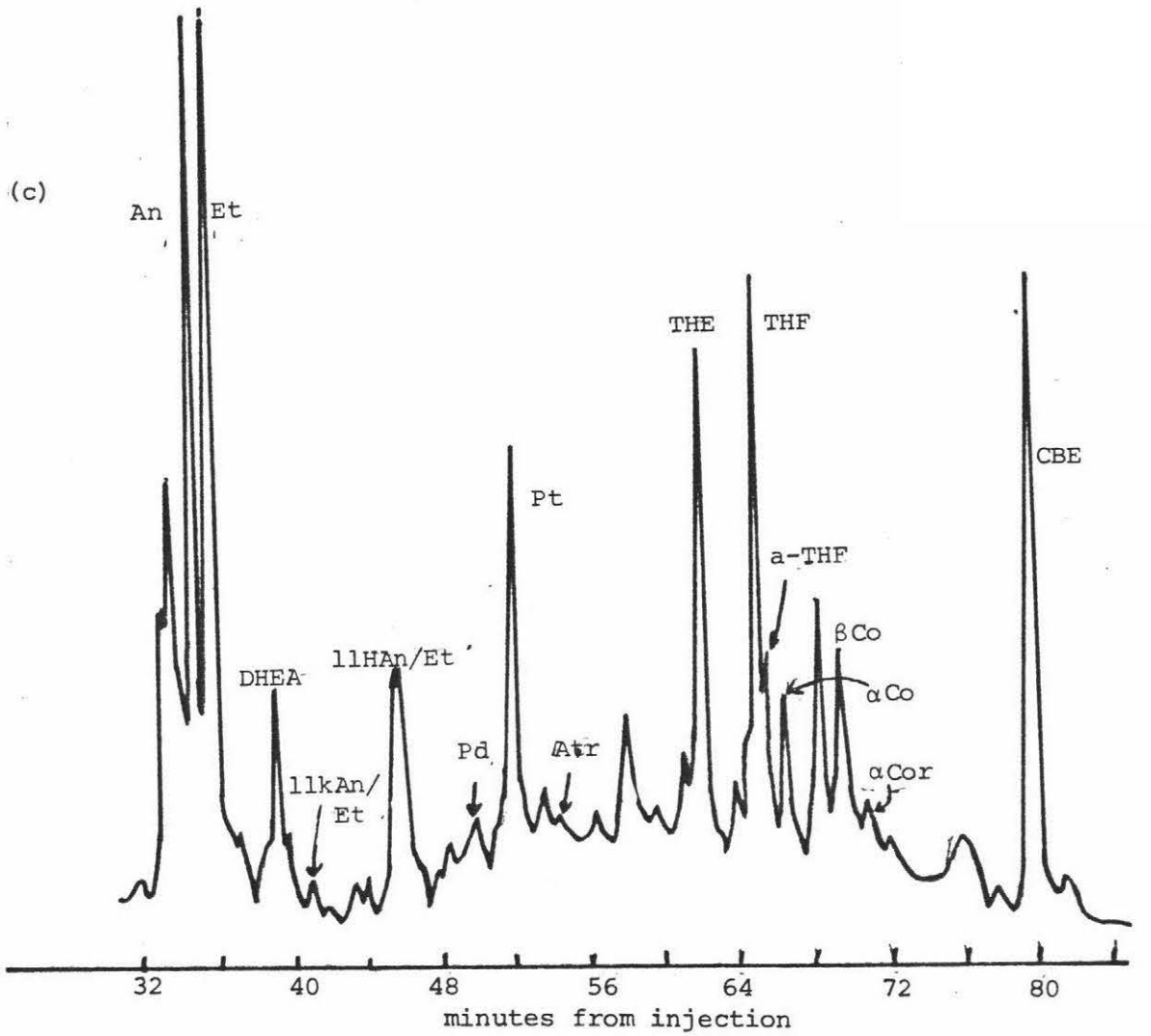


5 µl Sample (a), control day I. Range: 10^{-11} amp, attentuation: x16



5 µl Sample (b), control day I. Range 10^{-11} amp, attenuation: x16.

Figure 4viii (continued)



5 µl sample (c), control day I.

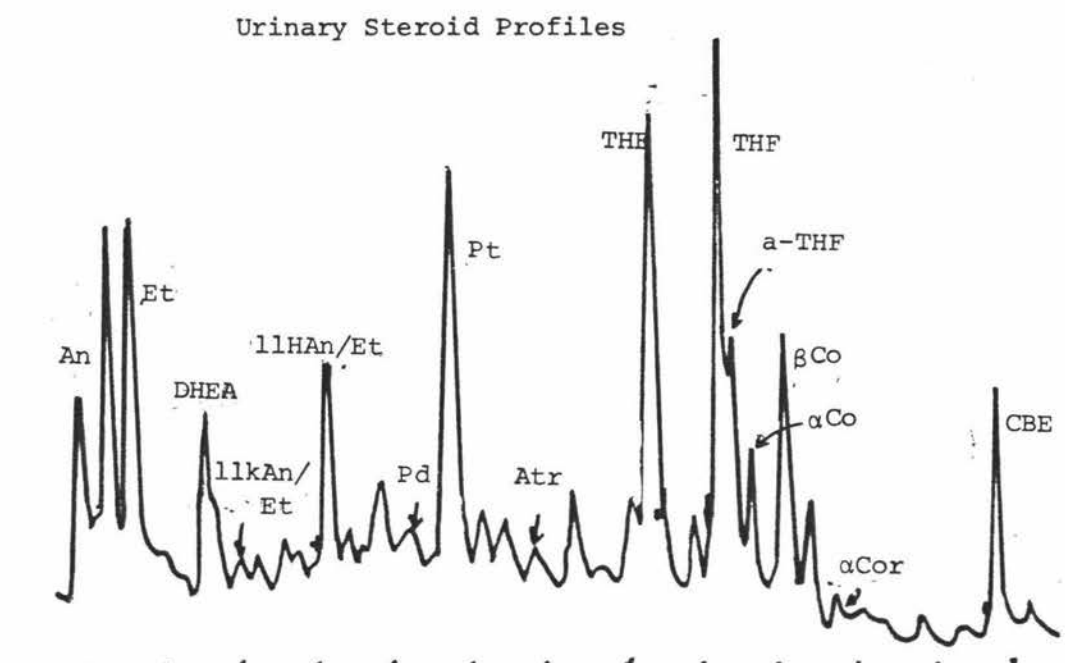
Range: 10^{-11} amps; attenuation x16.

Figure 4ix

Alcohol Load Experiment

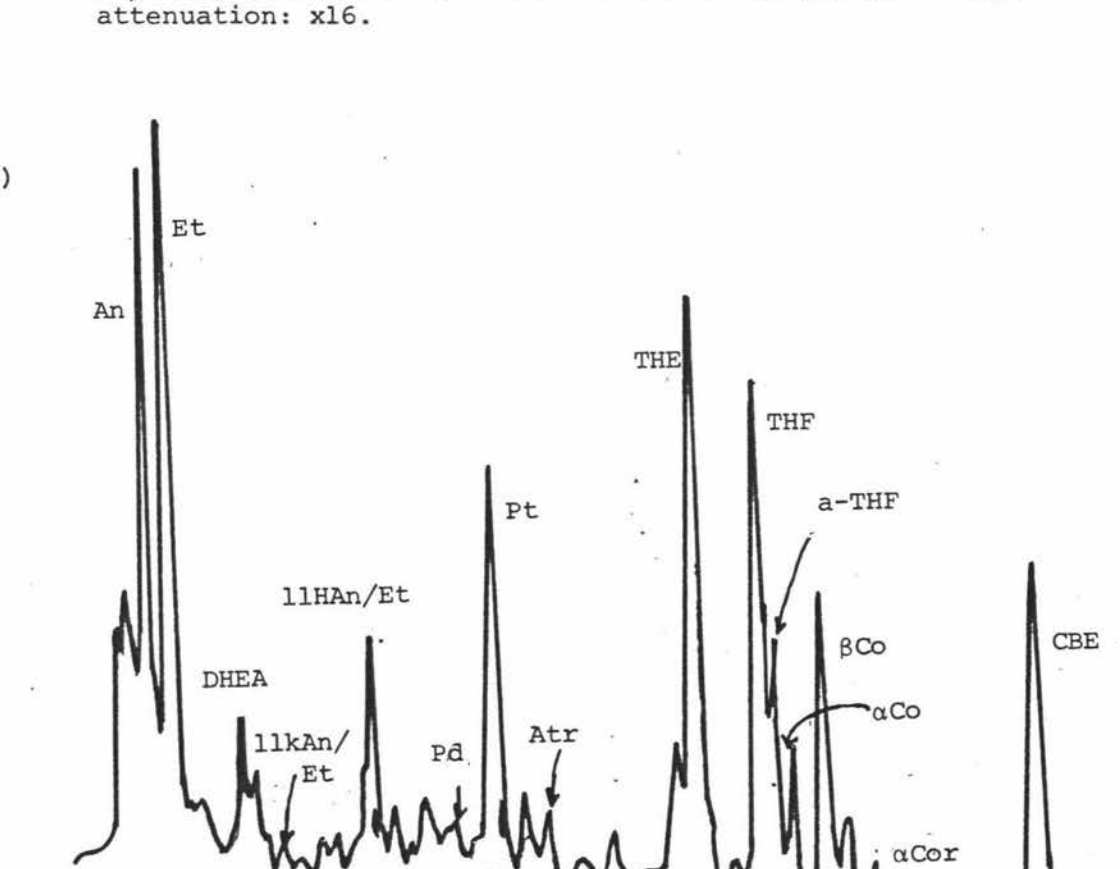
Urinary Steroid Profiles

(a)



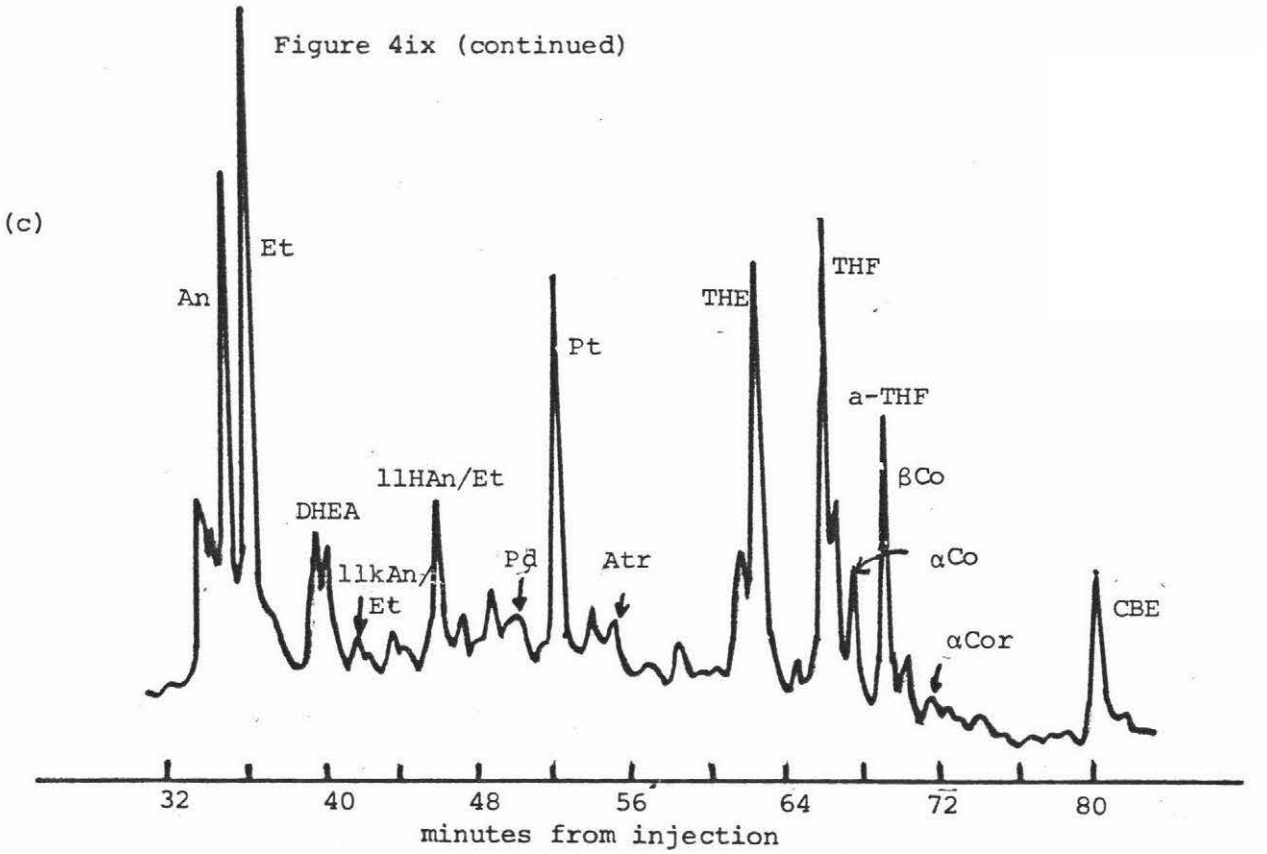
5 µl Day II sample (a): alcohol load. range: 10^{-11} amp; attenuation: x16.

(b)



5 µl Day II sample (b): alcohol load. Range: 10^{-11} amp; attenuation: x16.

Figure 4ix (continued)

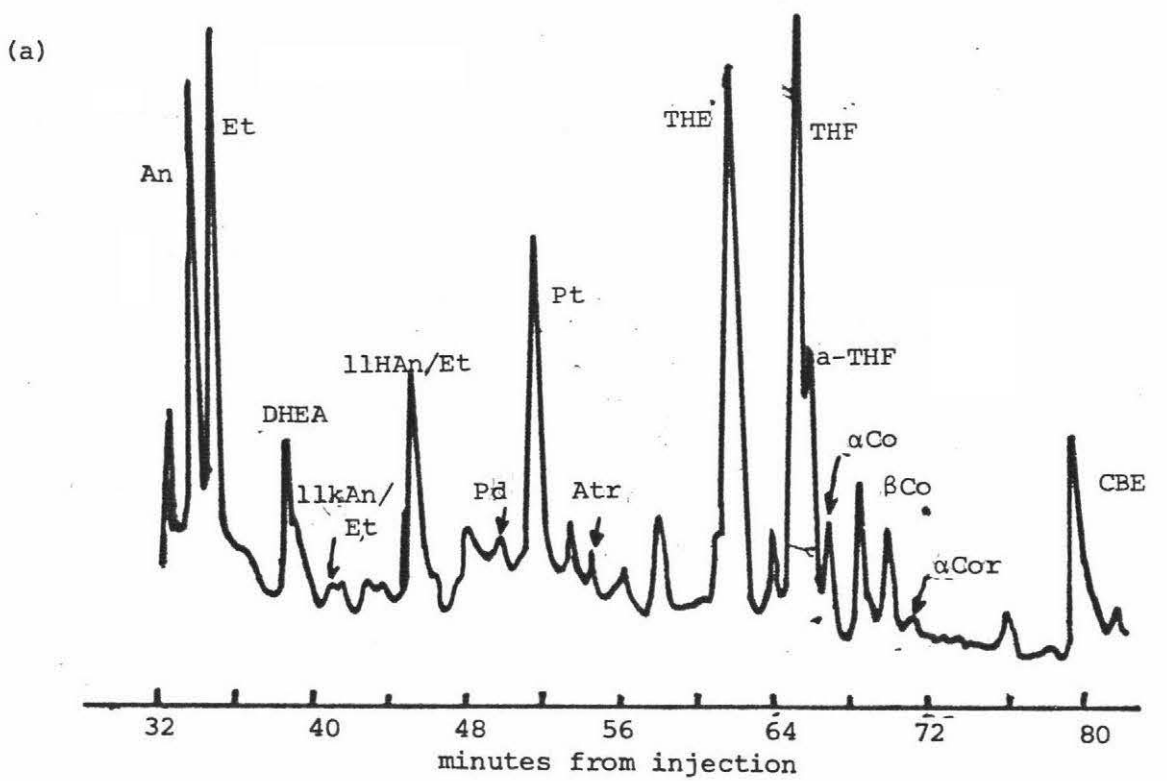


5 µl Day II sample (c): alcohol load. range 10^{-11} amp; attenuation x16.

Figure 4x

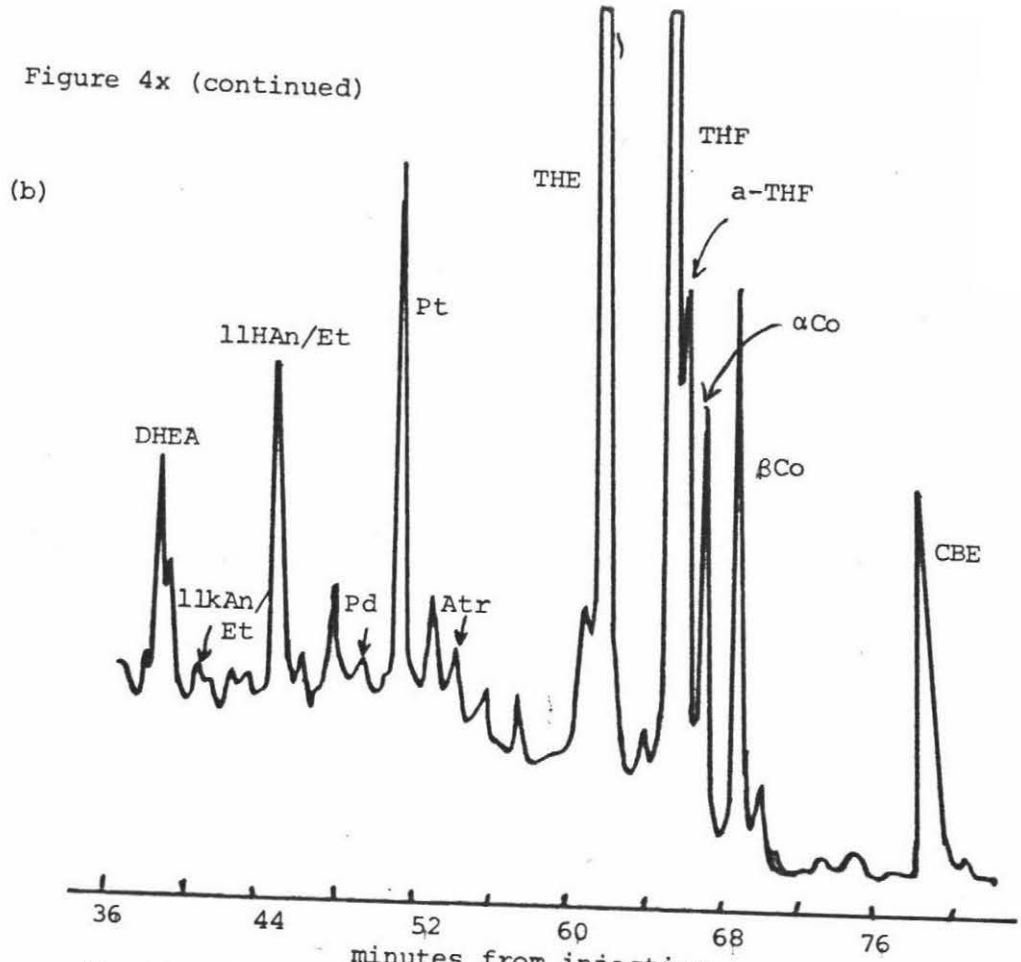
Alcohol Load Experiment

Urinary Steroid Profiles

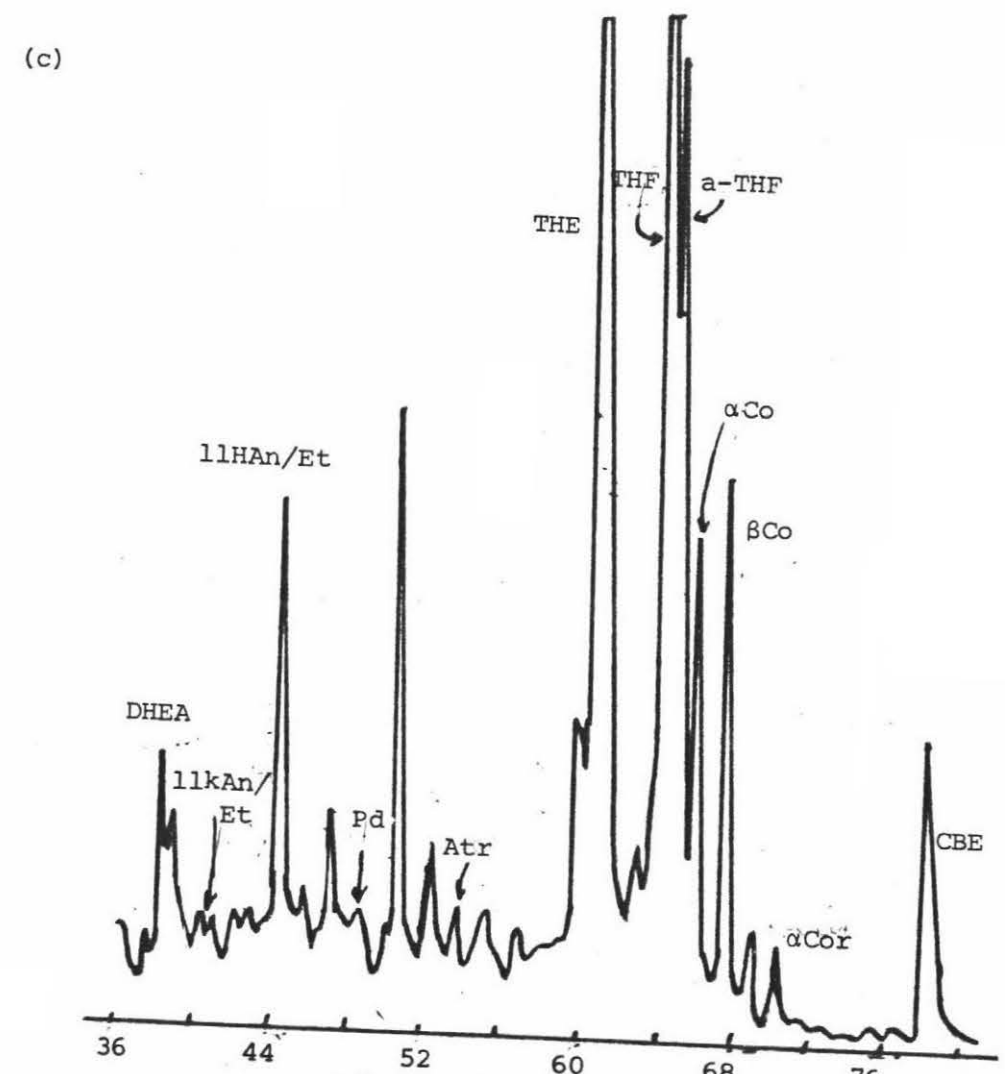


5 µl Day III sample (a): control. range: 10^{-11} amp; attenuation x16

Figure 4x (continued)



5 µl Day III sample (b): control.



5 µl Day III sample (c): control. (b) and (c): range 10^{-11} amp; attenuation x16.

Table 4viii

Alcohol Loading Experiment: Ratios of Steroid Pairs, Based on Peak Height/CBE Ratios

Steroids	Ia	IIa	IIIa	Ib	IIb	IIIb	Ic	IIc	IIIc
An/Et	1.3	1.0	0.92	1.2	1.0	1.4	1.0	0.75	
An+Et/DHEA	7.44	3.78	6.05	6.06	9.05	7.33	8.0	7.97	
(11HAn/Et)/(11KAn/Et)	5.4	5.5	8.5	5.3	6.3	6.4	5.7	4.5	8.9
(THF+a-THF)/THE	1.97	1.65	1.63	1.82	1.32	1.05	1.57	1.65	
αCor/αCo	0.55	0.30	0.46	0.63	0.42		0.35	0.20	0.24
αCo/THE	0.28	0.32	0.21	0.29	0.26	0.26	0.33	0.36	0.21
αCor/(THF+a-THF)	0.11	0.05	0.06	0.10	0.08		0.07	0.04	0.08
C-19/C-21	0.78	0.56	0.71	1.02	0.81	0.49	1.05	0.38	
Pt/Pd	7.11	8.82	4.85	10.71	11.0	10.0	9.23	7.78	7.92
Atr/DHEA	0.40	0.23	0.32	0.19	0.45	0.33	0.17	0.37	0.37

Table 4ix

Alcohol Loading Experiment: Ratios of Steroid Pairs, Based on Peak Area/CBE Ratios

Steroids	Ia	IIa	IIIa	Ib	IIb	IIIb	Ic	IIc	IIIc
An/Et	1.35	0.81	0.80	1.06	0.85		0.92	0.64	
(An+Et)/DHEA	10.17	2.71	4.0	7.70	13.14		7.62	9.67	
(THF+a-THF)/THE	1.31	1.56	1.28	1.60	1.11	1.02	1.24	1.42	1.18
αCo/THE	0.10	0.22	0.08	0.07	0.17	0.17	0.18	0.22	0.12
C-19/C-21	0.82	0.60	0.63	1.22	0.82		1.18	0.91	

Although no dramatic changes in steroid metabolism were obvious in this experiment, comparison of the ratios of some steroid pairs gives some indications of what enzyme systems or pathways may be affected during alcohol metabolism:

1. Changes in reduction at carbon-5 are reflected by the ratios of

(i) Androsterone to etiocholanolone: An/Et is decreased on day II (alcohol load) relative to the first control day. On the second control day, III, the An and Et peak areas were not measured quantitatively. A similar effect is shown by the comparison of peak height ratios. Results would suggest that during alcohol metabolism, reduction of the 4:5 double bond favours the formation of the 5β isomer (Et) over the 5α (An).

(ii) Androsterone plus etiocholanolone to dehydroepiandrosterone: An+Et/DHEA. Comparison of both the peak area and peak height ratios to those of the internal standard show that in the first sample (a), taken between 10 am and noon, the ratio drops, relative to the control days, i.e. reduction of the double bond is decreased, however, in the two later samples (b) and (c) the ratio is increased.

2. Reduction at the 11-position is reflected by three measured ratios

(i) 11HAn + 11HEt/11KAn + 11KEt: In this instance only peak heights were compared. This ratio shows a rise in the second sample (b) on day II relative to day I, and a decrease in (a) and (c) relative to the controls.

(ii) α Cortol/α Cortolone (heights only): In all samples the ratio appears to decrease on the alcohol day relative to the controls, indicating a decrease in reduction (-OH ⇄ =O) at the -11 position.

(iii) THF + a-THF/THE: Comparison of the areas shows a drop in the reduced (11-OH) steroids in II(b), relative to day I, but a general rise in samples (a) and (b) and a rise in sample (c), so that no firm conclusions can be drawn.

3. Changes in the reduction of the oxygen function at the 20 position are reflected by

(i) α Cortolone/THE: On the alcohol day both the area and height ratios increase relative to the control samples.

(ii) Cortol/THF + a-THF: shows a decrease during alcohol metabolism.

4. "Desmolase" activity is reflected by the ratios of total C-19/C-21 steroids: the ratio appears to be decreased during alcohol metabolism, suggesting decreased side-chain cleavage.

5. Hydroxylation at the 17 position is reflected in the ratios of

(i) Pt/Pd: generally, this appears to rise on day II, relative to days I and III, implying an increase in 17-hydroxylase activity.

(ii) Atr/DHEA: reflects 17 reduction coupled with 16-hydroxylation, the ratio tends to increase during alcohol metabolism.

The general trends shown by these results are summarised in Table 4x.

Table 4x

Summary of Alcohol Loading Experiment

Enzyme Activity	Effect of Alcohol Metabolism
5 Reductase	Increased (5α > 5β)
11β-hydroxysteroid dehydrogenase	Generally decreased
20α-hydroxysteroid dehydrogenase	Inconclusive (1 up, 1 down)
Desmolase	Decreased
17 Hydroxylase	Increased
17 Reductase + 16 Hydroxylase	Increased

"Alcoholic" Female

Three 24 hr urine samples were obtained from a 58 year old, post menopausal woman with a history of alcohol abuse, admitted to hospital with a measured blood alcohol level of approximately 250 mg%. The patient admitted to consuming approximately 20 oz of brandy per day for 15 years; then 40 oz a day for the 2 months prior to admission.

Sample I was collected during the first 24 hr following admission.

Sample II was collected between 72 and 96 hr following admission.

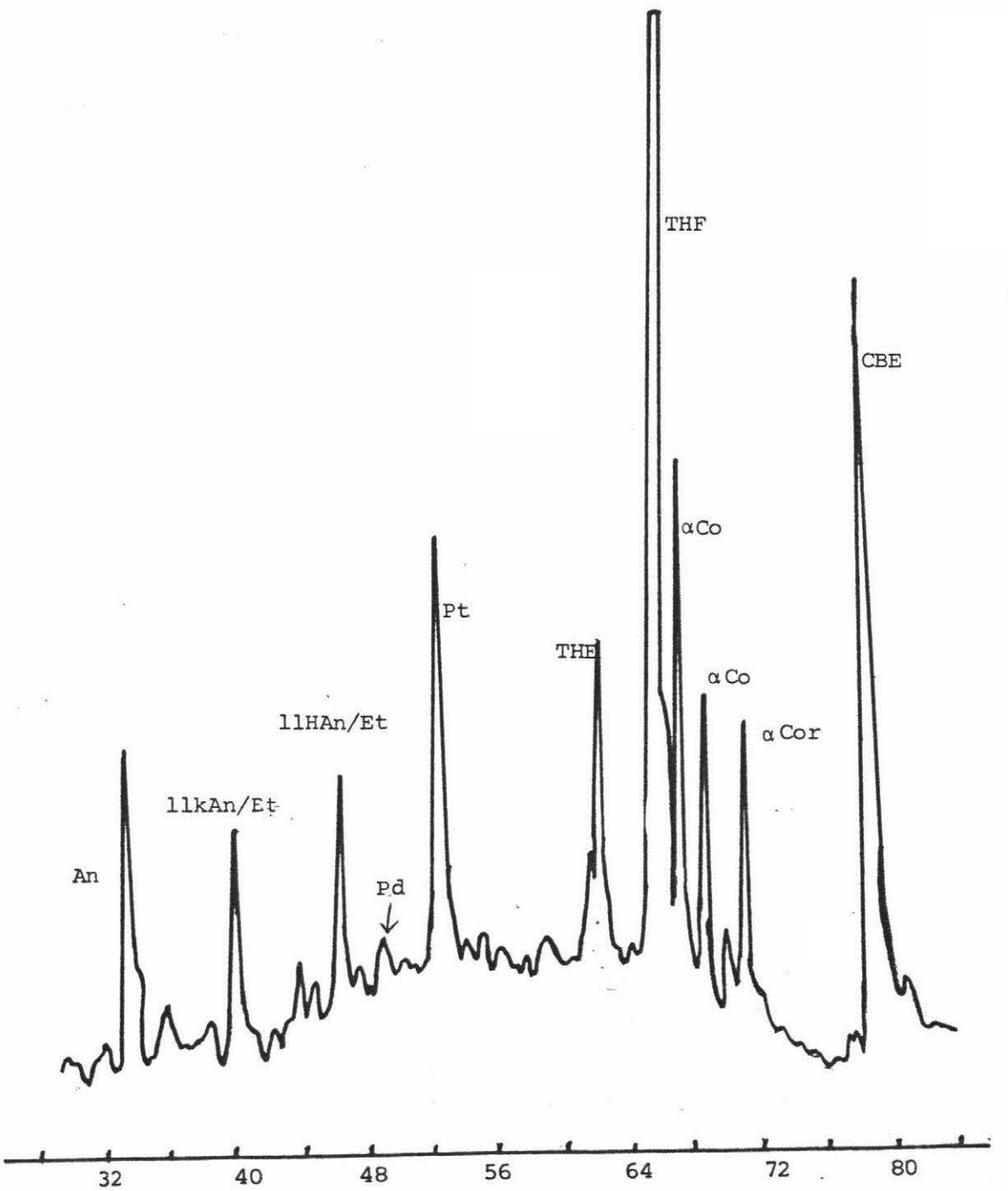
Sample III was collected one week after admission.

Volumes of all collections were measured, then diluted with water to 2 litres. 50 ml were taken from each diluted collection for hydrolysis, extraction and derivatisation as previously described. The chromatograms

Figure 4xi

Alcoholic Female Experiment

Urinary Steroid Profile: Day I (high blood alcohol)

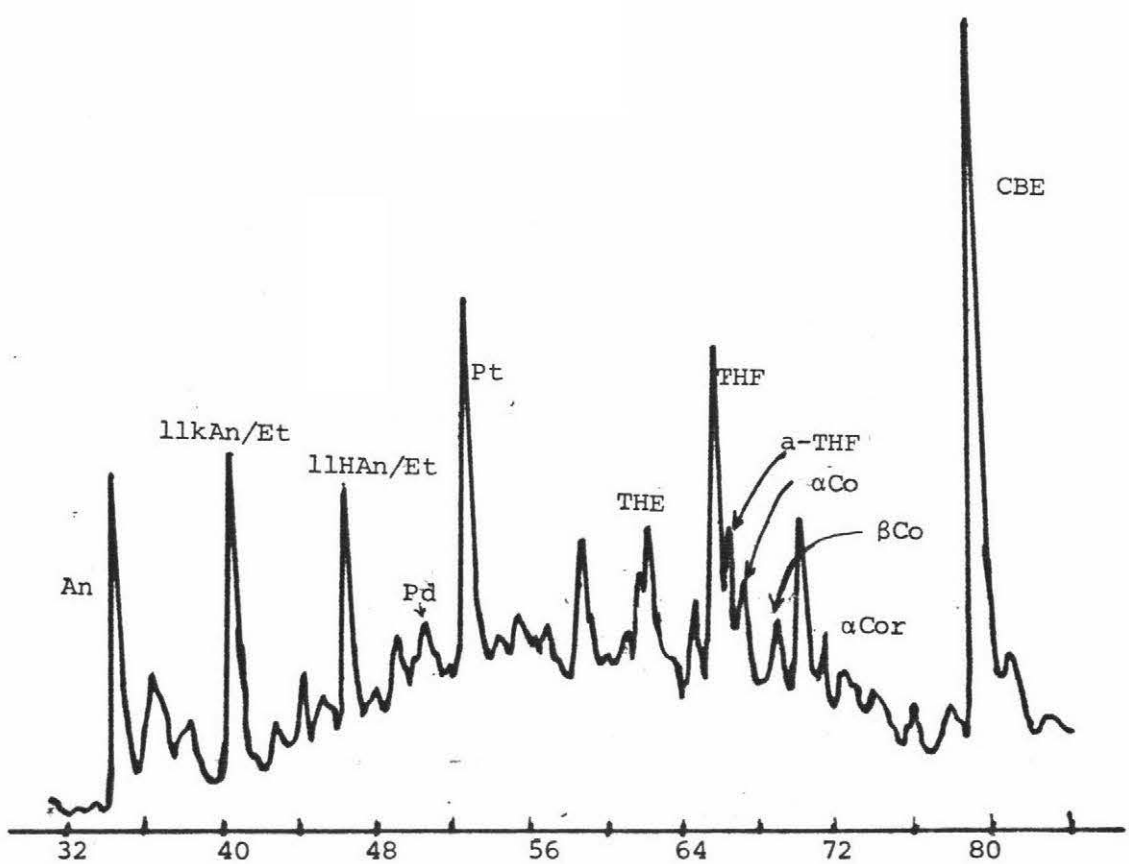


minutes from injection

5 µl sample, oven 200° for 12 min. then programmed at 1°/min.
Range: 10^{-11} amps; attenuation: x8; chart 15 cm/hr.

Figure 4xii

Alcoholic Female Experiment
Urinary Steroid Profile: Day II (control)



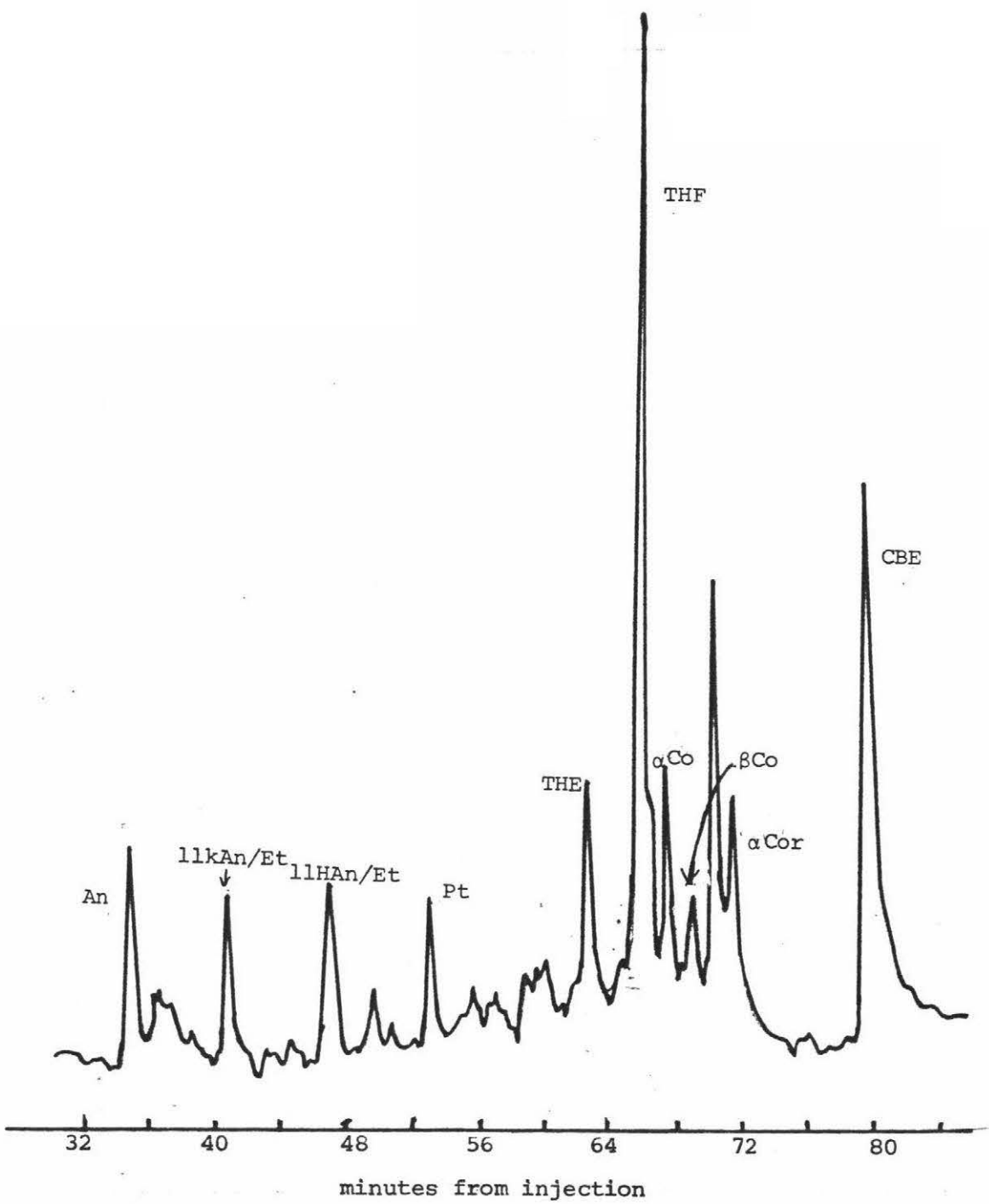
minutes from injection

5 µl sample; oven: 200° for 12 min. then programmed at 1°/min.
Range: 10^{-11}; attenuation: x8; chart: 15 cm/hr.

Figure 4xiii

Alcoholic Female Experiment

Urinary Steroid Profile: Day III (control)



5 µl sample; oven at 200° for 12 min. then programmed at 1°/min.
Range: 10^{-11} amp; attenuation: x8; chart: 15 cm/hr.

obtained from the three analyses are shown in Figs 4xi, 4xii and 4xiii, and the results are summarised in Table 4xi.

Table 4xi

Alcoholic Female Experiment: Ratios of Steroid Peak Areas/CBE

Steroid	Day I	Day II	Day III
An	0.33	0.38	0.20
Et	0.13	0.23	
11KAn/Et	0.21	0.33	0.17
11HAn/Et	0.21	0.24	0.23
Pd	0.07	0.05	
Pt	0.42	0.43	0.17
THE	0.30	0.07	0.25
THF	1.48	0.32	1.1
a-THF		0.14	
αCo	0.37	0.09	0.17
βCo+βCor	0.19	0.05	0.07
αCor	0.14	0.01	0.13

All ratios are based on the mean of three determinations.

The effects of alcohol metabolism and subsequent "drying out" on steroid metabolism were obtained by comparing the ratios of steroid to internal standard for selected steroid pairs on the three days. These comparisons are shown in Table 4xii. The ratios obtained tend to show a marked variation between samples I and II, and a distinct similarity between samples I and III. This could indicate that for this patient, "drying out", represented by collection II, produced a condition of stress and affected corticosteroid metabolism more acutely than a return to metabolic "normality", represented by collection III.

Table 4xiii shows a comparison of the amounts of the individual steroids excreted in each collection with normal literature values (Makin, 1975). In general there does not seem to be one single day on which all measured steroids approximate normal values. At the conclusion of the experiment at least some of the steroids under investigation were being excreted in quantities which were distinctly different from normal.

Table 4xii

"Alcoholic" Female Experiment

Effects of alcohol metabolism and subsequent "drying out" on steroid metabolism are shown by comparing ratios of steroid to internal standard, for selected steroid pairs on the three days.

Enzyme System	Steroids	Day I	Day II	Day III
11 reduction	$\frac{\text{11HAn/Et}}{\text{11KAn/Et}}$	1.0	0.73	1.4
	$\frac{\text{THF+a-THF}}{\text{THE}}$	4.9	6.5	4.4
	$\frac{\alpha\text{Cortol}}{\alpha\text{Cortolone}}$	0.38	0.10	0.77
20 reduction	$\frac{\alpha\text{Cortolone}}{\text{THE}}$	1.2	1.3	0.68
	$\frac{\alpha\text{Cortol}}{\text{THF+a-THF}}$	0.10	0.02	0.12
"Desmolase"	$\frac{\text{total C-19}}{\text{total C-21}}$	0.26	0.98	0.35

Table 4xiii

"Alcoholic" Female Experiment Steroid Excretion

Steroid	Day I	Day II	Day III	"Normal"*
An	2.2 mg/day	2.5 mg/day	1.3 mg/day	1.4 mg/day
Et	0.72	0.12		1.8
11KAn/Et	0.81	1.3	0.65	0.68
11HAn/Et	0.88	1.0	0.96	0.70
Pd	0.38	0.27		0.55^{1},5.0^{2}
Pt	0.98	1.0	0.40	0.90
THE	2.5	0.57	2.0	2.8
THF	8.7	1.9	6.5	2.2
a-THF		0.51		0.3
αCo	0.95	0.23	0.44	1.15(total Co)
αCor	0.45	0.03	0.42	0.23(total Cor)

* Normal female values, Makin (1975): ^{1}Fallopian phase: ^{2}Luteal phase

Conclusion

The results obtained from the experimental subjects suggest that while no firm conclusions can be drawn from these observations alone, the method developed is useful for the purpose intended. A similar method is in fact, being used in Horning's laboratory (Pfaffenberger and Horning, 1977) to study the relative activities of various steroid reductions.

The pilot study described here shows that changes in steroid excretion patterns due to alcohol are subtle and point to the need for observations on greater numbers of carefully controlled cases to confirm or disprove the suggestion of alcohol-induced shifts in the cases observed here.

CHAPTER 5

6-BETA-HYDROXYCORTISOL EXCRETION DURING ALCOHOL LOADING

INTRODUCTION

Microsomal Oxidation of Alcohol, Drugs and Steroids

Hepatic microsomes have been variously implicated in the oxidative metabolism of ethanol, drugs and steroids. The metabolism of alcohol has been shown to be affected by certain drugs (Chakraborty, 1978) as has that of cortisol (Lipsett, 1971) and this section was undertaken in an attempt to determine whether one particular pathway of cortisol metabolism was affected by the presence of alcohol.

Alcohol Metabolism

Hepatic metabolism of alcohol has been known for many years, but it recently became apparent (reviews by Isselbacher, 1977; Badawy, 1978) that several pathways may be involved. The major mechanism is the reaction with the cytoplasmic enzyme alcohol dehydrogenase (ADH) plus NAD^+ as cofactor to produce acetaldehyde and NADH. Two other systems: catalase, using hydrogen peroxide as cofactor and the M.E.O.S. (Microsomal Ethanol Oxidizing System) described by Lieber and de Carli (1972) may be involved, though their significance in the total metabolism of alcohol remains unclear.

Liver microsomes have been reported to contain a distinct ethanol oxidizing system (Orme-Johnson and Zeigler, 1965; Lieber and de Carli, 1968a). Because the activity of the system was increased with alcohol feeding (Lieber and de Carli, 1968b) it was suggested that the M.E.O.S. was of pharmacological significance and explained the ability of chronic alcoholics to metabolise alcohol faster than "normal" subjects (Goldberg, 1943; Isbell et al., 1955) it was also suggested that an increase in the activity of the NADPH dependent M.E.O.S. following ethanol feeding was associated with increased activity of the other NADPH dependent

microsomal drug detoxifying enzymes, explaining the observed increase in drug metabolism and resistance of alcoholics to various drugs (Lieber and de Carli, 1970). M.E.O.S., indeed resembles drug metabolising enzymes in:

(1) requiring NADPH and molecular oxygen;

(2) ethanol induces changes in the absorption spectra of microsomal haem proteins similar to those caused by drugs, including barbiturates (Lieber, 1966; Rubin et al., 1971).

Drug and Steroid Metabolism

Conney and Burns (1962) have reviewed the conditions and factors which affect the activity of liver microsomal drug metabolising enzymes. They note a stimulatory effect of certain foreign compounds and the influences of hormones, age, sex, and nutritional status. Adrenalectomy has been reported (Remmer, 1958a and b) to lower the activity of enzymes which oxidize hexobarbital; the activity was restored following administration of prednisone. The metabolism of barbiturates by rat liver was shown to be accelerated by the administration of cortisone or prednisone.

It is possible that drug metabolising enzymes play a role in the metabolism of naturally occurring substrates such as steroids, since both functions are localised in the hepatic microsomes and require NADPH. The similarities between liver microsomal oxidative drug metabolising enzymes and steroid hydroxylases have been pointed out by Kuntzman et al. (1964). Conney and Klutch (1963) isolated an androgen hydroxylating system from rat liver microsomes, which required NADPH and oxygen to hydroxylate testosterone in the 2,6,7 and 16 positions, and noted significant stimulation of the system by phenylbarbital and chlorcyclizine.

Cortisol Metabolism: The Role of 6β-Hydroxycortisol

The cortisol metabolite 6β-hydroxycortisol (6βOHF) has received considerable investigation over the past two decades. The first reported 6β-hydroxylation of steroids was by Lieberman et al. (1950) who identified 6β-hydroxypregnandiol in human urine. 6β-hydroxylases were subsequently identified in hog adrenal (Haines, 1952), in the corpus

luteum of cow ovary (Hayano et al., 1953) and in rat liver tissue (Axelrod and Miller, 1954). Burstein et al. (1954) reported the isolation and identification of 6βOHF from human urine following administration of cortisol to an adrenalectomised patient and also from a three day collection of late pregnancy urine.

Following these initial reports, 6βOHF was shown to occur in all human urine and its concentration to vary under a number of pathological and physiological conditions as well as the influence of exogenous agents. Thrasher et al. (1969) report normal excretion values of 0.35±0.20 mg/24 hr for males; 0.26±1.1 mg/24 hr for females and 0.18±0.05 mg/24 hr for children, and showed a significant positive correlation of 6βOHF excretion with those of total 17-hydroxycorticosteroids (17-OHCS) and creatinine. They suggest that the ratio: 6βOHF/17-OHCS is a better index of cortisol metabolism than the 6βOHF level alone.

Katz et al. (1962) noted 6βOHF as the most abundant unconjugated corticoid in all human urines studied at that time. In an attempt to elucidate its physiological role they found statistically significant differences in the output of 6βOHF by normal males and females and a distinct variation in level through the female menstrual cycle.

Other physiological and pathological factors which further research has shown to be associated with altered excretion of 6βOHF are outlined in Table 5(i).

Possible explanations for the observed changes in 6βOHF excretion under various conditions have been advanced: evidence suggests (Frantz et al., 1960; Touchstone and Blackmore, 1961; Katz et al. 1962; Burstein and Klaiber, 1965) that 6-hydroxylation represents an alternative path for cortisol metabolism, rendering the steroid more water soluble and suitable for excretion without the necessity for A-ring reduction and glucuronide conjugation, so that the pathway may assume increased importance if any of these steps are interfered with. In particular pharmacological agents such as barbiturate drugs may induce microsomal hydroxylases which favour the alternative route for cortisol metabolism in man while not exerting an important influence on its metabolic rate (Lipsett, 1971; Burstein and Klaiber, 1965; Southern et al., 1969).

The likelihood of the altered excretion pattern being due to a change in cortisol binding proteins is ruled out by the observation

Table 5i

Factors Reported as Being Associated with Increases, or Relative Increases in Excretion of 6β-Hydroxycortisol

Factor	Reference	Comments
Estrogen	Katz et al (1962)	Administration to normal males
Hypertension	ibid.	
Liver damage	ibid.	
Hepatic cirrhosis	Cohn et al (1961)	Associated with decreased conjugation of cortisol metabolites
Cancer	Werk et al (1964a)	6βOHF more than 40% of total 17-OHCS
Pregnancy	Burstein et al (1954) Frantz et al (1960;1961) Katz et al (1962) Dixon and Pennington (1968)	Also isolated 20-hydroxylated derivatives of 6βOHF
Newborn	Ulstrom et al (1960); Ducharme et al (1970) Daniilescu-Goldinberg et al (1973); Daniilescu-Goldinberg and Giroud (1974)	Possibly due to spillover from maternal circulation or inability to form glucuronide conjugates
o,p'-D.D.D* (insecticide)	Bledsoe et al (1964) Southren et al (1966a and b)	Diverts cortisol into 6β-hydroxylase pathway thus inhibiting cortisol action
Barbiturates	Burstein and Klaiber (1965) Kuntzman et al (1968) Southren et al (1969)	Associated with increases in other polar $C_{21}O_6$ steroids Increases microsomal *in vitro* 6β-hydroxylation of cortisol Effect present 91 days after treatment terminated
Diphenylhydantoin	Werk et al (1964b) Thrasher et al (1969)	
Phenylbutazone	Kuntzman et al (1966)	
Glucose-6-phosphate dehydrogenase deficiency	Borkowski et al (1962)	

cont....

Table 5i continued

Factor	Reference	Comments
ACTH administration	Touchstone and Blackmore (1961) Frantz et al (1961)	
Hypothyroidism	Yamaji et al (1969)	Suggested as secondary to impaired 4-5 double bond reduction

* o,p'-D.D.D: 2,2-bis(2-chlorophenyl-4-chlorophenyl)-1,1-dichloroethane.

(Bledsoe et al., 1964) that trans-cortin levels were unchanged following 2 weeks treatment of patients with o,p'-D.D.D.

Direct adrenal secretion is not a popular explanation though Touchstone et al. (1959) demonstrated the production of 6βOHF *in vitro* by human adrenal incubates in autologous plasma, and showed an increased production of this, and steroids more polar than cortisol, following the addition of A.C.T.H. to the medium. They suggest (Touchstone and Blackmore, 1961) direct adrenal secretion as a possible explanation for increased excretion of 6βOHF.

Katz et al. (1962) measured specific activities of cortisol metabolites after administration of labelled cortisol and cortisol secretion rate in normal males before and following estrogen treatment. These were found to be very similar, suggesting that 6βOHF was derived from cortisol metabolism rather than direct adrenal secretion, since the radioactive "pool" was not diluted. Thrasher et al. (1969) note the possibility of at least some 6βOHF, particularly in normal subjects, being derived from direct adreno-cortical production. 6βOHF was shown (Dixon and Pennington, 1969) to be a secretory product of the normal human adrenal.

6β-Hydroxycortisol as an Indicator of Hepatic Microsomal Oxidizing Capacity

Some workers (Menzebach, 1973; O'Malley et al., 1973; Smith and Rawlins, 1974) have investigated the possibility of using the excretion of 6βOHF as an index of liver drug metabolising capacity, but so far these have been unsuccessful, largely due to the considerable physiological variations in 6βOHF output. Roots et al. (1977) attempted, unsuccessfully, to correlate the impaired induction of drug metabolising enzymes as a result of liver damage by alcoholic cirrhosis with urinary 6βOHF levels.

The Aim of This Study

From the foregoing sections it is clear that hepatic microsomes are intimately involved in the metabolism of steroids, drugs and ethanol. This work was undertaken in an attempt to elucidate the effects, if any,

of alcohol ingestion on the metabolism of cortisol.

In addition to the common microsomal site of metabolism of these compounds, encouragement to undertake the study was gained from the following evidence:

1. The possibility that drugs and steroids are oxidatively metabolised by the same or similar enzyme systems (Kuntzman et al., 1964).
2. The inducibility of these enzymes by steroids, drugs and alcohol (Conney and Burns, 1962; Joly and Hetu, 1975; Valla et al., 1976.
3. The altered metabolism of drugs in the presence of ethanol (Chakraborty, 1978).
4. Altered patterns of steroid metabolism observed in cases of liver cirrhosis (Cohn et al., 1961).

The main volume of work involved the establishment of methods for measuring corticosteroid excretion, in particular total 17-OHCS and 6βOHF. Two methods are described for 17-OHCS and their suitability assessed. Isolation and quantitation of 6βOHF were based, primarily, on the method described by Thrasher et al. (1969). Other similar methods described in the literature include: Burstein et al. (1954); Touchstone et al. (1959); Frantz et al. (1960); Frantz et al. (1961), Bledsoe et al. (1964); Werk et al. (1964a); Burstein and Klaiber (1965); Berthold and Staudinger (1966); Kuntzman et al. (1968); Yamaji et al. (1969); Fukushima et al. (1969) and (1971); Ducharme et al. (1970); Hall et al. (1971) and Daniilescu-Goldinberg and Giroud (1974). All use solvent extraction (usually ethyl acetate) and separation by thin layer or paper chromatography, or combinations of the two.

MATERIALS AND METHODS

MATERIALS

Unless otherwise specified all chemicals were reagent grade or better and obtained from May and Baker Ltd, British Drug Houses Ltd or Sigma Chemical Co. Ltd.

All solvents were redistilled before use, unless a more rigorous treatment is indicated.

β-Glucuronidase was supplied by Sigma Chemical Co. Ltd, St Louis, Mo., U.S.A. as a crude extract from *Helix pomatia* containing approximately 100,000 Fishman units of glucuronidase activity per ml (one Fishman unit defined as the amount of enzyme hydrolysing 1.0 µg of phenolphthalein glucuronide per hour at pH 5.0 and 37°C) and 3690 µmolar units of aryl sulphatase activity per ml (using nitrocatechol sulphate as substrate, pH 5.0, 37°C).

Silica gel GF-254 was from E. Merck, Darmstat, Germany.

6βOHF was supplied by Steraloids Inc., Pawling, N.Y., U.S.A. THE, THF and Blue Tetrazolium were from Mann Research Laboratories, N.Y., U.S.A.

6β-hydroxy(1,2-^{3}H)cortisol and (1,2,6,7-^{3}H)cortisol were obtained from the Radiochemical Centre, Amersham, U.K. The radioactive concentration of ^{3}H-6βOHF was 1.0 mCi/ml in benzene:ethanol (9:1 v/v), specific activity 49 Ci/mmol. 50 µl aliquots were removed and diluted to a working concentration of approximately 2 µCi/ml with ethanol. ^{3}H cortisol had a radioactive concentration of 1.0 mCi/ml and specific activity 95 Ci/mmol; an aliquot of the stock was diluted to give a working solution containing approximately 1 µCi.

Scintillation fluid was made by dissolving 0.4% PPO (2,5-diphenyloxazol) and 0.04% POPOP (1,4-bis[2-(5-phenyloxazol]benzene), w/v in redistilled toluene.

All water used was deionised and/or glass distilled.

METHODS

Collection of Samples:

Complete 24 hour urine samples were collected in dry plastic containers, without preservative. These were stored at 4°C during and immediately following collection. If it was not possible to process the sample within 48 hours of the collection being completed, the entire sample, or representative aliquots, were frozen for up to three weeks prior to analysis.

Determination of 17-Hydroxycorticosteroids

Method I (hydrolysis method)

This was developed from those described by Kuntzman et al (1968) and Sunderman (1960), with internal recovery correction using tritiated cortisol.

Approximately 20,000 cpm ^{3}H-cortisol were added to a 10 ml aliquot of urine and to each of two scintillation vials (for determining total added counts). The pH of the urine was adjusted to 5.0±0.2 with acetic acid and incubated in a shaking waterbath at 37°C, with 20,000 units (200 µl) of β-glucuronidase, for 24 hours. The pH was checked at least once during the incubation period. The continuing activity of β-glucuronidase in the medium was checked semiquantitatively by incubating a 100 µl aliquot of hydrolysate with 0.5 ml 0.001 M phenolphthalein glucuronide buffered at pH 5.0, at 37°C for 30 min. Addition of sodium hydroxide, sufficient to make an alkaline solution, resulted in the production of the characteristic pink colour of free phenolphthalein, indicating that the enzyme was still active.

10 ml of cold acetate buffer, pH 5.0 was added to the hydrolysate which was then transferred to a 250 ml separatory funnel and extracted by shaking for 10 min with 100 ml chloroform. The extract was washed once with 10 ml 0.1 M NaOH, then twice with 10 ml aliquots of distilled water. Three 20 ml aliquots of extract were removed to 50 ml round bottomed flasks, evaporated to dryness (Buchi "Rotovapor") and retained for quantitation by the Porter-Silber reaction.

Two 2 ml aliquots were also removed to scintillation vials which, together with those taken initially for "total counts", were evaporated under a stream of dry nitrogen and redissolved in 10 ml scintillation fluid for the determination of procedural losses.

The Porter-Silber Reaction: (Silber and Porter, 1954)

The reagents: (a) Alcoholic sulphuric acid, 64% H_2SO_4:EtOH (2:1)
(b) Phenylhydrazine (BDH, AR), 1 mg/ml in (a).

1 ml (a) was added to one dried extract to act as "blank"; 1 ml (b) was added to each of the others. The flasks were mixed, stoppered and held overnight in the dark at room temperature. The reaction mixtures were transferred to 1 ml cuvettes and their absorbances read against the "blank" in a Unicam SP 500 spectrophotometer at 372, 412 and 452 nm. The Allen correction was applied to each reading:
$A_{corrected} = A_{412} - \frac{1}{2}(A_{372}+A_{452})$ and the steroid concentration determined by interpolation from standards analysed simultaneously.

Standard Curve: Duplicate aliquots containing 0, 1, 2, 5, 10 and 20 µg THE were evaporated to dryness under dry nitrogen. 1 ml phenylhydrazine reagent (b) was added to each tube and these were stored overnight, in the dark at room temperature. A typical standard curve is shown in Fig. 5i. The curve is linear between 0 and 20 µg THE and highly reproducible (Table 5ii), so that for routine analyses only one duplicate standard (2-5 µg, THE) was used.

Results were corrected for procedural losses by counting the aliquots removed to vials (Packard Tri-carb liquid scintillation counter, with automatic external standard) and determining the percentage of total added counts which were extracted. The method showed a 90±5% recovery of added ^{3}H-cortisol over five separate assays so that in routine work 90% recovery was assumed and the results corrected accordingly.

Figure 5i

Porter-Silber Reaction for 17-Hydroxycorticosteroids

Typical Standard Curve.

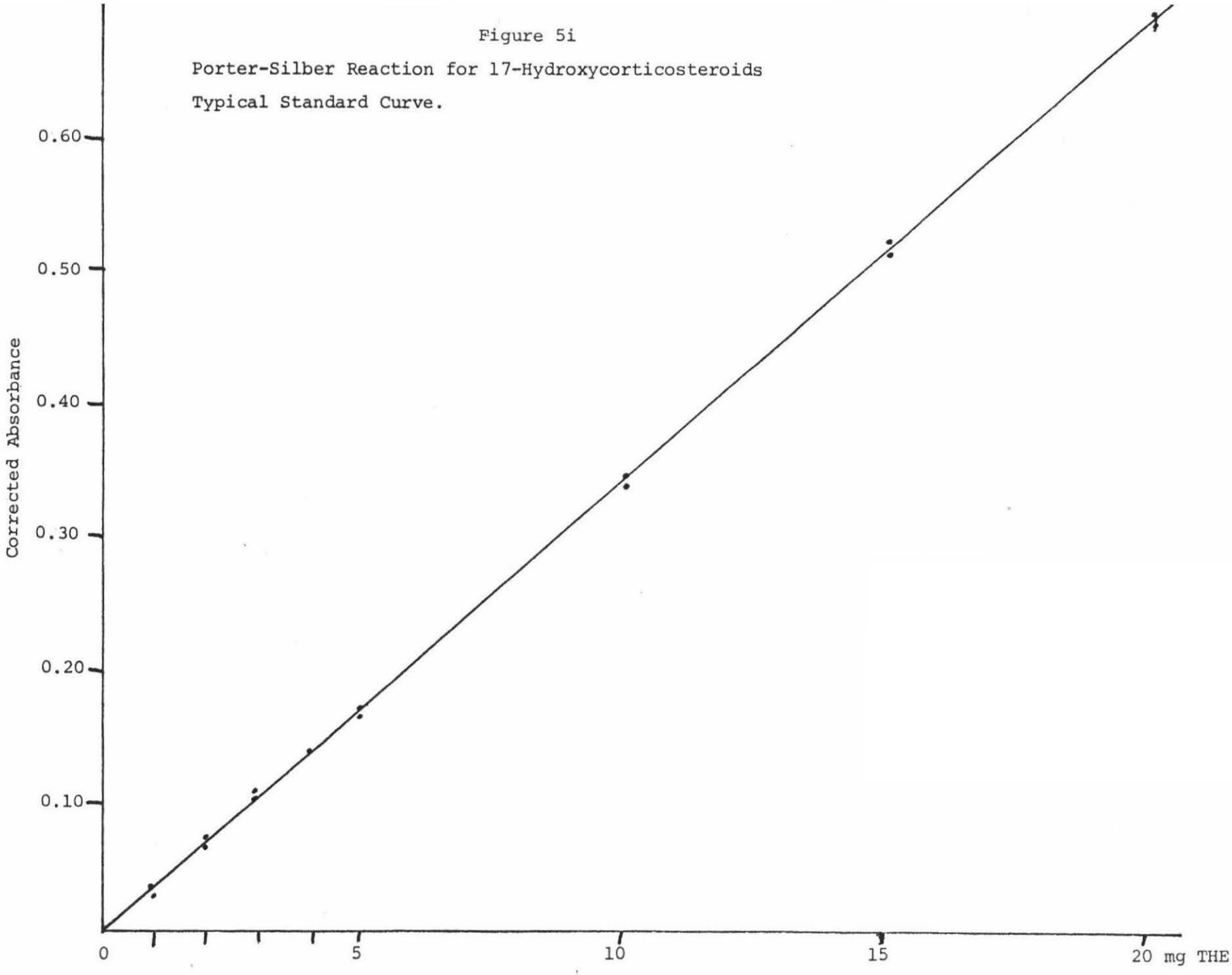


Table 5ii

Reproducibility of Standard Curve for Porter Silber Reaction

mg THE	1	2	3	4	5	10	15	20
Absorbance*	0.034	0.067	0.105	0.139	0.172	0.344	0.523	0.692
	0.030	0.070	0.107	0.139	0.164	0.338	0.514	0.685
	0.034	0.067	0.103	0.137	0.169			
	0.032	0.066	0.109	0.139	0.178			
	0.032	0.070	0.104	0.138	0.171			
	0.035	0.070	0.106	0.139	0.174			

Values are grouped, showing within-assay duplicates.

* Absorbances after applying the "Allen Correction":

$$A_{412} - \tfrac{1}{2}(A_{372}+A_{452})$$

Method II (column method)

The method of Ariyoshi and Osawa (1976) was adapted as follows: A neutral polystyrene resin Amberlite XAD-2 (BDH) was soaked overnight in water and washed well. Approximately 5 ml resin was packed into a glass chromatography column (1 cm x 30 cm) and washed with:

1. Three volumes of distilled, deionised water.
2. One volume of redistilled methanol.
3. Ten volumes of water.

The flow rate was maintained at approximately 0.5 ml/min.

The volume of resin was increased over the 3 ml used by Ariyoshi and Osawa, as early trials using a comparable volume of urine showed considerable overloading, with 20-30% of added 3-H cortisol being eluted during the loading of the sample. The volume of urine applied to the column was also decreased from 30 to 25 ml.

Approximately 30,000 cpm tritiated cortisol were added to 25 ml of a 24 hour urine sample. The pH was adjusted to 2.0 with 1 M sulphuric acid and the solution transferred to a 30 ml screw capped test tube. Approximately 0.5 g kaolin (BDH, previously activated in an oven at 110°C for one week) was added to the capped tube and shaken horizontally (Shaker, Analite Pty, Australia) for approximately 45 min.

In view of the highly coloured column eluates previously obtained, a more rigorous kaolin treatment was indicated than the three minutes' stirring of urine and kaolin suggested by Ariyoshi and Osawa.

The tube was then centrifuged to settle the suspended kaolin, and a 20 ml aliquot of urine removed. This was adjusted to pH 7 with 1 M NaOH and loaded onto the column.

The column was washed with 15 ml water and the urinary steroid conjugates eluted with 10.0 ml ethanol:water (80:20). The column was regenerated by washing with 5 volumes of absolute ethanol and 10 volumes of water.

Three 1 ml aliquots of the column eluate were taken and evaporated for assay by the Porter-Silber reaction, as described for the hydrolytic method, one aliquot serving as a blank. Two 0.1 ml aliquots were removed to scintillation vials, where they were evaporated and redissolved in 10 ml toluene scintillant for recovery counting.

Comparison of hydrolytic and column methods

12 ml urine was "spiked" with approximately 30,000 cpm ^{3}H-cortisol, hydrolysed as described for Method I and 10 ml of the hydrolysate extracted into 100 ml chloroform as previously described. Three 20 ml aliquots were taken for Porter-Silber reaction and two 2 ml aliquots for recovery counting.

25 ml of the same 24 hour urine sample was analysed by the column method and the results compared (Table 5iii).

The column method produced a very high Porter-Silber blank in spite of prolonged treatment with kaolin, suggesting that the results of the colour reaction were less reliable than those given by the almost colourless extracts of the hydrolysed urine. However the lower result for 17-OHCS concentration, given by the first method could have been due to incomplete hydrolysis of steroid conjugates.

The recovery of added ^{3}H-cortisol was approximately 10% higher with less deviation between duplicates in Method I than Method II, indicating a more efficient extraction of free steroids. The unavailability of any labelled steroid conjugates, however, prevented any conclusions as to the comparative efficiencies of the methods when the hydrolytic process was considered.

In a second experiment 2 µg of unlabelled cortisol per ml of urine was added to each of two aliquots of the 24 hour urine used above, and the samples were assayed by each of the two methods together with an identical "unspiked" sample for each method. In Method I, 84% of the added, free steroid was recovered (Table 5iii). On the other hand, the column method produced a blank so high that the Porter-Silber chromogens could not be measured, indicating that the method was not reliable for routine assays without further modification.

It was concluded that free steroids could be extracted from urine hydrolysates by chloroform with a relatively high efficiency. Experiments also showed that the β-glucuronidase enzyme was active throughout the course of hydrolysis. Since most established literature methods appear to favour a method based on organic extraction of hydrolysed urine and indicate incubation times and enzyme concentrations similar to those used here Method I appeared to be more reliable for the experiments proposed in this work.

The reproducibility of the two methods is shown in Table 5iii. The hydrolytic method indicates a 4% variation between duplicates in the same assay, but 33% between the mean values of two successive assays. The column method shows nearly 10% difference between intra-assay duplicates and the second assay could not be quantitated, suggesting that while neither method was particularly reliable, the hydrolytic method was probably the more useful of the two.

Table 5iii

Comparison of Two Methods for Determining
Urinary 17-Hydroxycorticosteroids

Hydrolytic Method vs. Column Method

	Estimated 17-OHCS Concentration	Recovery ^{3}H-6βOHF	Recovery 6βOHF
Hydrolytic			
assay I	2.75 µg/ml	95%	
	2.86 µg/ml	92%	
assay II	1.99 µg/ml		84%
Column			
assay I	5.64 µg/ml	82%	
	6.21 µg/ml	88%	
assay II	Not readable		-

Extraction and Purification of 6β-Hydroxycortisol from Urine

Extraction

Aliquots corresponding to approximately 10% of the total aliquots were taken for 17-OHCS assay.

6βOHF is highly polar and literature reports suggest that it is not excreted as a glucuronide or sulphate conjugate; consequently extraction was routinely performed without prior hydrolysis.

Before extraction, approximately 20,000 cpm ^{3}H-6βOHF were added to correct for procedural losses and 150 mg anhydrous sodium sulphate dissolved per ml of urine. The sample was transferred to a one litre separatory funnel, washed once with two volumes redistilled dichloromethane, then extracted twice with two volumes of redistilled ethyl acetate for 10 minutes each.

The combined extracts were washed 2-3 times with 1/20 volume 1 M NaOH containing 15% Na_2SO_4 (w/v) until the wash was clear; then once with 1/20 volume 1 M acetic acid containing 15% Na_2SO_4 (w/v); and finally, once with 1/100 volume 15% Na_2SO_4 (w/v).

The extract was taken to dryness (Buchi "Rotovapor") and redissolved in approximately 10 ml ethyl acetate:methanol (1:1) (Kuntzman, 1968), both solvents being dried over calcium chloride and redistilled before use. The extract was then filtered through Whatman No.1 filter paper into a 15 ml conical test tube to remove excess, solid Na_2SO_4. The ethyl acetate-methanol solution was concentrated under a stream of nitrogen to a volume of approximately 100-200 µl.

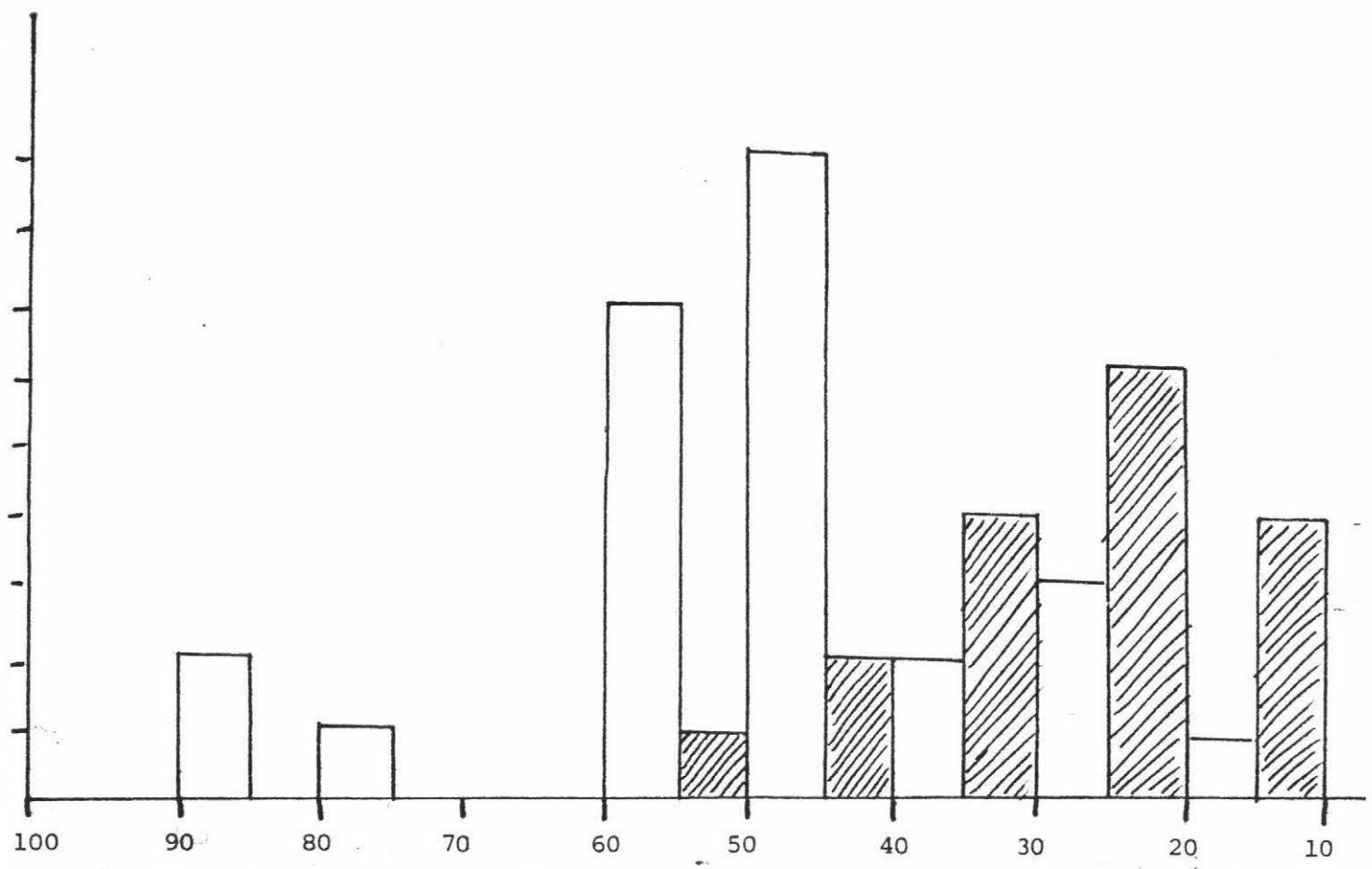
Purification

The extract was applied as a narrow band to a Silica Gel GF-254 thin layer chromatography (TLC) plate, together with a spot containing approximately 10 µg 6βOHF.

The plate was developed in chloroform containing 20% methanol (v/v), air dried and the position of 6βOHF in the extract determined under short wavelength ultraviolet light with reference to the standard marker. The band was carefully scraped from the plate into a screw-capped 15 ml test tube and the steroid eluted by shaking the silica gel for 15 min. with each of two 10 ml aliquots of ethyl acetate:methanol (1:1). The two aliquots were combined and evaporated to dryness. The extract was then

Figure 5ii

Recovery of ^{3}H, 6β-Hydroxycortisol after TLC (25 assays)
and Paper Chromatography (17 assays)



% Recovery ^{3}H 6β-Hydroxycortisol

Open bars represent TLC assays

Shaded bars represent PC assays

redissolved in 200 µl MeOH/EtOAc and 50 µl removed to a scintillation vial for recovery counting (1); two 50 µl aliquots were removed for the Porter-Silber reaction (2); and 50 µl for the Porter-Silber "blank" (3).

Aliquot (1) was evaporated and redissolved in 10 ml toluene scintillant.

(2) were evaporated and redissolved in 1 ml Porter-Silber reagent, each.

(3) was evaporated and redissolved in 1 ml EtOH/H_2SO_4.

The Porter-Silber reaction was continued as outlined for the 17-OHCS determination.

Results were corrected for procedural losses according to the recovery of ^{3}H-6βOHF: this was found to vary between 87 and 20% (mean: 48%) for TLC separation alone and 52 to 10% (mean: 29%) when a paper chromatography (PC) step was added (Fig. 5ii).

Recovery of unlabelled 6βOHF added to urine was estimated in three separate assays and ranged from 89-96%, after results were corrected for recovery of tritiated 6βOH cortisol (See Table 5iv).

The variation between duplicates in seven different assays is shown in Table 5v. More than half of the assays show less than 5% variation while three show 12, 16 and 20% each.

Table 5iv

Recovery of Unlabelled 6β-Hydroxycortisol

	Spike added	µg 6βOHF in unspiked urine	µg 6βOHF in spiked urine	% spike recovered
Assay 1	50 µg	13.1	58.5	93
Assay 2	50	53.1	91.8	89
Assay 3	50	117	161	96

Table 5v

Intra-assay Difference in 6β-Hydroxycortisol
Assay of Seven Urine Samples

*Duplicate Assays		*Mean	% Difference from Mean
586	476	531	20
793	766	780	3
936	798	867	16
3070	3450	3260	12
428	415	422	3
604	582	593	4
993	1015	1004	2

*6βOHF excretion in µg/24 hr.

Conjugated 6β-Hydroxycortisol

Experiments were performed to determine whether a significant amount of 6βOHF was excreted as conjugates. This was done both with fresh urine and with the residue which remained after the extraction of the free steroid.

Method I

Free and free-plus-conjugated 6βOHF were determined simultaneously in two identical urine aliquots: One sample was extracted and purified by the routine method for the free steroid, while a second sample was submitted to overnight hydrolysis with 1,000 units of β-glucuronidase/ml of urine at pH 5.0 and 37°C, prior to an extraction and purification procedure identical with that of the first sample.

Both TLC eluates were quantitated by the Porter-Silber reaction and corrected for the recovery of ^{3}H-6βOHF, added prior to hydrolysis and extraction. In only one experiment was total 6βOHF (2.52 mg/24 hr) greater than free (2.41 mg/24 hr) and this difference of 5% is well within the experimental error of the method.

Method II

Following extraction of free 6βOHF, as outlined previously, the urine residues were retained, together with the first two NaOH washes. These were combined and washed once with a half volume of diethyl ether. The ether layer was discarded, and nitrogen was bubbled through the urine to remove traces of solvent. The pH was adjusted to 5.0±0.2 with acetic acid, 1,000 units β-glucuronidase per ml of urine were added and the mixture incubated overnight at 37°C. The hydrolysate was then extracted and purified by the same method used for free 6βOHF. No band corresponding to 6βOHF was detected on the TLC plate.

Any conjugated 6βOHF present in urine is probably less than the variation between free 6βOHF estimations and was ignored in routine assays.

Specific Activities

Assays were routinely performed with the only purification step subsequent to extraction being a single thin layer chromatographic separation. A series of experiments were performed in which further purification by paper chromatography (PC) was employed in order to check the purity of the 6βOHF eluted from the TLC plate.

TLC eluates were evaporated to dryness and redissolved in 0.2 ml ethyl acetate:methanol (1:1). 20 µl was taken for counting and 20 µl for estimation by the Porter-Silber reaction; the remainder was streaked onto the origin of a Whatman 3 MM paper chromatograph together with a sample (5-10 µg) of authentic 6βOHF in ethanol. The chromatogram was equilibrated overnight with the stationary phase of EtOAc:$CHCl_3$:MeOH:H_2O (1:3:2:2) and developed by descending elution with the mobile phase (Frantz et al, 1961).

Portions of the chromatograms were visualised by viewing under ultra-violet light or by staining with Blue Tetrazolium (BTZ) as follows: Papers were soaked in a trough containing BTZ reagent (5 ml of BTZ, 5 mg/ml in formamide + 500 ml 2 M NaOH) for 5 min., then washing with 2% acetic acid and distilled water, 5 min. each (Kuntzmann et al, 1968).

The area of paper corresponding to 6βOHF was cut out and eluted by shaking with ethyl acetate:methanol (1:1) for 15 min. The eluate was evaporated and redissolved in solvent, an aliquot being taken for

counting and the remainder for the Porter-Silber reaction.

In a few cases a second paper chromatographic separation was performed in toluene:MeOH:H_2O (16:3:1) (Thrasher et al, 1969), but recoveries were not sufficiently high for any significant estimations to be made.

It was found, however, that a reasonably constant specific activity was obtained between the initial TLC separation and one subsequent paper chromatogram. The results shown in Table 5vi suggest that the specific activity of 6βOHF is not increased by a second chromatography step to a degree which would justify the increased error and decreased recovery resulting from such a step.

Table 5vi

Specific Activities of ^{3}H-6β-Hydroxycortisol following Thin Layer and Paper Chromatographic Separations

Experiment	Specific Activity*		% Increase in S.A.
	After TLC	After PC	
1	222	268	20
1a	436	468	7
2	68.0	70.9	4
2a	71.3	79.3	11
3	267	353	32
3a	328	568	42

* Specific activity in counts, min^{-1}, μg^{-1}.

Variation in specific activity between TLC and PC is smaller than the variation between duplicates in the same assay.

RESULTS AND DISCUSSION

Normal Level of 6β-Hydroxycortisol

A 24 hour urine specimen was obtained from a male subject with no apparent physiological abnormalities, and an aliquot representing 10% of the total output was extracted and analysed for 6βOHF by the routine methods outlined previously. A 10 ml aliquot was simultaneously analysed for 17-OHCS by the hydrolytic method. Duplicate analyses were performed and the results corrected for procedural losses by calculating the recovery of radioactive markers.

The values obtained are shown in Table 5vii and compare well with the standard literature values. Recovery of tritiated 6βOHF was between 43.3 and 39.5% compared with 89% tritiated cortisol recovered in the 17-OHCS determination. 50 µg unlabelled 6βOHF was added to a third aliquot and after correction of the result for labelled steroid, 89% of the added unlabelled steroid was recovered.

Table 5vii

Excretion Levels of 6β-Hydroxycortisol and 17-Hydroxycorticosteroids by a Normal Subject

Assay	6βOHF	17-OHCS	6βOHF:17-OHCS
(i)	586 µg/24 hr	5022 µg/24 hr	
(ii)	476 µg/24 hr	4927 µg/24 hr	0.095-0.117
Mean	531 µg/24 hr	4975 µg/24 hr	0.107 µg/24 hr

Alcoholic Subjects

Subject (1)

A 24-hour urine collection was obtained from an alcoholic subject upon admission to hospital with an acutely elevated blood alcohol level (Sample I). A second specimen (Sample II) was obtained one week later after the patient had undergone treatment and "drying-out".

Aliquots from the two collections were analysed for 6βOHF and 17-OHCS as described above. Since there was some variation in duplicates, the assay of 6βOHF on the first specimen was repeated on a frozen aliquot a week later when the second collection was analysed.

The ratio 6βOHF:17-OHCS in the first collection varied between 0.706 and 5.35, with a mean of 2.47 over eight combinations of assay results as shown in Table 5viii. The measured concentration of 6βOHF corresponds to that fraction of 17-OHCS extracted by EtOAc and "17-OHCS" the fraction extracted by chloroform, thus it is possible for the 6βOHF:17-OHCS ratio to be greater than one since EtOAc is the more polar solvent. Combinations of two assays for each 6βOHF and 17-OHCS estimate in the second urine gave a mean ratio of 0.056 with a range between 0.0507 and 0.0615.

Table 5viii

Levels of Urinary 6β-Hydroxycortisol Obtained from Alcoholic Subject (1)

	Sample I (with alcohol)		Sample II (without alcohol)
	Assay 1	Assay 2	
6βOHF*	2515	1168	428
	2403	3880	415
17-OHCS*	1651		6940
	725		8190
6βOHF:17-OHCS	0.706-5.35**		0.051-0.062***
	(mean: 2.47)		(mean: 0.056)

* in μg/24 hr

** combinations of eight assay results

*** combinations of four assay results

Though there is much variation in the assay values for 6βOHF in "Sample I", all are more than double those recorded in "Sample II". Conversely the values obtained for 17-OHCS are at least five times greater in the second collection. Although the accuracy of results, particularly for 6βOHF, must remain doubtful, the trends suggest that at a high blood alcohol level the patient displayed a ratio of 6βOHF:17-OHCS which was, on average, more than 20 times that observed in a normal person. On withdrawal from alcohol, the patient's ratio fell to approximately half that of the normal subject.

The initial conclusion from this pilot study was that active alcohol metabolism represented a condition where 6βOHF was excreted as the almost exclusive metabolite of cortisol and far exceeded the production of the usual chloroform-extractable 17-OHCS. In fact the 17-OHCS levels measured here were at most one third of the expected normal value.

In the second collection the measured 6βOHF:17-OHCS ratio fell dramatically. This was due to a marked increase in 17-OHCS production since the 6βOHF levels remained in the normal range.

These results alone suggest that at both high and low alcohol levels this patient showed a supranormal adrenal activity and that in the presence of high ethanol levels cortisol was metabolised to the unconjugated, polar steroid 6βOHF. When blood alcohol dropped, the extra cortisol appeared to be metabolised via the normal route, presumably to conjugated THF, THE etc. These findings do not, however, rule out the possibility of direct adrenal secretion of 6βOHF in the high alcohol state, nor can all the effects be unequivocally and directly attributed to alcohol without fuller consideration of the patient's state of health at that time.

Urinary 6βOHF levels were measured again a month later when the same subject was readmitted to hospital with high blood alcohol. This time, however, the results failed to follow the above pattern (Table 5ix). The measured excretion of 6βOHF was only slightly elevated above normal in the initial collection and, in fact, showed a marked increase in the second collection. The fact that the second collection was made only three days after the first, compared with nine days in the first series of observations may be significant: 6βOHF levels may be increased even more during the initial "drying-out" period and take at least a week to return to a normal level.

Subject (2)

A similar experiment with a second alcoholic patient showed an initial 6βOHF excretion rate of 593 µg/24 hr, rising to 867 µg/24 hr after six days (Table 5ix). The first level is essentially normal and the second not excessively elevated.

Table 5ix

6β-Hydroxycortisol Levels in Alcoholic Subjects

Subject	Collection	Initial Assay*		Repeat Assay*	
(1)	1	793	766		
	2	4140			
(2)	1	604	582		
	2	936	798	960	1595
(3)	1	993	1015	232	
(4)	1	3070	3450	4700	

* in µg/24 hr

Further Subjects

Initial collections were obtained from two further subjects during the "high alcohol" period and mean 6βOHF levels of 1004 and 3260 µg/24 hr respectively (Table 5 ix) were obtained. Both levels showed a distinct increase over normal, but without follow-up collections no significant conclusions could be drawn. Without more careful monitoring of patients and samples, which was not possible in these cases, it is difficult to draw any conclusions as to the possible meanings of measured levels. The lack of 17-OHCS levels measured in the later patients precludes direct comparison of ratios.

Conclusions

The accuracy of results obtained in the foregoing experiments came under a considerable amount of doubt when the measurement of 6βOHF levels was repeated on frozen samples of a number of these urines. As shown in Table 5ix, most of the results of the second assay bore very little relation to those of the first, and indicated that further developmental work should be done on the assays before any significance could be firmly attached to their results. Indications were that the steroid was not always cleanly separated from the others by a single thin layer chromatograph, and it was decided that if the assay was to be further refined and developed it should also be extended to include the separation and measurement of a number of specific steroids. This section of the work is outlined in Chapter 4.

Unfortunately the GLC system used was unable to measure either cortisol or 6βOHF although Horning et al (1974) report the quantification of both steroids by selective ion detection with a gas chromatograph-mass spectrometer-computer system. Initial attempts to couple the GC system described in Chapter 4 to a Micromass 12F mass spectrometer were discontinued when it became apparent that the high temperatures necessary to separate 6βOHF in the GC gave problems in interfacing this instrument to the spectrometer for the estimation of 6βOHF. In retrospect it would seem that the most feasible method for determination of urinary 6βOHF is one based on an improved TLC system.

CHAPTER 6

CONCLUSION

This work represents the development of a number of methods and a series of pilot studies which could form a useful basis for the study of hormone-alcohol relationships, although limited significance may be attached to the isolated results obtained here.

Chapter 2 details a readily usable and reliable radioimmunoassay for plasma cortisol. The two pilot studies involving normal and alcoholic subjects suggest a possible relationship between concentrations of cortisol and alcohol in human blood. The studies undertaken here, however, are not sufficient to unequivocally establish the exact nature of this relationship.

Any further studies in this area would need to be carefully designed, not only to remove the contributions of natural diurnal rhythms but to minimise, as far as possible, individual variations among subjects. Hospital patients admitted in acute alcohol intoxication compose an extremely heterogeneous group, displaying large individual variations in clinical histories and pathologies, nutritional, psychological and sociological statuses; thus careful screening and grouping of subjects would be necessary before any significance could be attached to results obtained from such a group. Alcohol loading of normal volunteer subjects is very much easier to control but one must be cautious in extrapolating results obtained from light and moderate drinkers to the situations of heavy and habitual drinkers.

Results reported in the literature show both increases and decreases in plasma cortisol levels in response to alcohol ingestion. Since plasma cortisol concentration reflects a large number of different physiological and psychological stimuli, experiments designed to establish a direct relationship between blood alcohol levels and cortisol secretion rates must control for any other such influences.

Chapter 3 reports attempts to obtain steroid excretion profiles by extraction of urine and purification using a series of successive thin layer chromatographic separations. While this appeared on the basis of early trials to be a useful approach, low yields of steroids made any quantitation very difficult. In general, the gas chromatographic separations,

outlined in Chapter 4, represent a much more useful technique for obtaining urinary steroid profiles.

The glass capillary GLC method described in this work was used to produce urinary steroid profiles very similar to those shown in the literature and involves the relatively simple adaptation of a conventional gas chromatograph to take a high resolution capillary column. The technique has several advantages over the TLC method in that it is able to estimate comparative concentrations of a large number of individual steroids from one, relatively crude, urine extract in a single separation step. This represents a considerable saving in time and increase in recovery which, when coupled with the use of suitable internal standards, allows reasonably accurate quantitation of individual steroids.

The GLC method proved disappointing, however, in being unable to estimate two steroids of particular interest in this study, i.e. cortisol and 6β-hydroxycortisol, without considerable modification of the available equipment. It would therefore, in this context, appear that urinary cortisol levels could be more easily and reliably estimated by the RIA method described for plasma cortisol, and that 6βOHF could more feasibly be estimated by a refinement of the TLC methods described in Chapters 3 and 5, using labelled steroid as an indicator of position and recovery.

Urinary steroid profiles were obtained from four individual subjects (Chapter 4): those derived from 24 hour collections from a normal male and a normal female closely resemble corresponding profiles shown in the literature. Unfortunately, time was not available to assess the variability of profile studies from a large number of normal subjects, and this data is not yet available in the literature. Consequently, the significance of differences in the profile from the alcoholic female studied could not be assessed, or the changes observed in the alcohol-loading of a normal male. While it may be concluded that alcohol does not produce gross disturbances in the excretion of the measured steroid metabolites, the possibility of more subtle differences was not ruled out. Greater numbers of carefully controlled experiments and subjects would be required before any observed changes could be judged significant.

Although there are no literature references which specifically relate 6βOHF excretion to alcohol intake, the established relationships between microsomal oxidation systems for alcohol, drugs and steroids, outlined in the introduction to Chapter 5, suggest that this is more than

possible. Comparison of results obtained from a normal subject with those from the first alcoholic subject tend to support the hypothesis that cortisol metabolism is diverted into the 6βOHF pathway by the simultaneous metabolism of alcohol. The failure of further observations to support this may be due to the irreproducibility of the analytical method or to variations in the collection of urine from hospital patients. In either case, further developmental work must be done on the TLC method for separating and estimating 6βOHF and attempts made to standardise clinical conditions and urine collection protocols before the initial observations can be supported or disproved.

This work has been useful in establishing methods for estimating individual and groups of corticosteroids, i.e. by RIA, TLC and GLC. Pilot studies on normal and alcoholic subjects point to a number of possible and probable relationships between alcohol and corticosteroid hormones, although no definite conclusions can be made without further observations. The study reinforces suggestions that experiments designed to unequivocally establish effects of ethanol on cortisol metabolism must be carefully controlled to minimise and, if possible, eliminate all spurious influences.

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