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FACTORS AFFECTING THE EXCRETION OF URINARY STEROID
METABOLITES IN MAN

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in
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ABSTRACT

A gas liquid chromatograph (GLC), adapted to accept a "Support Coated Open Tubular" (SCOT) capillary column, was used for profiling of the neutral steroid hormone metabolites from human urine specimens. Following hydrolysis with β-glucuronidase to release the steroid metabolites from their conjugates, they were extracted with organic solvents and converted into methoxime-trimethylsilyl derivatives for separation on the GLC column.

Initial work involved determining basal data on consecutive days and also during the twenty four hour period. The circadian variation in this experimental work , showing a biphasic pattern of steroid metabolite excretion, with a maxima in the morning, and a second smaller maxima in the afternoon.

The effect of cold stress on metabolite excretion was examined, however the results obtained were inconclusive.

Dexamethasone was administered, 0.5 mg per six hours for forty eight hours, to induce suppression of ACTH secretion. The expected pattern of decresed 17 hydroxycorticoid excretion, and to a lesser extent 17 oxosteroid excretion was observed.

Alcohol loading was examined, with varying levels of intoxication. One subject maintained a blood alcohol level of approximately 50 mg% for three hours, and the urine specimens were collected at two hour intervals. The excretion rate of the steroid metabolites increased a few hours after ethanol loading, compared to the control. THE, THF and aTHF showed the greatest increments, and this may possibly be due to increased hepatic A ring reductase activity resulting from increased NADH:NAD$^+$ ratio. A detailed examination of excretion rates and plasma cortisol concentration would be required to prove that ethanol causes this action on the A ring reductase activity.

Three twenty-four hour specimens were collected from a subject with a history of alcohol abuse over the previous ten years. On Day 2 of the three day experiment, a 26 ounce bottle of Scotch was consumed, but other than an increase of all steroid metabolites on the day of alcohol loading, no significant observations were made.

Three urine specimens from alcoholics admitted to the Palmerston North Detoxification Unit were analysed. No 11OHEt could be detected in any of these three patient's urine, however the β-glucuronidase used in this analysis had a low activity resulting in incomplete hydrolysis, and basal

data for these patients would be required to prove that the absence of 11OHEt was a result of alcohol intoxication.

One of the alcoholic patients showed high levels of cortisol metabolites, probably the result of stress of hospitalization.

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ABBREVIATIONS

STEROIDS

Abbreviation	Trivial Name	Systematic Name
An	Androsterone	5α-androstan-3α-ol-17-one
Et	Etiocholanolone	5β-androstan-3α-ol-17-one
DHEA	Dehydroepiandrosterone	5-androsten-3α-ol-17-one
*11KAn	11-Ketoandroterone	5α-androstan-3α-ol-11β,17-dione
*11KEt	11-Ketoetiocholanolone	5β-androstan-3 -ol-11β,17-dione
11OHAn	11-Hydroxyandrosterone	5α-androstan-3α,11β-diol-17-one
11OHEt	11-Hydroxyetiocholanolone	5β-androstan-3α,11β-diol-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
THS	Tetrahydro-11-deoxycortisol	5β-pregnan-3α,17α,21-triol-20-one
THE	Tetrahydrocortisone	5β-pregnan-3α,17α,21-triol-11,20-dione
THB	Tetrahydrocorticosterone	5β-pregnan-3α,11β,21-triol-20-one
THF	Tetrahydrocortisol	5β-pregnan-3α,11β,17α,21-tetrol-20-one
aTHF	Allotetrahydrocortisol	5α-pregnan-3α,11β,17α,21-tetrol-20-one
αC'one	α-Cortolone	5β-pregnan-3α,17α,20α,21-tetrol-11-one
#βC'ol	β-Cortol	5β-pregnan-3α,11β,17α,20β,21-pentol
#βC'one	β-Cortolone	5β-pregnan-3α,17α,20β,21-tetrol-11-one
αC'ol	α-Cortol	5β-pregnan-3α,11β,17α,20α,21-pentol

*11KAn and 11KEt coelute and are collectively referred to as 11KAn/Et

#βC'ol and βC'one coelute and are collectively referred to as βC/C

GENERAL

ACTH	Adrenocoticotrophic hormone
TSIM	Trimethylsilyl imidazole
MO-TMS	Methoxime-trimethylsilyl
GLC	Gas liquid chromatography
C24	Hydrocarbon: n-tetracosane
C32	Hydrocarbon: n-dotriacontane

OH	Hydroxy
SCOT	Support Coated Open Tubular
GC-MS	Gas Chromatography-Mass Spectrometry
GLT	Glass Lined Tubing
μl	Microlitre
ml	Millilitre
μg	Microgram
g	Gram
mm	Millimeter
M	Molar
^{o}C	Degree Celcius
ID	Internal Diameter
min	Minute
mv	Millivolt
Na_2SO_4	Sodium Sulphate
$CHCl_3$	Chloroform
HMDS	Hexamethyldisilazane
cm	Centimeter

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CHAPTER 1

INTRODUCTION

A human urine sample contains many steroid metabolites which are the end products of a complex set of metabolic reactions involving more than one organ and more than one control mechanism (Polvani et al, 1967). The urinary steroids contribute to the end products of processes which start with the synthesis of steroids in the adrenal gland, gonads and the fetoplacental unit, continues through with secretion of the steroids by the glands, their metabolism in various body tissues, and finally ends with the excretion of the metabolic products through the kidneys.

Although some excretion occurs into bile, some in faeces and probably minute amounts of some steroids in sweat, the most important route of elimination is the urine. About 75-95% of the label of administered ^{14}C-cortisol is found in urine (Vestergaard, 1978).

By looking at whole body steroid metabolism "through the kidneys", one is therefore likely to see changes due to alteration in kidney or liver function, adrenal or gonadal function, pituitary or hypothalamic function or external environment.

Table li shows the normal secretion rates of steroid hormones in man.

Steroid Hormone Catabolism

Hormonally-active steroids are hydrophobic substances and the catabolic reactions not only inactivate, but also render the steroid molecule more hydrophillic. The catabolic reactions occur mainly, although not exclusively, in the liver. To render the steroid molecules even more water soluble, the majority of the catabolic products of secreted steroid hormones are conjugated as glucuronides and sulphates before being excreted in the urine. The steroids containing the 5-ene-3β-hydroxy configuration are usually found as sulphates, while the majority of the other steroids are found as glucuronides (Table lii).

The metabolism of the neutral steroids, progesterone, androgens and corticosteroids is presented in a simplified form in Fig. li.

One of the structural features of the majority of the active secreted steroid hormones - with the exception of the estrogens - is a

Table 1i

Mean Secretion Rates of Steroid Hormones in Man

Steroid	Secretion Rate (mg/day) Men	Women	
Cortisol	20.00	17.00	
Corticosterone	2.30	-	
Doexycorticosterone	0.24	0.50	
11-Deoxycortisol	0.34	-	
Aldosterone	0.19	0.14	
Pregnenolone	9.00	-	
17-OH Pregnenolone-S	5.00	-	
Progesterone	0.60	2.90	
Testosterone	6.90	0.35	
Epi-testosterone	0.22	-	
5α-Dihydrotestosterone	0.32	0.075	
4-Androstenedione	1.80	3.40	
DHEA	3.00	0.70	
DHEA-S	5.90	7.70	
Estrone	0.11	0.11	(FP)
Estrone-S	0.77	0.095	(FP)
		0.183	(LP)
Estradiol-17 β	0.06	0.116	(FP)
		0.205	(LP)

FP = follicular phase
LP = luteal phase
S = sulphate

Reproduced from Makin (1975)

Table lii

Principal Urinary Metabolites and Conjugates of Some Steroids

Steroid	*Principal metabolites*	*Principal conjugate present*
Cortisol and cortisone	tetrahydrocortisol(THF)*, tetrahydrocortisone	G
	(THE)*, cortols and cortolones,	G
	11-oxygenated-17-oxosteroids e.g.	
	11-hydroxyandrosterone,	G, S
	cortisol	unconjugated
Corticosterone	tetrahydrocorticosterone (3α,11β,21-trihydroxy-5β-pregnan-20-one),*	G
	corticosterone	unconjugated
DOC	tetrahydroDOC (3α,21-dihydroxy-5β-pregnan-20-one)*	G
11-Deoxycortisol	tetrahydro-11-deoxycortisol	G
21-Deoxycortisol	3α,17-dihydroxy-5β-pregnan-20-one*	G
Aldosterone	tetrahydroaldosterone*	G
6β-Hydroxycortisol	6β-hydroxycortisol*	unconjugated
Pregnenolone	5-pregnenediol,	S
	pregnanediol	G
17-Hydroxypregnenolone	5-pregnenetriol	S
Progesterone	pregnanediol,	G
	3α-hydroxy-5β-pregnan-20-one	G
17-Hydroxyprogesterone	pregnanetriol,	G
	3α,17-dihydroxy-5β-pregnan-20-one	G
Testosterone	androsterone, aetiocholanolone,	S, G
	epi-androsterone,	S, G
	testosterone	G
4-Androstenedione	androsterone, aetiocholanolone,	S, G
	epi-androsterone,	S, G
	testosterone	G
DHAsulphate	DHAsulphate	S
DHA	DHAsulphate,	S
	androsterone, aetiocholanolone, epi-androsterone	S, G
Oestradiol-17β	oestradiol-17β, oestrone, oestriol	G
Oestrone	oestradiol-17β, oestrone, oestriol	G
Oestriol	oestriol	G

* Indicates unique metabolite

G = glucuronide; S = sulphate.

12

Reproduced from Makin (1975)

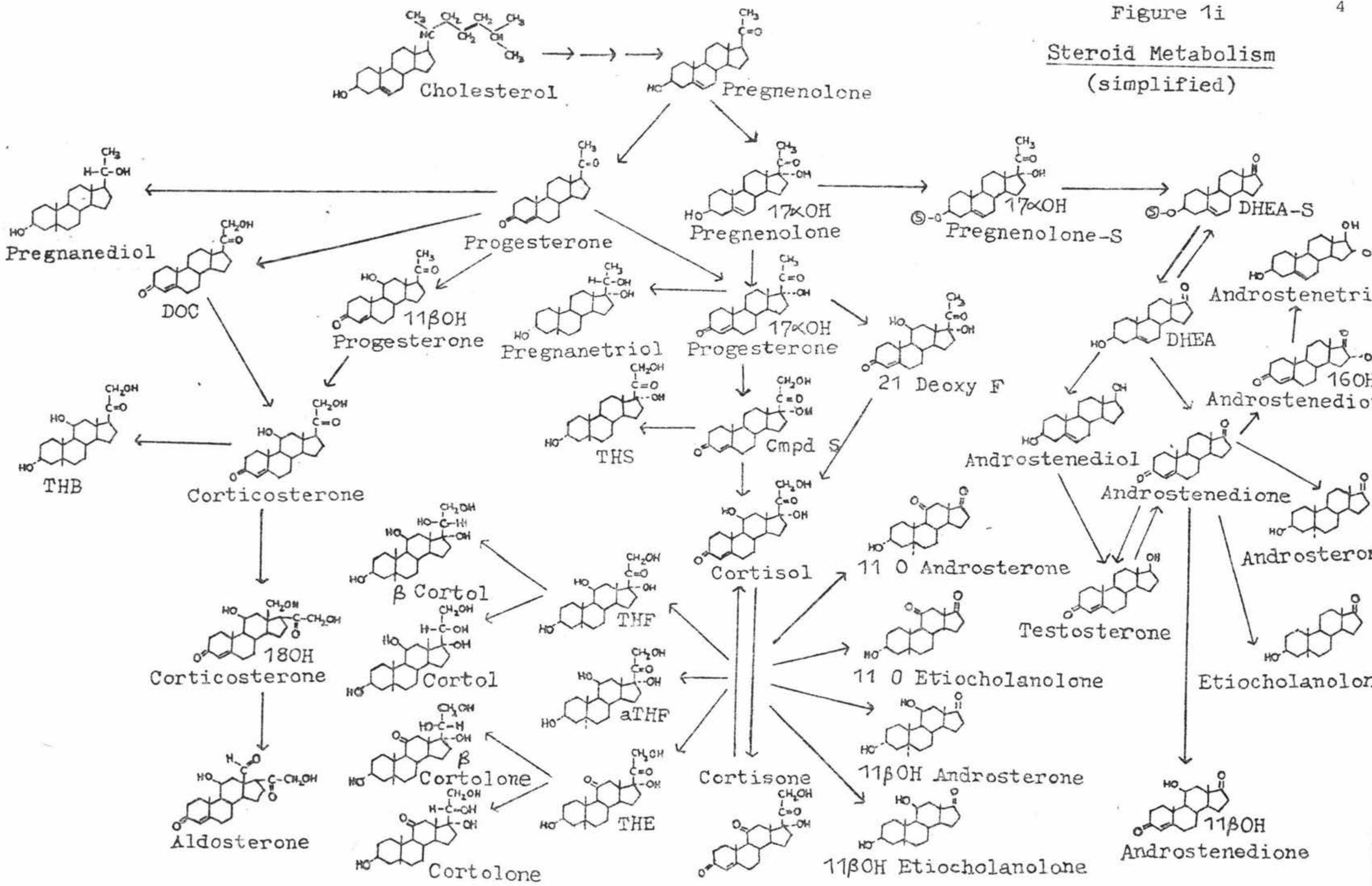


Figure 1i
Steroid Metabolism
(simplified)
Cholesterol
Pregnenolone
Progesterone
17αOH
Pregnenolone
17αOH
Pregnenolone-S
DHEA-S
Pregnanediol
DOC
11βOH
Progesterone
Pregnanetriol
17αOH
Progesterone
21 Deoxy F
DHEA
Androstenetri
16OH
Androstenedio
THB
Corticosterone
THS
Cmpd S
Androstenediol
Androstenedione
Cortisol
11 O Androsterone
Androsteron
β Cortol
THF
Testosterone
18OH
Corticosterone
Cortol
11 O Etiocholanolone
Etiocholanolon
aTHF
β
Cortolone
Cortisone
11βOH Androsterone
Aldosterone
THE
Cortolone
11βOH Etiocholanolone
11βOH
Androstenedione

4-ene-3-oxo configuration in the A ring. Reduction of the A ring and the 3-oxo group, finally resulting in the production of tetrahydro-reduced inactivated compounds with a 3-hydroxyl, takes place in two stages. Complete reduction of the 4-ene-3-oxo in this way could produce four isomeric metabolites, the 3α-OH steroids predominate in man. Further reduction of the 20-oxo group in the side chain of the cortico-steroids occurs. 17-Hydroxylated C-21 steroids can be converted by oxidative side chain cleavage into C-19 17-oxosteroids.

Steroids reduced at C-3 and C-20,as well as in ring A, form the bulk of the metabolites of corticosteroids, but in addition to the major catabolic routes, other more minor reactions may occur. Oxido-reduction of the 11-oxo function, further hydroxylation at C-6β or at C-16α, and reduction at C-20 without reduction in ring A or at C-3 may occur.

Methods for Measurement of Urinary Steroids

Although the initial separation and concentration of steroids is aided by the fact that the unconjugated compounds are extractable with organic solvents, quantitation of individual metabolites by classical methods has involved many stages of separation using partition, adsorption, thin-layer and paper chromatography and chemical fractionation. Clean separations are necessary due to the low specificity of the colourimetric and fluorimetric methods available for the final measurement.

When attention is focused on only one or two steroids, the technique of radioimmunoassay has the advantages of simplicity, sensitivity and a specificity which often avoids the need for sample purification. On the other hand its applicability is limited by the availability of specific antisera and radiolabels for each steroid to be measured.

For profiling of steroid samples the above methods are inapplicable either because they are too specific (eg. radioimmunoassay) or they lack specificity (eg. colourimetric tests). When a large number of urinary steroids are to be assayed simultaneously, GLC methods provide virtually the only qualitative and quantitative approach.

Steroid Profiling by GLC

Prior to the use of Support Coated Open Tubular (SCOT) capillary columns, conventional packed columns were used to separate mixtures of biologic steroids. A great deal of this work was performed by

E. C. Horning and co-workers. Urinary Steroids (Horning and Horning, 1970) documents methods prior to the introduction of capillary columns. In 1960 VandenHeuvel, Sweeley and Horning prepared thin film columns (1-3% of phase), with a thermally stable liquid phase (SE-30, a methyl siloxane polymer) that could be used for the separation of a number of steroids. The development of the hydrogen flame ionisation detection system around 1958, with a high degree of sensitivity greatly aided the development of the current systems used for steroid profiling by GLC.

Metal capillary columns have long been used, particularly in the petroleum industry, but were found to destroy many biologic samples including many steroids, necessitating the use of all-glass apparatus.

Grob overcame the initial problems of the liquid phases forming microdroplets when heating and cooling, by etching and modifying the glass surface. The use of these columns for steroid work is evaluated by Vollmin and Curtius (1971) and Ros and Sommerville (1971). The deposition of an adherent film of particulates to form a "rough wall" (thereby increasing the amount of liquid phase) forms the basis of the SCOT column (German et al, 1973).

A principal advancement in SCOT capillary columns was discovered by German and Horning (1973), by the development of a procedure for depositing a film of SE-30 (or other phase) on silanized glass. The film contained fine particles of silanized silicic acid (Silanox), suspended in the phase. The surface of the glass was therefore modified (not etched) by silanization in order to decrease the absorption of organic compounds on the glass surface. A modified injection splitter system was developed for this system, which had a short precolumn in the injector. This insured gas phase splitting rather than aerosol splitting, as well as trapping non-volatile substances which are often present in derivatised biochemical samples (German and Horning, 1972; German et al, 1973).

One of the SCOT column advantages lay in its ability to accept and resolve large samples of complex mixtures containing compounds present in widely varying amounts. Columns ranging from 30 to 60 meters in length and with internal diameters of 0.3 mm show a resolving power of 50-150,000 theoretical platés (Pfaffenberger and Horning, 1977).

With the commercial production of SCOT capillary columns, the investigations of urinary steroid profiles accelerated. Although the methods for the preparation of the samples for GLC did not change

radically, many minor changes have now resulted in a "cleaner" chromatogram with improved resolution (Luyten and Rutten, 1974; Pfaffenberger and Horning, 1975; Schomburg et al, 1976; Shackleton and Honour, 1976; Fantl and Gray, 1977; Pfaffenberger and Horning, 1977; Phillipou et al, 1978).

The development of gas chromatography-mass spectrometry (GC-MS) and gas chromatography-mass spectrometry-computer (GC-MS-CO) coupling has greatly enhanced the study of biologic steroid samples (Axelson and Sjovall,1974; Sjovall, 1975; Setchell et al,1976; Stillwell et al, 1973).

Factors Affecting Steroid Excretion Rates and Patterns

Various physiological factors, endogenous or exogenous substances may interfere with either synthesis, secretion or metabolism of the steroids and may quantitatively or qualitatively change the patterns of excretion of the hormone metabolites. Many factors affecting the excretion of steroid hormones in urine are summarized by Vestergaard (1978), encompassing body characteristics, drugs, stress, debilitating diseases and disorders.

Urinary total 17-oxosteroid excretion has been shown to increase in both sexes from infancy to a maximum in the twenties, with a slow decrease thereafter (Kaiser et al, 1964). Different absolute values have been observed between males and females, but it has become clear that most of the difference is due to man being bigger and heavier, as well as having more of an active cell mass than woman. Lindbolm (1973) found the excretion of 17-oxosteroids and the secretion rate of cortisol to be practically identical expressed per kilogram for men and women. Work by Pfaffenberger and Horning (1977) indicates two different types of steroid metabolism in premenopausal females, one group displaying profiles closely resembling those of males.

The main changes in neutral steroid excretion during pregnancy is the appearance of large amounts of progesterone metabolites. The diversity of these metabolites did not become apparent until GC-MS methodology allowed their identification (Eriksson and Gustafsson, 1977).

Two clinical applications of GLC profiling of urinary steroids are the detection of ovulation by changes in progesterone metabolites and the detection of "missing enzymes".

The variation that occurs in healthy subjects under living

conditions is obviously of great practical importance because it enters into the evaluation of the significance of changes induced by any treatment or pathological change. The variability encountered in experimental collection of excretion data for steroids in urine can be divided into three catagories: that due to methodological variability; that due to periodicity or rhythmic change; and a leftover "random" variability. The main periodicity that has been revealed by studies of steroid excretion in man is the 24 hour periodicity and approximate monthly periodicity, particularly as it relates to the menstrual cycle. Seasonal, semi-annual and annual periodicity have also been recorded.

Diurnal Variation of Steroid Excretion

In 1943, 24 hour periodicity in the excretion of urinary total 17-ketosteroids was described, closely followed by reports of periodicity in the excretion of reduced corticosteroids (Pincus et al, 1948). Vestergaard and Leverett (1957) observed a double wave excretion pattern for total 17-oxosteroids when two hour sampling was utilised. Toccofondi et al (1970) found a circadian rhythm for DHEA, An and Et with peak excretion between 6 am and 2 pm. Cavalleri et al (1974) reported peaks for these same compounds between 8 am and 1 am and a clear circadian rhythm. Molino et al (1973) comparing two hour samples taken at midnight with similar samples taken between 8 am and 10 am found significant differences between the samples for most 17-oxosteroids (An, Et, 11KAn, 11OHAn), but not for 11KEt and 11OHEt. Both THF and THE were significantly higher in the morning, but THS and Pt were not.

A circadian rhythm has been shown to exist for free cortisol in urine, with a peak at 7-8 am and a second smaller maximum in the afternoon. Beale and Tyrrell (1974) found a marked variability in the excretion of cortisol metabolites (THE, THF, aTHF, cortols and cortolones) when one hour and three hour collection periods were used and marked differences in percentage distribution between metabolites at different times of the 24 hour period. A distinct diurnal rhythmicity was found in these studies also, with highest values for all metabolites in the early morning hours and a nadir in the middle of the night.

Many pathological states can disturb the normal periodicity or introduce periodicity not normally present, such as Cushing's Syndrome, blindness and depressive states (Vestergaard, 1978).

Effect of Nutritional Status

Nutritional factors not only directly exert important influences on the excretion of neutral steroids *per se*, but also indirectly through the effect disease may have on the nutritional status of a subject. Obesity appears to affect values of corticosteroids excreted in some subjects, dieting and starvation can reduce some of the 17-oxosteroids and, if pronounced, the excretion of all neutral steroids. Unless carried to exhaustion, exercise has little effect on the excretion of neutral steroids.

Effect of Stress

Stress of different kinds can very significantly affect steroid secretion, seen particularly in the increases of urinary steroid excretion of patients admitted to hospital. Psychological stress, including anxiety and the confrontation with difficult and novel life situations have been shown to activate the adrenocortical system and bring about increases in the excretion of neutral steroids in urine (Vestergaard, 1978).

Early studies using "cold" stress, by exposing the subjects to cold water (Kuhl et al, 1952) showed a marked increase in the excretion of total 17-oxosteroids. Later work as reviewed by Collins and Weiner (1968) has given ambiguous results as far as the excretion of neutral steroids are concerned both after cold and heat stress. Probably only severe conditions will activate the adrenal cortex to a marked degree.

Effect of Pharmacological Agents on Hepatic Steroid Metabolism

A number of non-hormonal drugs, through their action on microsomal enzymes in the liver, stimulate both the hydroxylation of other drugs and of steroids. Treatment of humans with phenobarbital, antpyrine and phenylbutazone markedly stimulates the metabolism of cortisol to 6βhydroxycortisol (Conney et al, 1973).

A variety of hormonal drugs, both naturally occurring and synthesized, have an effect on steroid metabolism and excretion. When cortisol and cortisone are administered, there is a suppression of 17-oxosteroid secretion due to negative feedback suppression of ACTH release. The synthetic analog dexamethasone has a similar effect, resulting in a decrease of 17-hydroxycorticosteroid excretion (Liddle, 1960).

Effect of Hepatic Redox Ratio

It is well documented that the NADH : NAD^+ ratio increases in the liver during ethanol oxidation (Forsander et al, 1958). Cronholm et al (1971) assumed that this increase would influence the reductive metabolism of steroids. Investigations along two main lines were carried out;

(1) analysis of the steroid redox couples in blood and urine before and after ethanol administration, and

(2) analysis of the incorporation of deuterium via reduced pyridine metabolites into steroids during the metabolism of $^2H_2$-ethanol.

When ethanol (0.1-1 g/kg body weight) is given to humans there is a rapid increase of the concentration of monosulphates of 5-androstene-3β,17β-diol, 5α-androstan-3α,17β-diol and 5α-androstan-3β,17β-diol. In several cases a simultaneous decrease of the corresponding 17-ketosteroids is observed.

Only steroids with a free 17β-hydroxyl group are increased by alcohol ingestion, indicating direct formation from the corresponding ketosteroid. The increase is very rapid and the rate of change appears to be limited by the rate of ethanol absorption upto a particular level.

The findings indicate that 5-androstene-3β,17β-diol sulphate and DHEA are in an equilibrium determined by the NADH : NAD^+ ratio at a site of interconversion in the liver.

The monosulphates of pregnenolone and 5-pregnen-3β,20α-diol may form another couple, however the ratio between the concentrations of these steroids changes less than the above case.

High concentrations of sulphates of pregnanolone and pregnanediol isomers are found in pregnancy plasma. For this reason ethanol has been given to pregnant subjects in order to study the effect on the ratio between 20-hydroxyl and 20-ketosteroid sulphates viz 5-pregnen-3β, 20α-diol and 3βhydroxypregnene-20-one. Although changes were small, the 20α-hydroxy/20-ketosteroids ratio consistently increased after ethanol administration (Cronholm et al,1969).

Effect of Ethanol on Steroid Secretion

In a study carried out by Mendelson et al (1971), a positive correlation was observed between ascending blood alcohol levels and

increase in serum cortisol values for the two subjects studied in an eleven day alcohol administration experiment. When ethanol dosage was progressively decreased, a parallel decrease occured in serum cortisol levels. This data is in agreement with animal studies, indicating that ethanol administration is associated with stimulation of adrenocortical secretory activity. This may be due to;

(1) Ethanol enhancing secretion of corticotropin via activation of neural pituitary circuits. Animal studies indicate hypophysectomized animals do not show an adrenocortical response after ethanol was administered. Humans with pituitary lesions do not have an increased serum cortisol level after ethanol administration, so it is likely ethanol stimulation of adrenocortical activity in man involves the neural-pituitary mechanism.

(2) The high serum cortisol levels may result from impairment in the catabolic metabolism of cortisol. Hepatic function appeared normal.

Margaff et al (1967) found cortisol secretion rate and excretion of total 17-hydroxycorticosteroids indicated adrenocortical function in alcoholic patients was not impaired, although excretion of the cortisol metabolites was significantly decreased. The findings suggested alterations in cortisol metabolism, rather than a disorder of adrenocortical function in the alcoholic patients.

Effect of Alcoholic Cirrhosis on Steroid Metabolism

The triad of alcoholic cirrhosis testicular atrophy and gynaecomastia was first observed by Silvestrini in 1926 (Fabre et al, 1973). He suggested that cirrhosis caused an endocrine imbalance, increasing the levels of circulating estrogens, producing testicular atrophy and gynaecomastia. But the total estrogen excretion and ratios of estrogens in patients with liver cirrhosis have been found to be normal.

Southren et al (1973) demonstrated a decreased plasma level and production rate of testosterone in alcoholic cirrhotic livers. It was observed that conversion of testosterone to androstenedione was accelerated and this accounted for the decreased levels of testosterone in plasma.

Galvao-Teles (1973) from their work suggested that a fall in unbound androgens rather than a rise in the unbound estradiol in plasma, leads to hypogonadism and gynaecomastia in man with chronic liver disease, especially those with alcoholic cirrhosis.

There is a marked increase in plasma level and production rate of androstenedione in men with cirrhosis of the liver. This steroid has been shown to contribute significantly to circulating estrogens in those patients. A seven fold increase in peripheral production of testosterone in cirrhosis was observed by Gordon et al (1975). There is a three fold increase in the conversion of plasma testosterone to plasma estrone, and an increased contribution of androstenedione to estrogens.

Alcohol was administered to normal male volunteers for periods of up to four weeks, resulting in an initial dampening of episodic bursts of testosterone secretion followed by decreases in both mean plasma testosterone concentration, production rate of testosterone and an increased metabolic clearance rate of testosterone in most subjects. This was due to a combined effect of a decreased plasma binding capacity and increased hepatic testosterone A ring reductase activity (Gordon et al, 1976).

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

β-Glucuronidase was supplied by Sigma Chemical Co., St Louis, U.S.A. as a crude extract from Helix Pomatia, containing approximately 100,000 Fishman Units of glucuronidase activity/ml (1 Fishman Unit defined as hydrolysing 1.0 gram phenophthalein glucuronide per hour at pH 5.0 and 37°C); and approximately 3,700 Molar Units of arylsulphatase activity/ml, using nitrocatechol as substrate at pH 5.0 and 37°C.

Androsterone, dehydroepiandrosterone, 11 ketoandrosterone, pregnanediol, tetrahydrocortisone, tetrahydrocoticosterone, tetrahydrocortisol, cortolone, β cortol, β cortolone and cortol were supplied by Mann Research Laboratories, New York, U.S.A.

Etiocholanolone, 11 ketoetiocholanolone, 11 hydroxyandrosterone, 11 hydroxyetiocholanolone, pregnanetriol,androstenetriol and THS were supplied by Sigma Chemical Co., St Louis, U.S.A.

Water was glass distilled and deionised by passage through an ion exchange resin.

Methoxyamine hydrochloride was prepared by Dr. D. R. K. Harding, Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, by the method of Hjeds (1965) and was stored dessicated.

All solvents were redistilled before use.

Pyridine was of analytical grade, supplied by Ajax Chemicals Ltd, Sydney, Australia.

Lipidex 5000 was supplied by Packard Instrument Co., Zurich, Switzerland.

Amberlite XAD-2 was supplied by Rohm and Hass Co., U.S.A.

Hydrocarbons were supplied by Applied Science Laboratories Inc., Penna., U.S.A.

Silyl 8 was supplied by Pierce Chemical Co., Rockford, Illinois, U.S.A.

METHODS

Gas Liquid Chromatograph

A Varian 2700 series gas liquid chromatograph was adapted to accept a glass SCOT capillary column (GSC SE/30, Scientific Glass Engineering Pty. Ltd, Melbourne, Australia). The column was of deactivated, neutral borosilicate glass, with a "Chromosorb" based support material, silanized and coated with Silicone SE-30.

During the course of this work, three SCOT columns were used. For the majority of the urinary steroid profiling, a 40 meter column was used showing an ex-factory efficiency of 40,000 theoretical plates. Two series of profiles were done on a 27 meter column with loss of a resolution of the THB, THF, aTHF and αC'one peaks in some instances.

Installation of a new column required conditioning at 200°C for 36 hours. Column bleed was then minimised by holding oven temperature at 280°C for 4 hours. It was found necessary to perform this latter step each week.

To minimise adsorption problems of polar samples, silation of glass-lined tubing (GLT) and the SCOT column was often necessary. Usually ten succesive 2 μl injections of Silyl 8 at 2 minute intervals with oven temperature at 200°C was utilised. It was necessary to clean the injector and detector after treatment with Silyl 8.

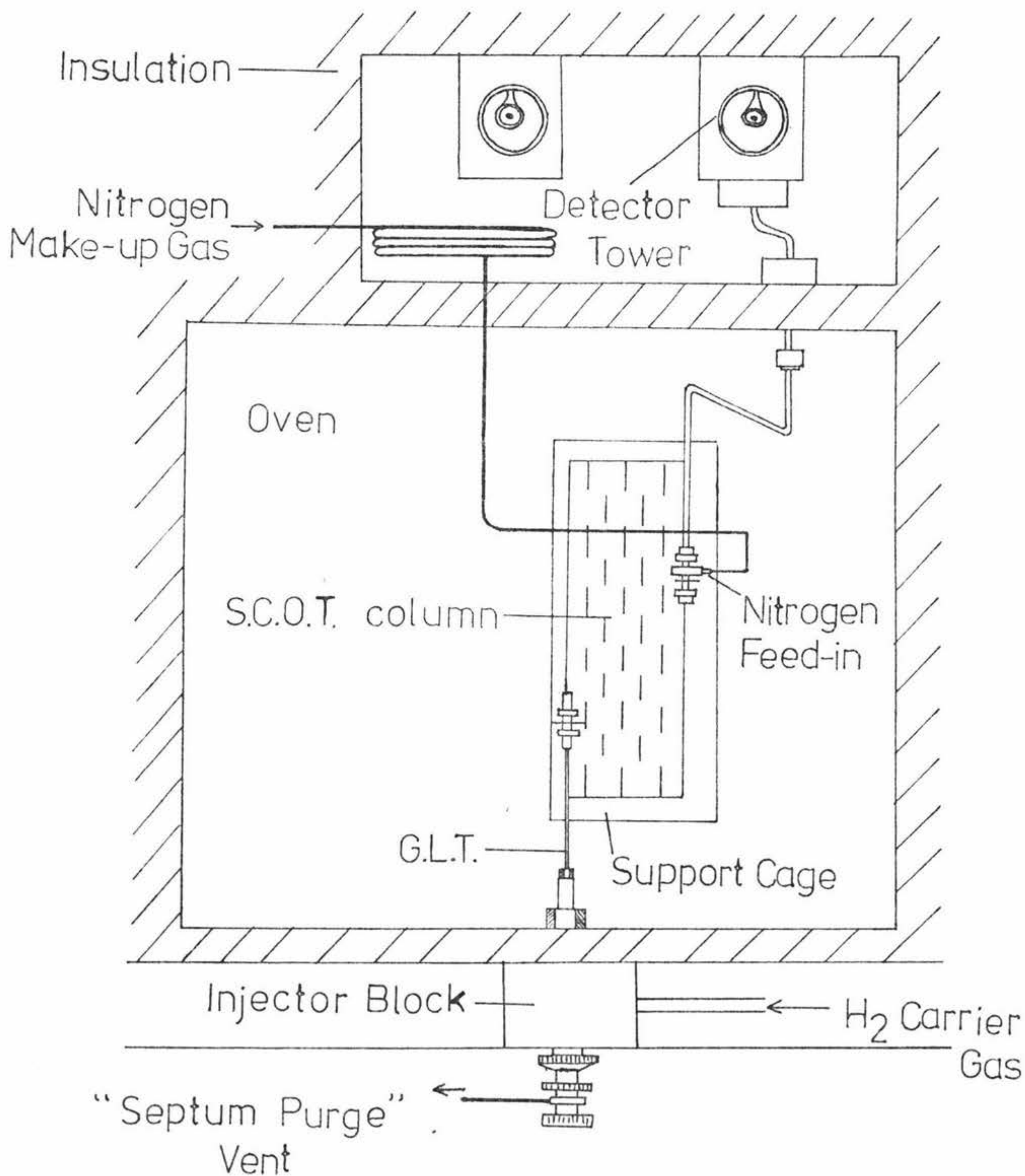
The column was connected to the injector and detector by stainless steel GLT, 0.5 mm internal diameter (ID). The column was housed in an SGE metal support cage. High temperature graphite ferrules were used to obtain airtight connections at all junctions except where the GLT enters the detector. Here teflon tape ensured an airtight connection, held in place by a spring loaded disc (Fig 2i).

Flow of hydrogen carrier gas was controlled by a two stage reduction. Initial reduction was achieved with a Negretti and Zambra precision gas pressure regulator, and secondary control using the GLC inbuilt gas flow controller. Carrier gas flow was maintained at approximately 6.5 ml/min (recorded with oven temperature at 200°C). Nitrógen make-up gas was fed in at the end of the column, where it couples on to the GLT to the detector. This is to compensate for the low carrier gas flow, so the samples are fed into the detector at a faster rate than the carrier gas

Figure 2i

Varian 2700 GLC with SCOT Column

Top View



allows and the peak broadening effect of dead space in the detector line is minimised. Nitrogen gas produced maximum sensitivity with a flow of approximately 60 ml/min. The copper tube carrying the nitrogen was coiled six times in the detector block to heat the nitrogen before joining the column effluent.

Air and hydrogen flow rates were maintained at approximately 300 and 40 ml/min respectively. All gas flows were measured using a bubble meter: carrier gas and nitrogen flow rates were measured from the flame assembly tip whilst the hydrogen and air flow rates were measured via the built-in flow diverters on the GLC.

Detector and injector block temperatures were maintained at approximately 300°C and 280°C respectively. For urinary steroid profiles the initial oven temperature was 200°C and programmed at 1°C/min from the time of injection. The flame ionization detector was used at a sensitivity of 10^{-11} amps/mv.

Injection System

A splitless injector system (Fig 2ii) was employed with a septum purge adapted by addition of a needle valve so that the purged gas flow could be maintained at approximately 2 ml/min. The injection system required regular weekly cleaning by washing with water,acetone and hexane by suction, and blowing dry with nitrogen. It was important that this cleaning was done regularly, since the injector proved to be a common cause of loss of reproducibility. Minute pieces of graphite ferrule and septum became lodged in the GLT of the injector system, and were responsible for the loss of reproducibility.

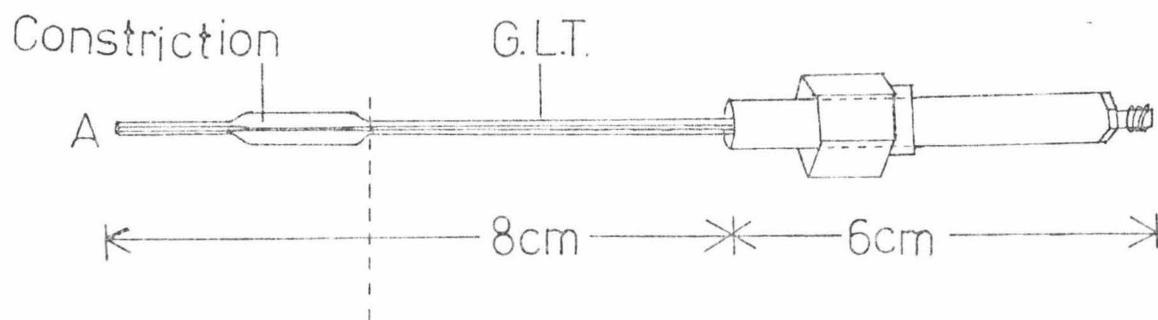
Septa were replaced prior to injector system being cleaned, and the injection hole made in the septum, so if any fragments became lodged in the system they could be removed when cleaned.

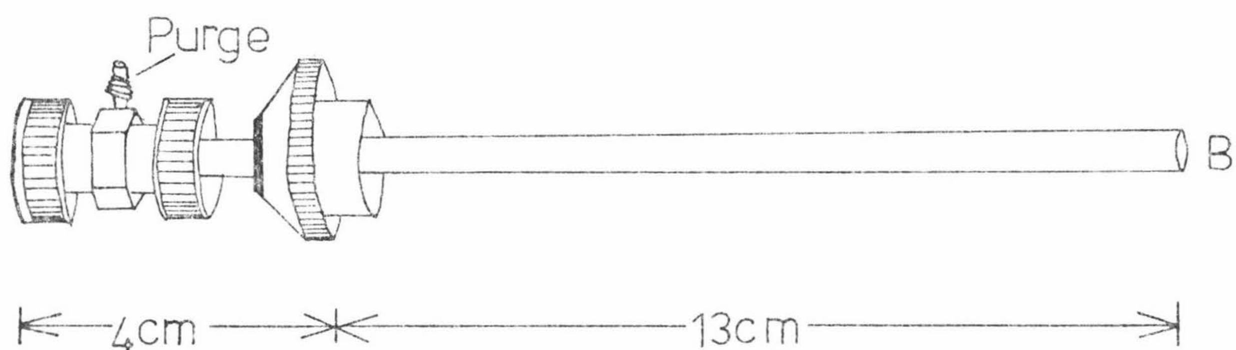
A 5 µl SGE syringe with guided plunger and 7 cm needle (0.12 mm ID) was used for sample injection.

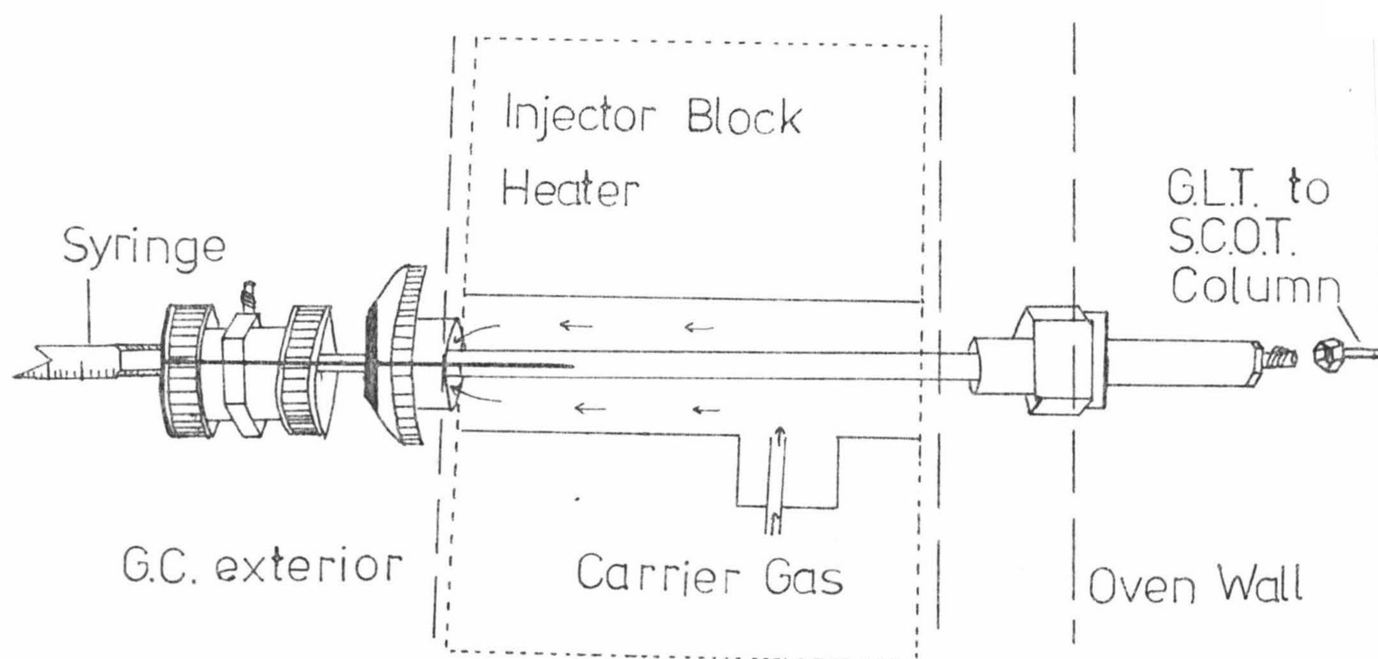
Prior to the splitless injector, a splitter injector was fitted to the GLC but was found to be unsatisfactory. The split ratio was inconsistent and although applying a back pressure to the vent line using a mercury column impróved the consistency of the split ratio, it did not bring it up to a satisfactory standard.

Figure 2ii

Injector







Detector

The flame ionization detector was used unmodified and cleaned at the same intervals as the injector. The detector was dismantled, the cathode probe, anode probe, flame assembly tip and collector cylinder were cleaned by sonication in water for one hour. The tower, detector base and detector cap were cleaned with acetone.

Integrator

Peaks were automatically intergrated by a Varian 477 integrator with digital base-line correction. Peak areas were printed out by a Victor Digimatic Printer. The settings for digital base-line correction, peak width half height and slope sensitvity were kept constant throughout the entire project.

Recorder

Peaks were recorded by a W + W Electronic Basel Chart Recorder with 1 mv full scale sensitivity.

Human Urinary Steroid Profiles

Methods of extraction and derivatisation of urinary steroids were based principally on those used by Phillipou et al (1978). All samples were carried out in duplicate as recommended by Shackleton and Honour (1976). In the following description of the final procedure used, numbers in parentheses refer to footnotes at the end of this section.

Urine Collection

Urine samples were collected in glass bottles, containing 15 grams boric acid preservative, (about 1 gram for 2 hours collection). The volume of each sample was recorded when collected.

If not processed immediately, urine was frozen in 250 ml glass bottles stoppered with aluminium foil lined corks. Bottles were half filled and frozen on an angle to prevent cracking.

Hydrolysis

A 10 ml aliquot of urine was pipetted into a 100 ml Erlynmeyer flask and diluted to 15 ml with distilled deionised water. Urine was transferred to XAD-2 columns (1), and washed into the column with three 5 ml aliquots of water, and run down to the top of the column with a drip rate of about 2 per second, this eluate was discarded.

100 ml Erlynmeyer flasks were rinsed with methanol and the steroids and steroid conjugates were eluted into them with 45 ml of methanol. The columns were allowed to run totally dry. Methanol was then evaporated on a rotary evaporator at 60°C.

To the flasks, 5 ml of acetate buffer (2), pH 4.4, was added and swirled. 670 µl of β-Glucuronidase (3) enzyme was added and incubated overnight for sixteen hours in a waterbath at 49°C. The flasks were stoppered to minimise evaporation.

Extraction

The hydrolysed extract was transferred into a 100 ml separating funnel with 15 ml ethyl acetate and shaken well three times for a

total of 2 minutes. When layers separated, the lower aqueous phase was run back into the Erlynmeyer flask and extracted again with a second 15 ml ethyl acetate. The aqueous phase was then run off and discarded. 15 ml of 10% $NaHCO_3$ was added to the combined extracts in the funnel, shaken for 2 minutes, releasing CO_2, then the lower aqueous phase run off and discarded (4).

Ethyl acetate extract was then poured into a 125 ml conical flask containing about 10 grams of anhydrous Na_2SO_4 and swirled. 100 µl of internal standards (100 µg/ml in hexane), n-tetracosane (C24) and n-dotriacontane (C32) was added at this stage.

The extract was poured into a 250 ml round bottomed quick-fit flask, excluding as much Na_2SO_4 as possible. The drying agent was washed with a further 25 ml ethyl acetate and poured into the same flask. The ethyl acetate was then removed on a rotary evaporator at $40^{o}C$.

The dry extract was then transferred to 10 ml glass stoppered quick-fit tubes (5) with three 2 ml aliquots of $CHCl_3$ and evaporated to dryness in a current of dry nitrgen with the tubes in a warm sandbath.

Derivatisation

To the free steroid extract, 50 µl of methoxyamine hydrochloride in pyridine (100 mg/ml) was added (6), using a Gilson adjustable pipette. The tube was kept at 60° for 30 minutes in a heating block (Multi-blok Heater, Lab-line Instruments, Melrose Park, Ill., U.S.A.) with the stopper held in place by a rubber band. Pyridine was then blown off with dry nitrogen, in a warm sandbath, leaving an oily residue.

50 µl of TSIM was then added (6) from a sealed vial using a Hamilton syringe, the tubes restoppered and heated for 2 hours in the heating block. The tubes were then removed and allowed to cool to room temperature.

Lipidex 5000 columns (7) were set up before completion of the TSIM treatment. The Lipidex 5000 in Lipidex solvent (cyclohexane: pyridine: dimethoxypropane: HMDS 194: 2: 10: 4) was placed in the column (16 x 1 cm), with a plug of sand in the bottom, using a pasteur pipette. Excess Lipidex was sucked out after settling, leaving about 8 cm of Lipidex 5000 in the column.

The derivatised residue was taken up in a 0.5 ml of Lipidex solvent, vortimixed and applied to a Lipidex column with a pasteur pipette. The eluate was collected in 10 ml conical quick-fit tubes. When the sample had entered the column, a further 1 ml of Lipidex solvent was added to the derivatisation tubes, vortimixed and put onto the columns, which were then allowed to run dry.

Solvent was then blown off with dry nitrogen over a warm sandbath and then derivatised steroids taken up in 120 µl of GLC solvent (hexane; pyridine; HMDS 98: 1: 1). 1 to 2 µl of the derivatised extract was used for injection into the GLC.

Footnotes

(1) XAD-2 Columns

"Clean-up" procedures are often employed for urine before or after hydrolysis, to remove phospholipids and other acidic emulsifying agents. The use of Amberlite XAD-2 is first described by Bradlow (1968), other resins used are encompassed in Horning and Horning (1970) and Luyten and Rutten (1974).

XAD-2 resin was conditioned by the method of Setchell (1976). 400 grams of resin was washed with stirring for 1-2 hours in a 2 litre beaker, with the following succession of solvents.

2 litres 2M NaOH

2 litres distilled deionised water

2 litres 2M HCl

2 litres distilled deionised water

2 litres acetone

2 litres ethanol

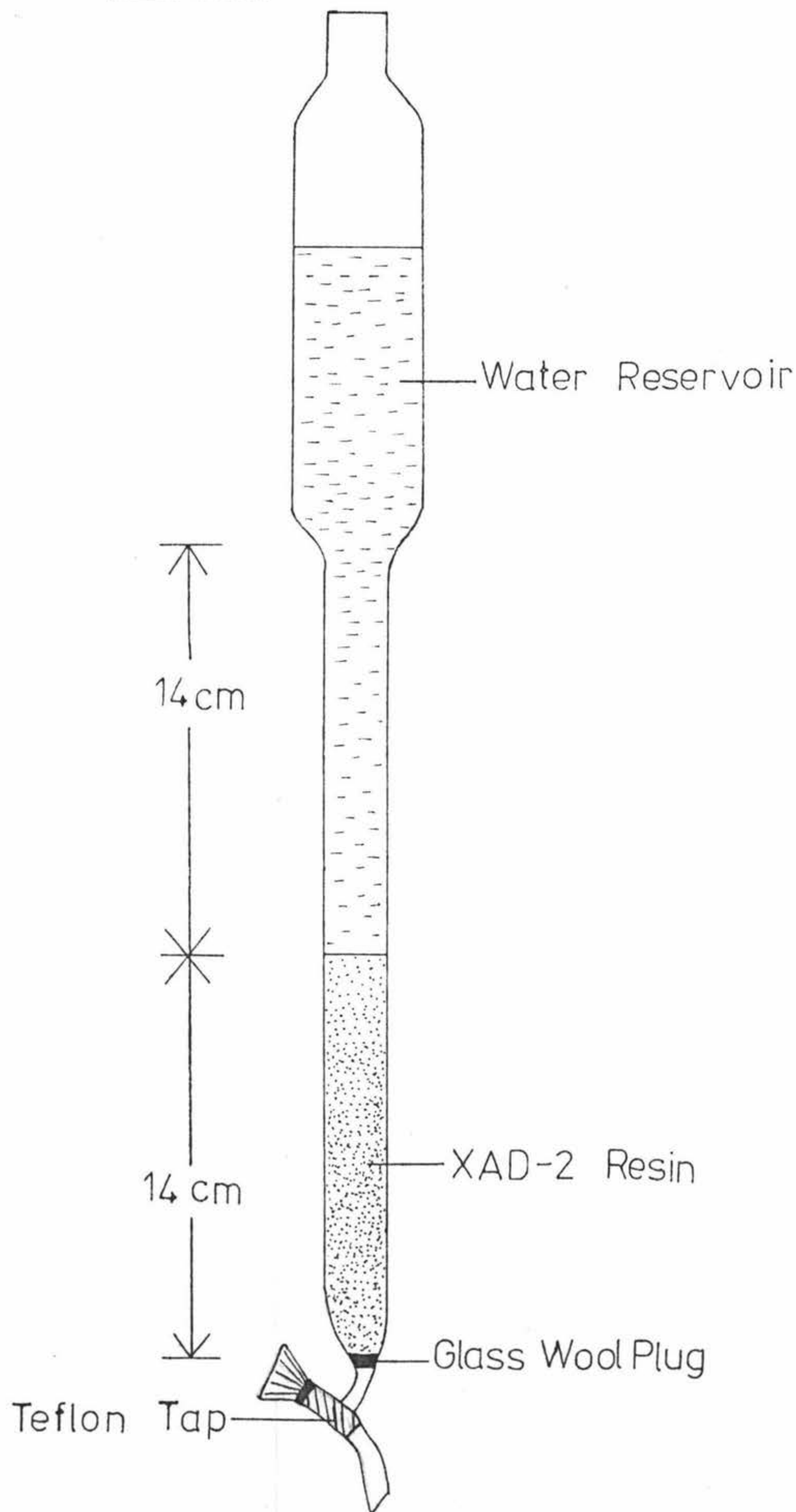
2 litres distilled deionised water

The resin was then boiled for 4 hours in water, rinsed with water and fines removed off the top by suction (a pipette connected to a water vacuum). Each solvent was removed largely by suction and the remainder by buchner funnel. The conditioned resin was stored in water.

The XAD-2 columns were packed as shown in Fig. 2iii. Regeneration of the resin was achieved by washing with 60 ml of methanol, then water, during which columns were repeatedly inverted until air bubbles were removed. They were then washed through with a reservoir of water and left filled with water.

Leunissen and Thijssen (1978) investigated the accuracy and reproducibility of essential steps in sample preparation, mostly in experiments where subjects had been administered ^{3}H-labelled cortisol. Steroids, eluted from XAD-2 resin, had a mean recovery of $94.5 \pm 5.5\%$. In the elution of steroids and steroid conjugates 40 ml of methanol was used, the use of 50 ml did not increase the recovery. However, when 15 ml of urine instead of 10 ml were passed through the XAD-2 column, significantly lower recoveries were found (87.5%). Care must therefore be taken to avoid overloading the column, particularly with urine samples of patients excreting abnormally large quantities of steroid conjugates.

Figure 2iii



(2) Acetic Acid Buffer, pH 4.4, 0.2 Molar

11.55 ml of acetic acid was added to 800 ml of water. Using NaOH this was adjusted to pH 4.4. The volume was made upto 1 litre and the buffer stored in stoppered bottles at 4°C.

(3) Hydrolysis

The two most widely used methods of hydrolysing the glucuronide and sulphate conjugates are boiling under strong acidic conditions and enzymic hydrolysis. The boiling acid method is not recommended since many steroids undergo structural alterations under these conditions (Horning and Horning, 1970). The preferred enzymic hydrolysis procedure is that utilising glucuronidase (a preparation from Helix Pomatia, containing both sulphates and β-glucuronidase). Hydrolysis conditions vary from one laboratory to another (Horning and Horning, 1970; Luyten and Rutten, 1974; Pfaffenberger and Horning, 1975; Shackleton and Honour, 1976; Fantl and Grey, 1977; Phillipou et al, 1978).

Wide differences in rate exist for the hydrolysis of steroid conjugates; the rate of enzymic hydrolysis of sulphates of 5α-H, 3α-ol steroids may be very slow, for example, and low yields of these steroids may result. The enzymic hydrolysis procedure is the least satisfactory step in carrying out quantitative analytical procedures for steroids (Horning and Horning, 1970).

For absolute qualitative analysis of steroid profiles enzymic hydrolysis should be followed by solvolysis, since sulphatases present in Helix Pomatia juice are not able to hydrolyse sulphate conjugates of a 3α-OH group in 5α steroids, and of 17 and 20 hydroxyl groups. In human urine these conjugates were found to be excreted in varying amounts. For ^{3}H-cortisol metabolites the mean recovery of hydrolysis plus solvolysis was 95.0 $\pm$ 8.3% (Leunissen and Thijssen, 1978).

(4) Extraction

Leunissen and Thijssen (1978) examined the purification of the urine sample (removal of acids) by washing the ethyl acetate fraction with aqueous sodium bicarbonate and water. They found the

alternative use of the anion exchanger, Amberlyst A-26, suffers from the disadvantage that the recovery of some steroids is not quantitative. Sephadex LH-20 overcame these problems but at the time was not commercially available.

Although some highly polar steroids were not completely recovered or were altered during the alkali wash, this method was found more satisfactory than the use of Amberlyst A-26. Recoveries of ^{14}C-androstenedione and DHEA-S from the alkali wash ranged from 96 to 101%.

For a continued procedure involving XAD-2 extraction, hydrolysis with solvolysis and alkali wash, applied to mixed steroid metabolites, the recovery of 80-96% agreed exactly with that predicted from multiplication of recoveries from the individual steps.

(5) Derivatisation Tubes

When washing the derivatisation tubes detergents should be avoided. The tubes were washed with acetone, then water, followed by sonication for 1 hour in water.

(6) Derivative Formation

In 1960 VandenHeuvel, Sweeley and Horning found that the side chain of free cortisone and related 17α-hydroxysteroids was lost through a thermal cleavage reaction during GLC. This observation was soon verified in other laboratories. A search for thermally stable derivatives was not entirely successful, until 1966 when Gardiner and Horning found that methoxime trimethylsilyl (MO-TMS) derivatives of cortisol and other related compounds were stable under GLC conditions.

The purpose of converting ketone groups to methoximes is to prevent enol ether formation during the silylation procedure. The reaction of steroid ketones with methoxyamine hydrochloride to form MO derivatives proceeds smoothly for relatively unhindered keto groups (3, 16, 17 and 20-one Groups), where as the reaction at positions 11 and 12 will not normally occur, or react very slowly. It is important that the reaction be carried out in anhydrous conditions; pyridine is believed to be the best solvent for the reaction (Horning and Horning, 1970).

Very few problems are associated with the preparation of derivatives of steroids with two or three functional groups: the major difficulties lie in working with steroid mixtures of biologic origin containing compounds with many (up to six) functional groups.

The rates of reaction of unhindered, moderately hindered and highly hindered hydroxyl groups are quite different; the reaction with TSIM adequately converts all hydroxyl groups in human urinary steroids, alone or in combination, to trimethylsilyl ethers while restricting enol ether formation for the 11-one group of tetrahydrocortisone to 2% or less (Pfaffenberger and Horning, 1975).

The resulting MO-TMS derivatives are thermally stable, easily volatized and show no indication of dehydration or compound loss when good GLC analytical practices are followed.

All 17-one steroids that have been studied yield a single MO derivative, but 3-one-4-ene steroids form syn- and anti- isomers. These isomeric derivatives are stable and are usually formed in a characteristic ratio for each steroid.

It was found to be extremely important to keep the concentration of methoxyamine hydrochloride constant since the relative amounts of syn- and anti- derivatives formed vary with the concentration (Shackleton and Honour, 1976).

Certain steroids, eg. tetrahydroaldosterone, are largely destroyed when TSIM is used as the silylating reagent. This can be overcome by using a milder silylation reagent eg. HMDS/TMCS (Shackleton and Honour, 1976). However MO-TMS derivatives are the most satisfactory ones known for studying mixtures of steroids by GLC (Pfaffenberger and Horning, 1977).

TSIM Preparation

32 grams HMDS, 32 grams imidazole flakes (in excess) and 250 ml toluene (dried over sodium and redistilled) were refluxed for 18 hours in a 500 ml round bottomed flask. A drying tube was attached to the top of the reflux column. The toluene was then removed in a rotary evaporator with a water bath temperature of about 50°C. TSIM was then distilled off at 5 mm mercury pressure (Hi-Vae pump with liquid-air trap) and collected at 75-80°C. A paraffin oil bath on a hot plate was used. Imidazole boils at 138°C at 12 mm mercury pressure.

(7) Lipidex 5000

In GLC systems involving liquid injection with stream splitting, the derivatives may be injected directly, without prior removal of reagents. Solvents and reagents must be removed when using a splitless injector owing to the low volatility of the silylating reagents, especially TSIM, and this is accomplished by the use of Lipidex 5000. It also removes interferring material which may have followed the steroids in previous purification steps (Axleson and Sjovall, 1974). This material probably had similar polarity to that of the steroids, but the MO-TMS reaction did not convert it into compounds having the same low polarity as the MO-TMS steroid derivatives. The latter could therefore be eluted from Lipidex 5000 ahead of the interferring material.

Lipidex 5000 can also be used prior to MO-TMS derivatisation to remove non-polar lipids from plasma and urine samples (Sjovall, 1975).

Lipidex 5000 is a hydroxyalkoxypropylated product of Sephadex LH-20, with the alkyl side chains covalently bound to the polysaccharide matrix. The average chain length of Packard Lipidex 5000 is 15 carbons and is approximately 50% substituted.

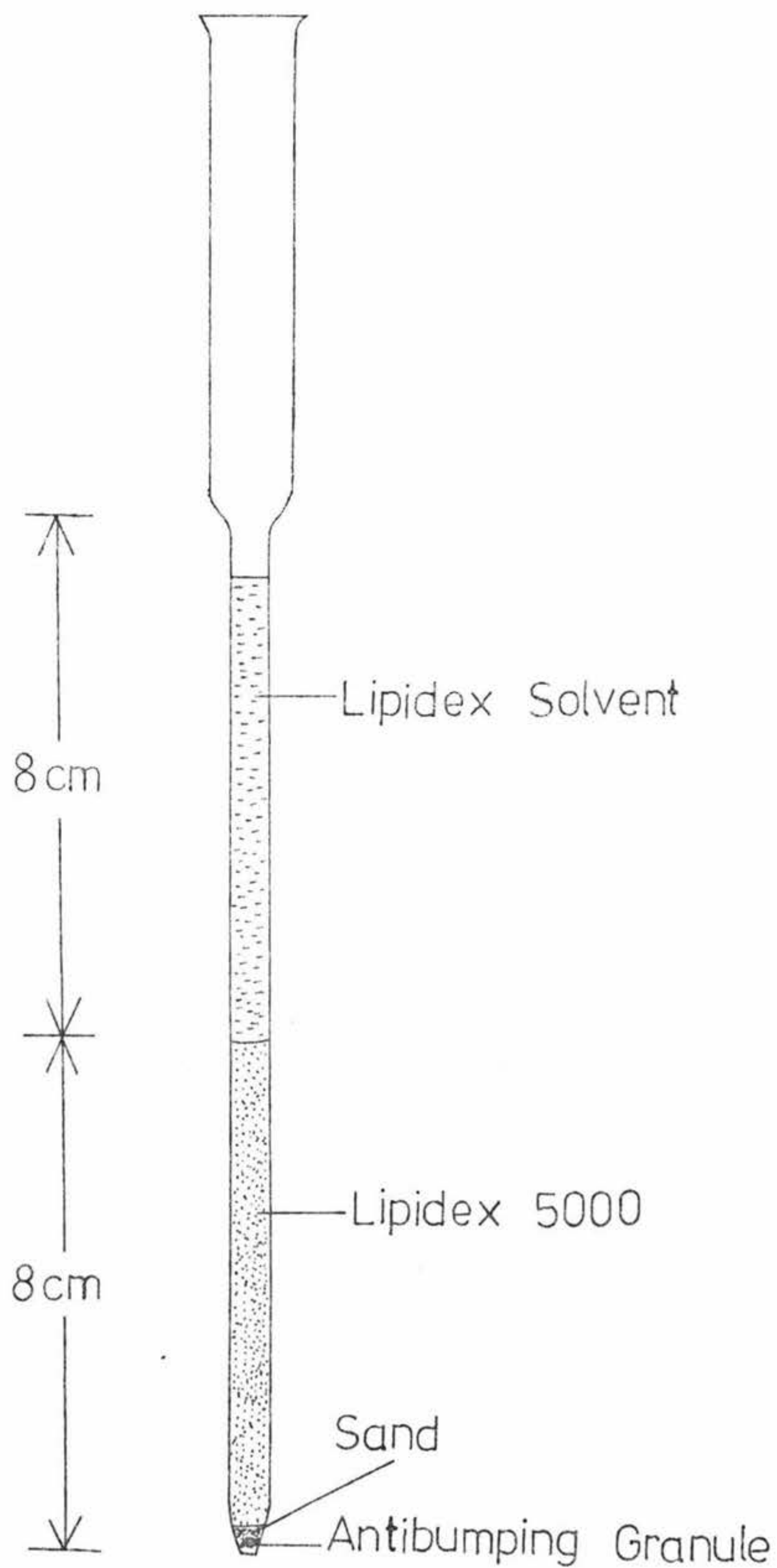
Preparation of Lipidex 5000

The Lipidex 5000 when received was stored in methanol. 10 grams was transferred to a centrifuge tube, centrifuged, and washed with about four volumes of Lipidex solvent, and centrifuged. The Lipidex 5000 was then slurried in about five volumes of Lipidex solvent and shaken to obtain a slurry, free from lumps of adhering gel beads and air bubbles

Lipidex 5000 columns were set up as shown in Fig. 2iv.

Figure 2iv

Lipidex 5000 Column



Steroid Standards

Identification and Estimation of Steroids by GLC

A quantitative estimation 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 a differing GLC response, so that separate "mass response factors" should be obtained experimentally for each steroid relative to a given standard. Variation in "mass response factors" may be attributed to differences in reference steroid purity, efficiency of derivatisation, detector response and column condition (Shackleton and Honour, 1976). 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.

Specific steroid peaks are positively identified with GLC-MS and although 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.

Two internal standards were used, n-tetracosane (C24) which elutes about 5 minutes before An, and n-dotriacontane (C32) which elutes about 5 minutes after αC'ol. Quantitation was achieved by comparing the area for 20 μg of steroid standards to 10 μg of C24, taken through derivatisation together (Table 2i).

The MO-TMS derivatives of the standard steroids were achieved using the method of Phillipou et al (1978).

Retention Times

The retention times for the nineteen neutral steroids extracted from urine weredetermined after injection of single compounds and mixtures, relative to the internal standard n-dotriacontane (C32) and measured from the time of injection. The relative retention time (relative to C32) was expressed as retention index, where C24 represents 2400 and C32 represents 3200. By plotting a graph of relative retention time verses retention index, the retention index for all the steroids can be determined. These Kovax indices were found to

Table 2i

Average Mass Response Factors

Steroid	Mass Response Factors
An	0.99
Et	0.94
DHEA	1.22
11KAn	0.68
11KEt	0.66
11OHAn	0.64
11OHEt	0.71
Pd	1.10
Pt	1.45
Atr	1.43
THS	1.23
THE	0.74
THB	0.67
THF	1.37
aTHF	0.95
αC'one	1.41
βC'one	0.68
βC'ol	0.56
αC'ol	0.56

be reproducible within 10 units under constant conditions independent of the amount of steroid (Table 2ii).

Little or no response in the overall system was found with 6β OH cortisol, cortisol, aldosterone, corticosterone, 6β OH cortisone, 11 dehydrocorticosterone, deoxycorticosterone and 17 OH progesterone. This confirms the observation of Axleson (1977) that many 3-keto-4-ene steroids are unstable in the system used.

The nineteen neutral steroids from urine were derivatised as previously described from the standards (Fig. 2v).

Identification of the steroids was based on the retention index already calculated, spiking the biologic sample with steroid standards, as well as comparison with published profiles. The order of elution and overall steroid profiles were very similar to those in the literature.

The MO-TMS derivatives of steroids are sensitive to traces of moisture, so contact with the atmosphere was kept to a minimum. The derivatised samples were kept in quick fit tubes in a dessicator, and only unstoppered to remove an aliquot for injection.

The ratio of peak areas for indiviual steroids to that of the internal standard was found to be quite reproducible for consecutive injections on the day of silylation and for two to three days following (Table 2iii). The ratio of the steroid standard mixture remained reproducible for a slightly longer period, but after five days this reproducibility diminished, with the last five steroids eluted showing greater variability than the rest. This can be attributed to the higher baseline and increased baseline drift observed with samples that have been stored for a few days.

Dilution of the derivatised sample, while maintaining a constant concentration of the internal standard, produces a fairly linear decrease in the ratios of steroids to internal standard (Evans, 1979). 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.

Table 2ii

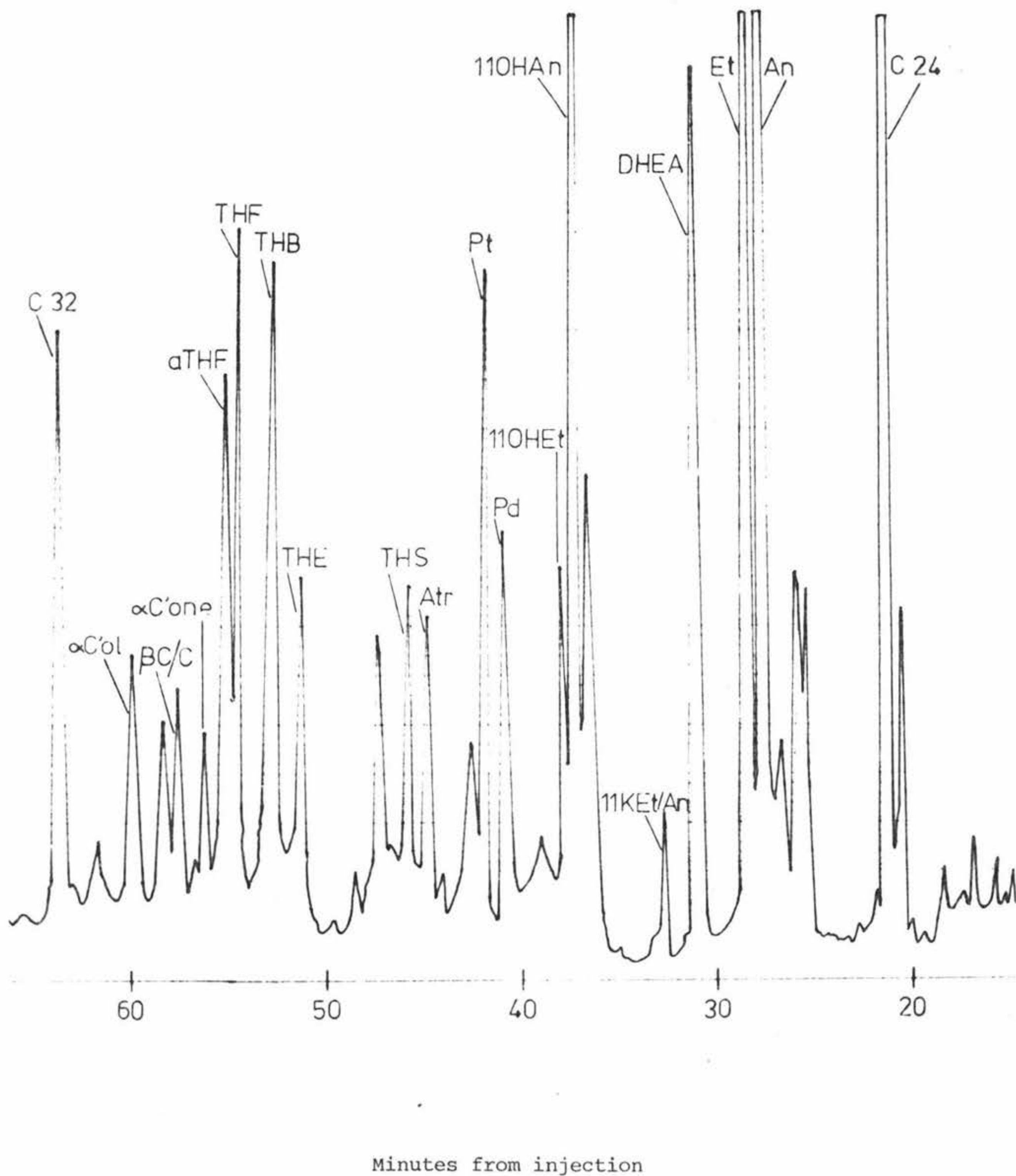
Retention Index of Steroids

Steroid	Retention Index
An	2510
Et	2523
DHEA	2583
11KAn	2608
11KEt	2608
11OHAn	2692
11OHEt	2705
Pd	2765
Pt	2788
Atr	2848
THS	2860
THE	2970
THB	2995
THF	3030
aTHF	3044
αC'one	3055
βC'one	3090
βC'ol	3090
αC'ol	3125

11KAn/Et and βC/C coelute

Figure 2v

GLC Profile of Synthetic Steroid Derivatisation Mixture



Minutes from injection

Table 2iii

Reproducibility of Steroids

Steroid	Day 1	Day 2	Day 3	Day 4
An	1.10	1.10	1.02	1.05
Et	1.77	1.75	1.96	1.73
DHEA	0.87	0.87	0.85	0.92
11KAn/Et	0.15	0.16	0.15	0.13
11OHAn	1.07	1.07	1.07	1.25
11OHEt	0.72	0.72	0.70	0.78
Pd	0.40	0.40	0.37	0.43
Pt	0.45	0.43	0.43	0.39
Atr	0.29	0.30	0.32	0.32
THS	0.27	0.27	0.28	0.27
THE	0.78	0.77	0.77	0.64
THB	1.29	1.32	1.31	1.12
THF	0.35	0.33	0.29	0.26
aTHF	0.44	0.43	0.38	0.48
αC'one	0.32	0.31	0.28	0.19
βC/C	0.45	0.42	0.38	0.32
αC'ol	0.78	0.74	0.64	0.41

CHAPTER 3

EXPERIMENTAL PROTOCOL, RESULTS AND CONCLUSIONS

(1) Ten Day Collection

A ten day collection provides an index of analytical and individual variation in the daily excretion of steroid metabolites.

Protocol

In order to assess day to day variation in steroid excretion, ten consecutive 24 hour urine samples were collected from an adult male. Urine collections of 1500 ml or less were made up to 1500 ml with water, and urine with a volume greater than 1500 ml was made up to 2000 ml (Table 3i).

Table 3i

Day	Urine Volume (ml)	Diluted Volume (ml)
1	1205	1500
2	1945	2000
3	1752	2000
4	1593	2000
5	1292	1500
6	1360	1500
7	952	1500
8	1043	1500
9	1332	1500
10	1500	1500

A ten ml aliquot of urine was prepared as previously described. A 27 meter column was used in this analysis with a loss of resolution of aTHF and αC'one, and in few instances THF.

Results

Steroid excretion rates (mg/24 hours) are presented in Table 3ii, and shown graphically in Fig. 3i. In all experiments the excretion rates presented are the average of multiple runs on duplicate samples.

Two of the chromatograms of standards and urine spiked with the standards are illustrated in Fig. 3ii.

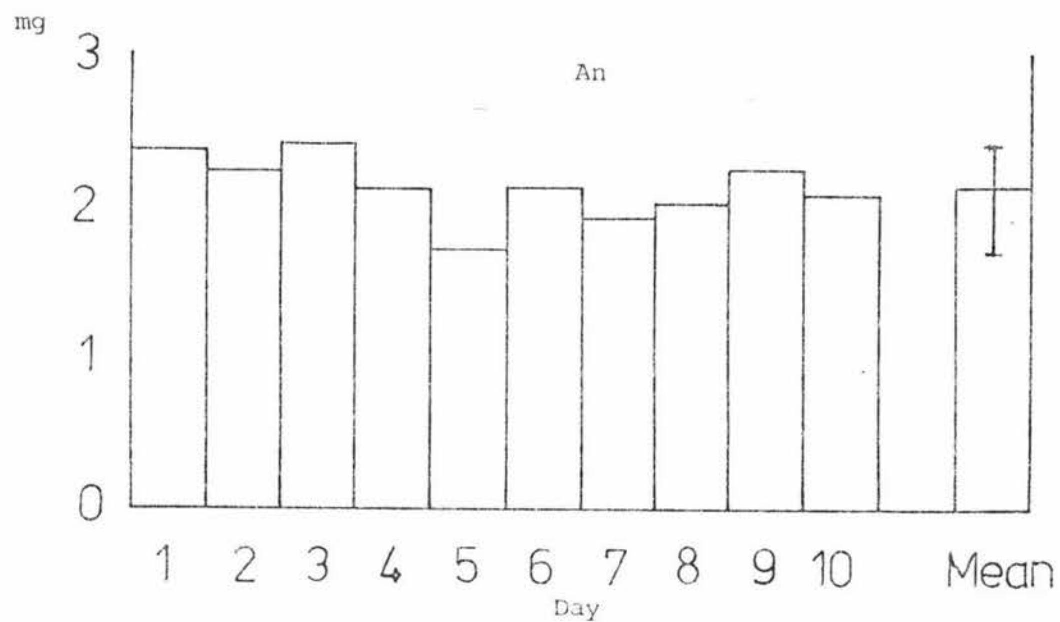
The average daily excretion of each steroid is presented in Table 3iii, with a comparison of the daily excretion from the literature.

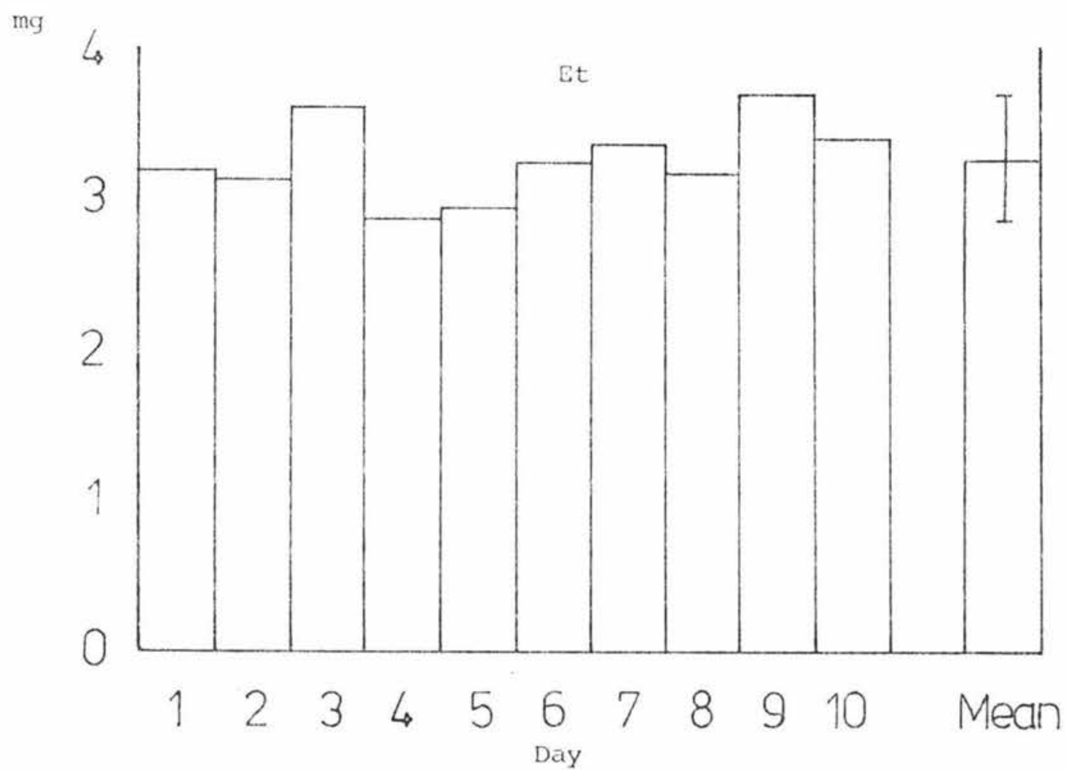
Table 3ii

mg excreted/day

Steroid	1	2	3	4	5	6	7	8	9	10
An	2.38	2.23	2.40	2.11	1.72	2.11	1.91	2.01	2.23	2.09
Et	3.18	3.12	3.59	2.86	2.92	3.22	3.34	3.16	3.68	3.39
DHEA	1.56	1.77	1.77	1.53	1.15	1.39	1.25	1.36	1.54	1.65
11KAn/Et	0.69	0.81	0.84	-	0.65	0.53	0.58	0.71	0.83	0.84
11OHAn	3.40	4.28	4.50	-	3.04	3.32	3.52	3.79	4.53	4.16
11OHEt	1.53	1.46	-	-	1.13	1.31	1.65	-	-	1.64
Pd	2.64	2.76	2.52	2.25	1.52	1.61	1.76	2.60	1.59	2.85
Pt	0.96	0.85	1.63	1.25	0.87	0.75	1.29	1.47	0.77	1.08
Atr	0.53	0.55	-	0.59	0.27	0.47	0.63	-	0.45	-
THS	0.52	0.87	0.48	0.56	0.40	0.73	0.49	0.64	0.45	0.62
THE	3.26	2.67	2.23	3.00	2.25	2.44	2.41	1.94	2.56	3.10
THB	1.27	1.45	1.44	-	0.95	0.97	-	1.14	1.43	-
THF	1.12	0.88	0.61	-	0.80	0.94	-	-	0.78	-
aTHF	-	0.55	-	-	0.56	-	-	-	-	-
βC/C	1.27	1.34	1.03	1.12	0.94	0.95	1.06	1.03	1.16	1.08
αC'ol	1.14	1.74	1.42	1.51	1.26	1.08	0.96	1.16	1.44	1.14

Figure 3i
Steroid Excretion Rates





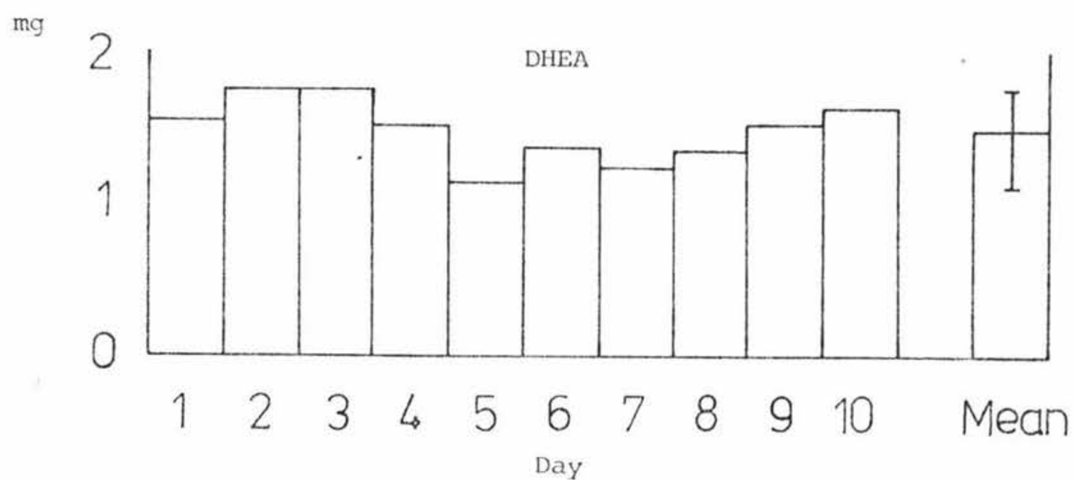


Figure 3i (cntd.)

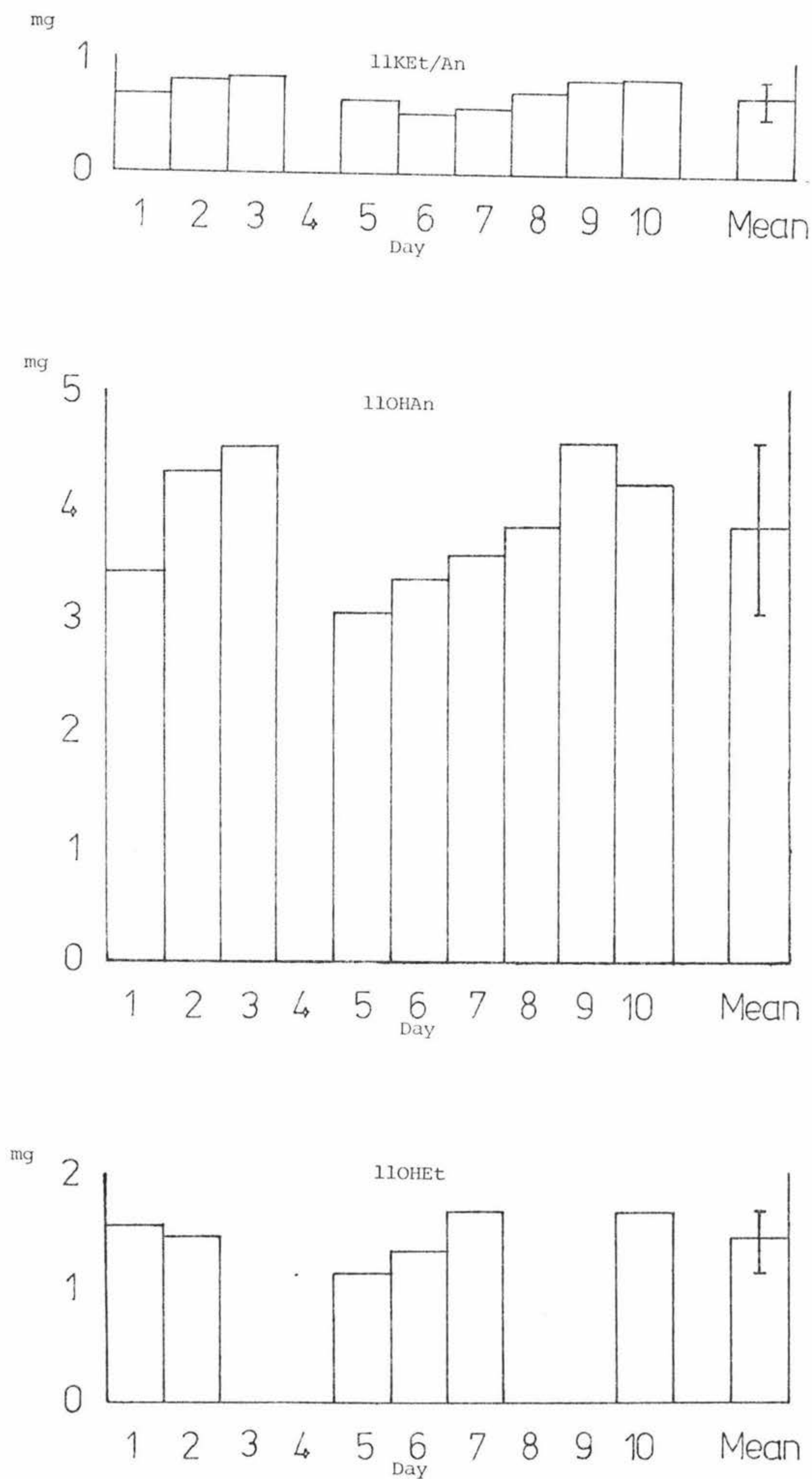


Figure 3i (cntd.)

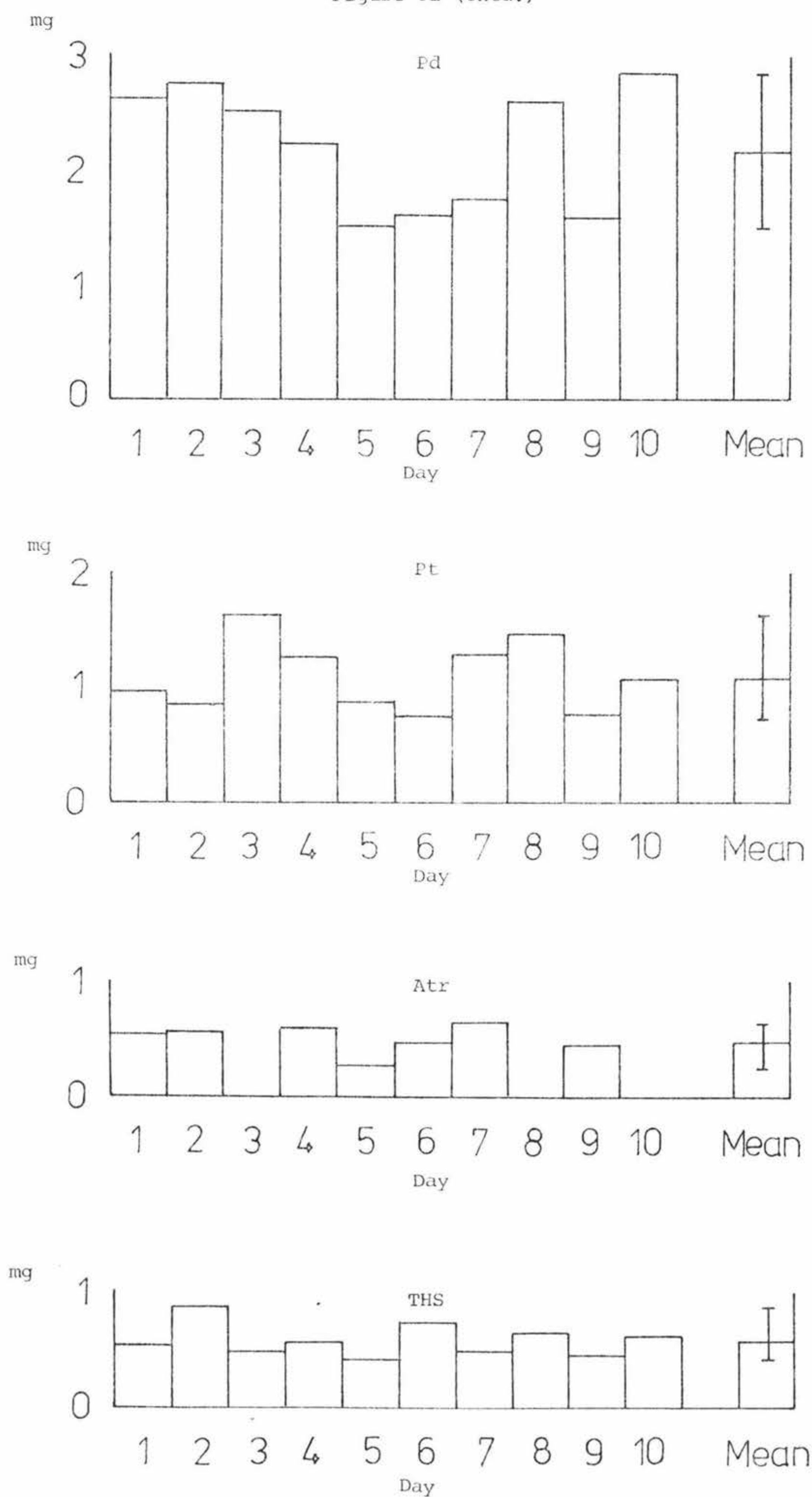
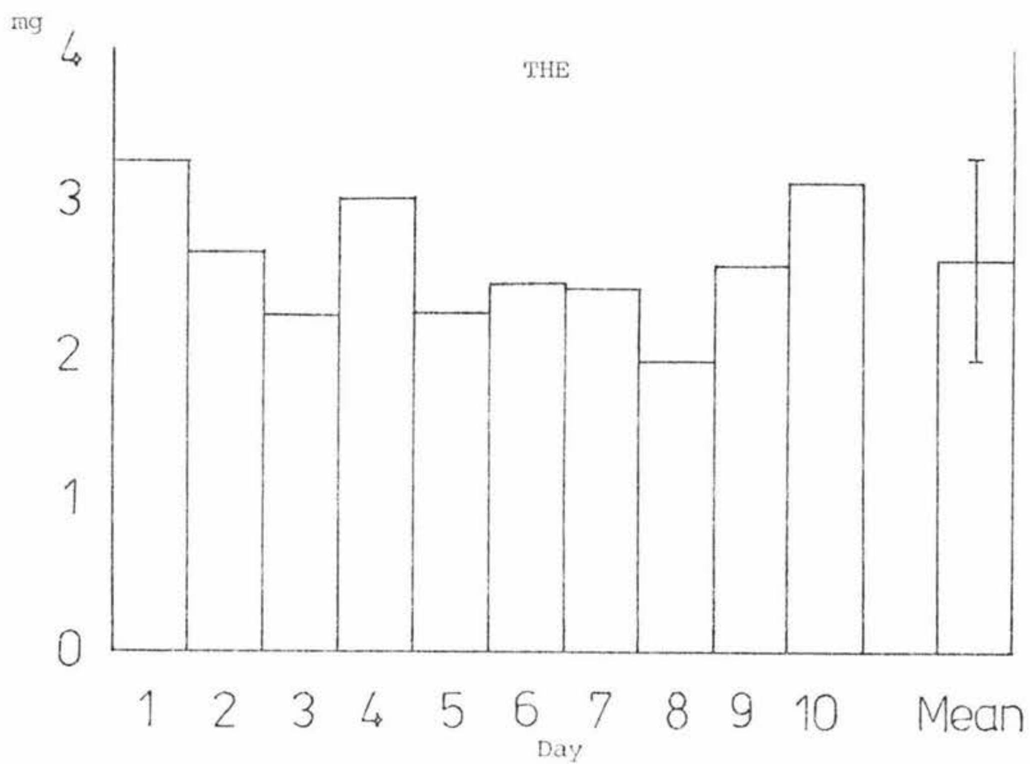


Figure 3i (cntd.)



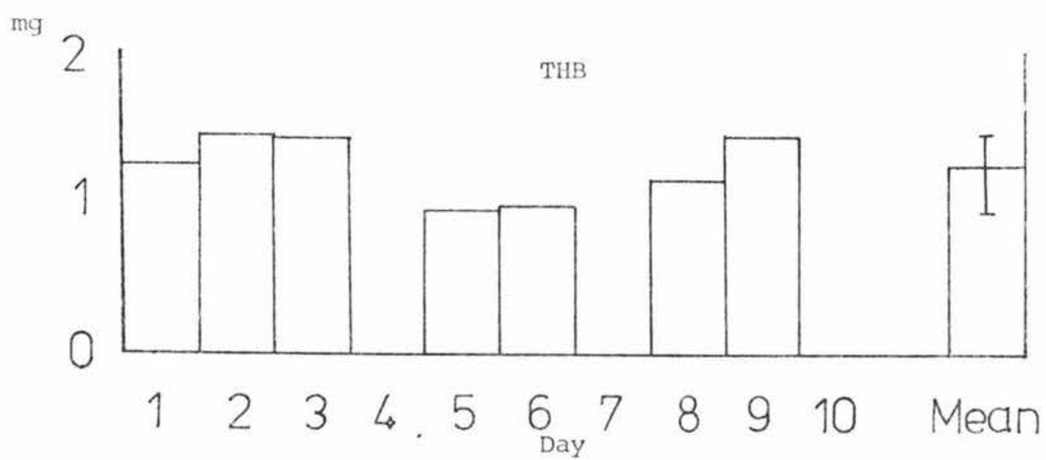
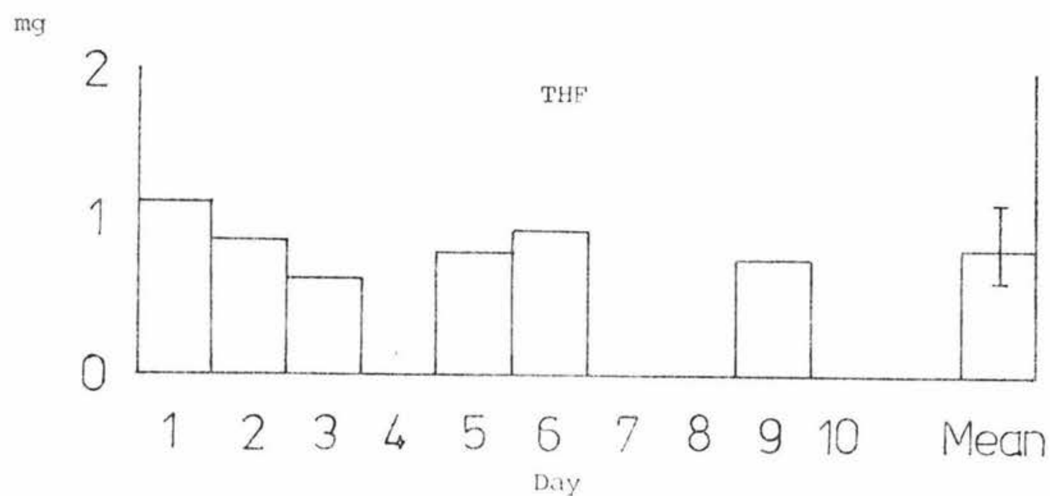
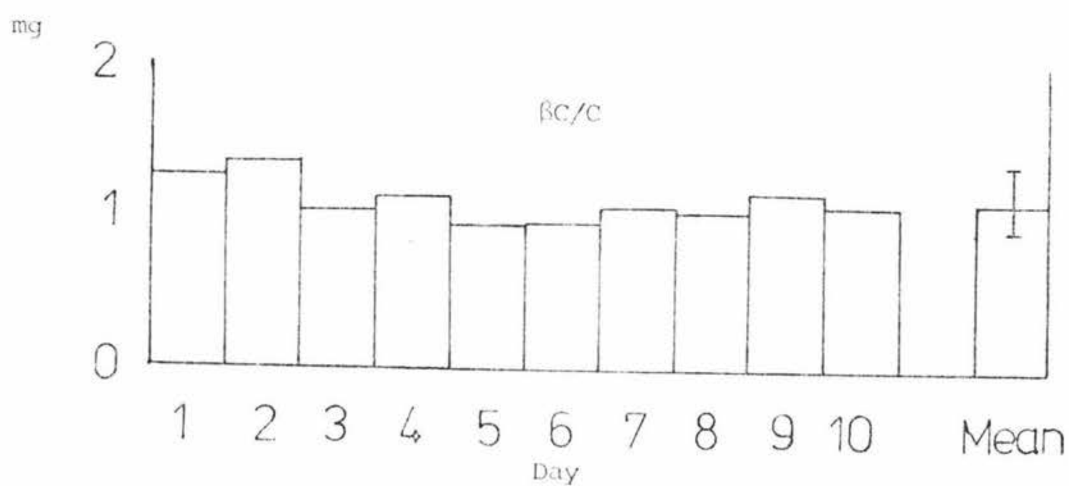
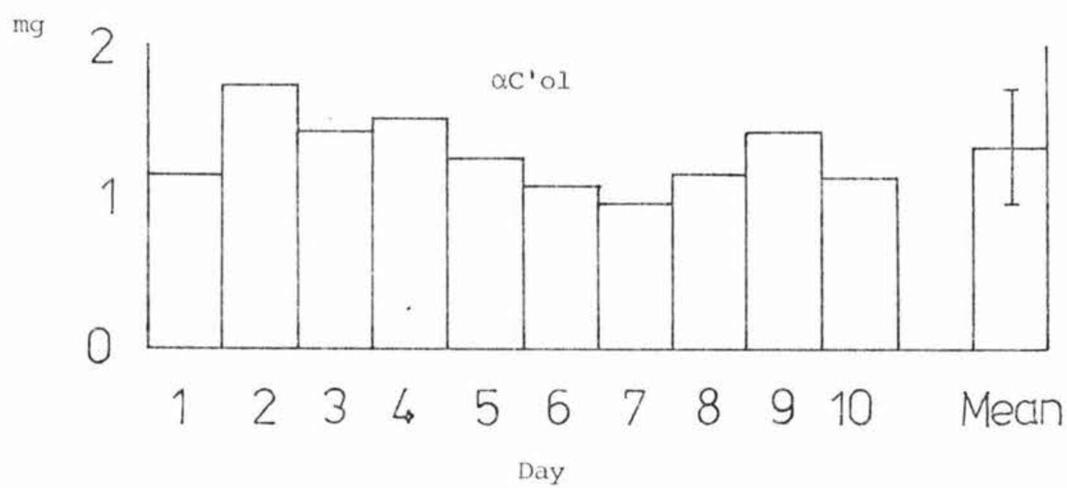


Figure 3i (cntd.)







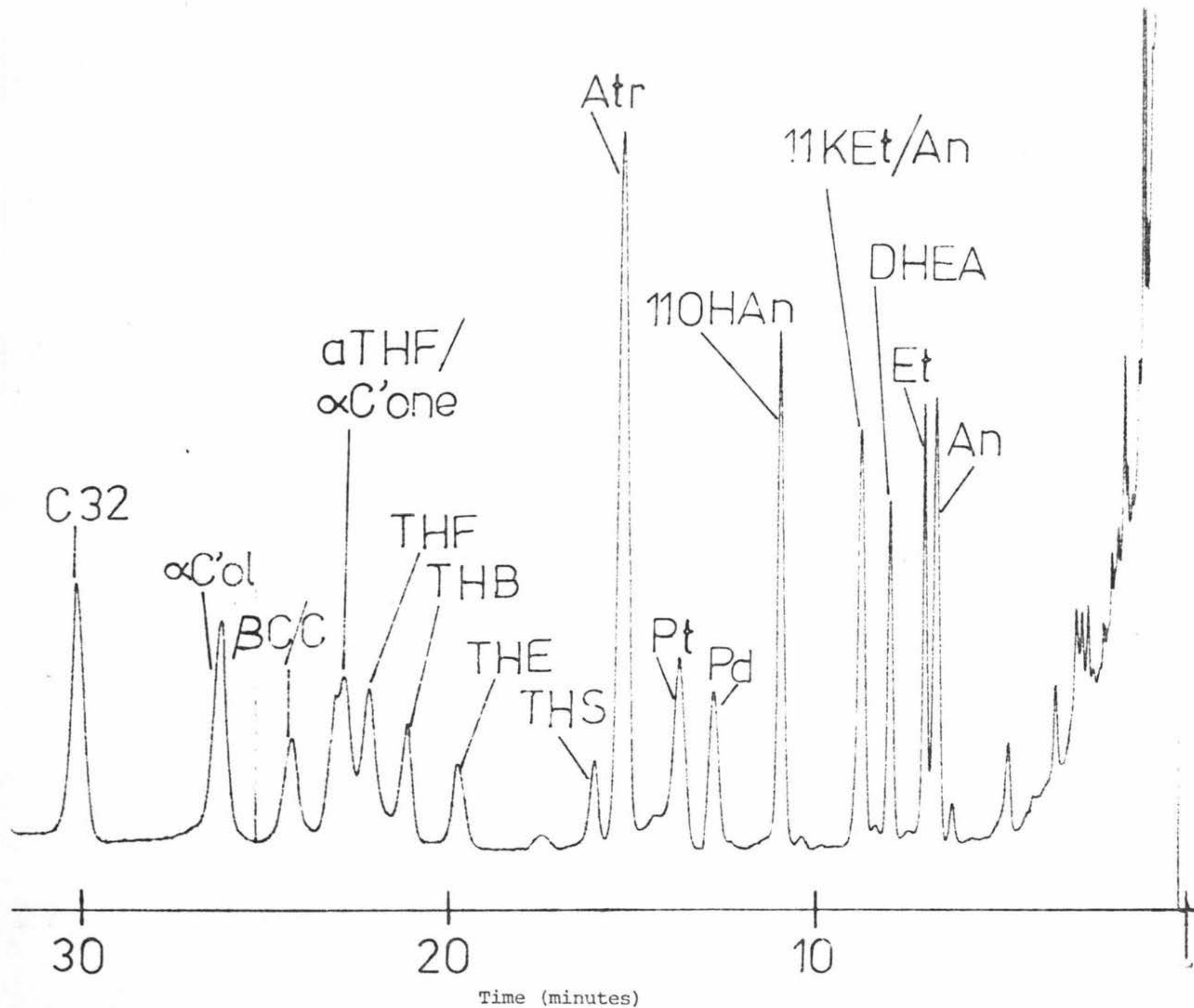
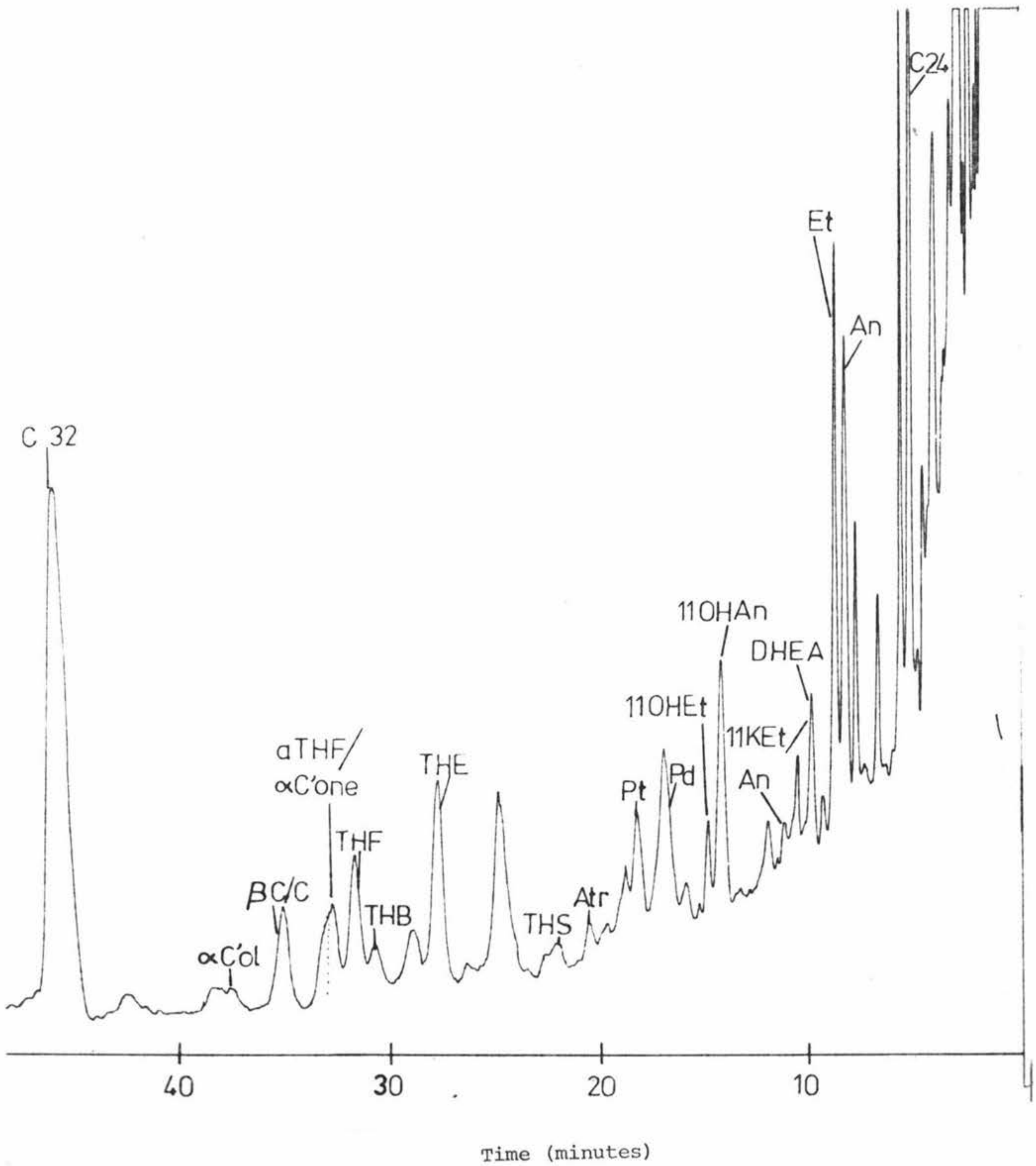


Figure 3ii(a)

GLC Profile of Synthetic Steroid Derivative Mixture

Figure 3ii(b)

GLC Profile of Steroids of Subject 1, Day 1

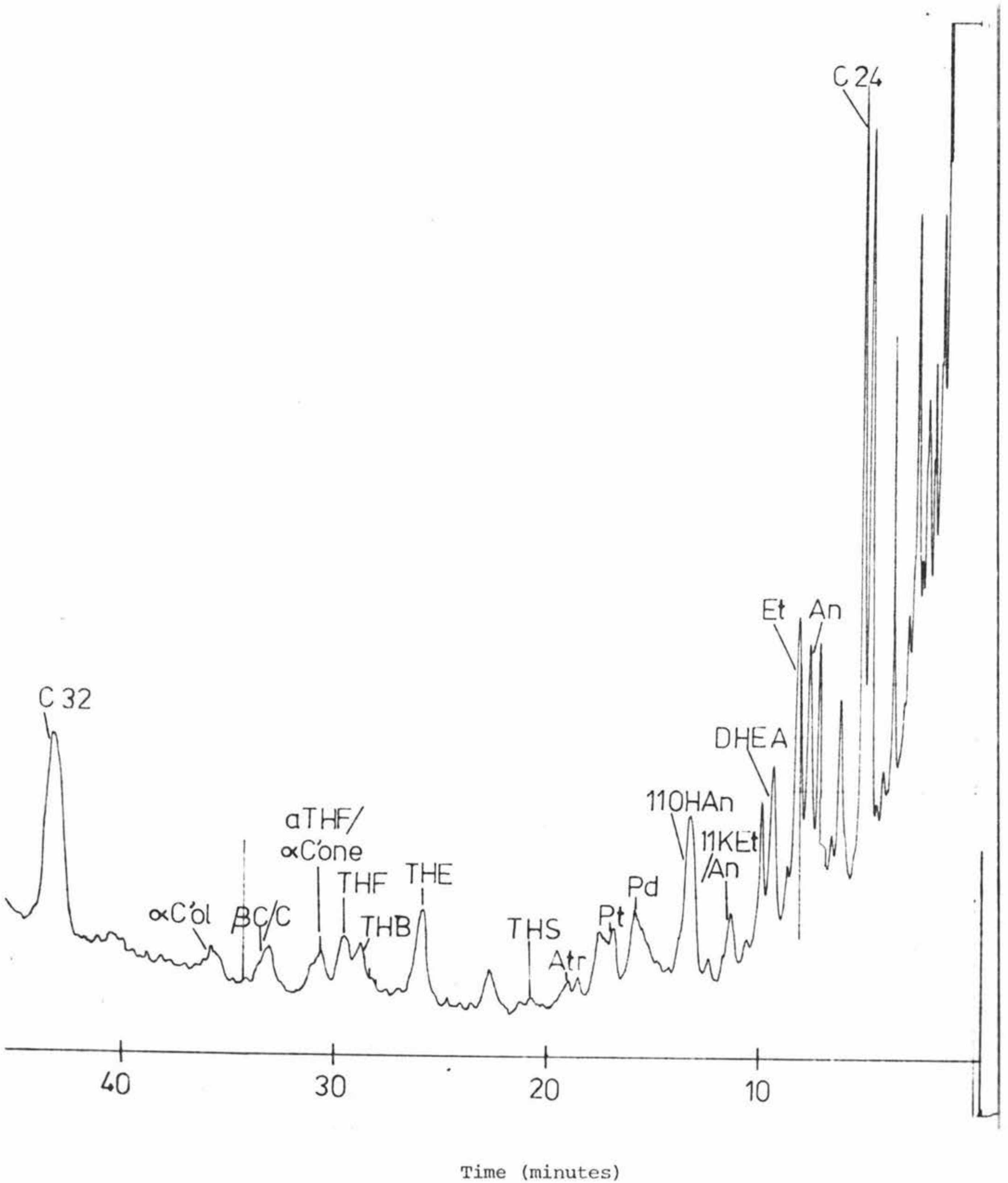


Time (minutes)

1.5 μl sample, 27 meter column

Figure 3ii(c)

GLC Profile of Steroids of Subject 1, Day 2

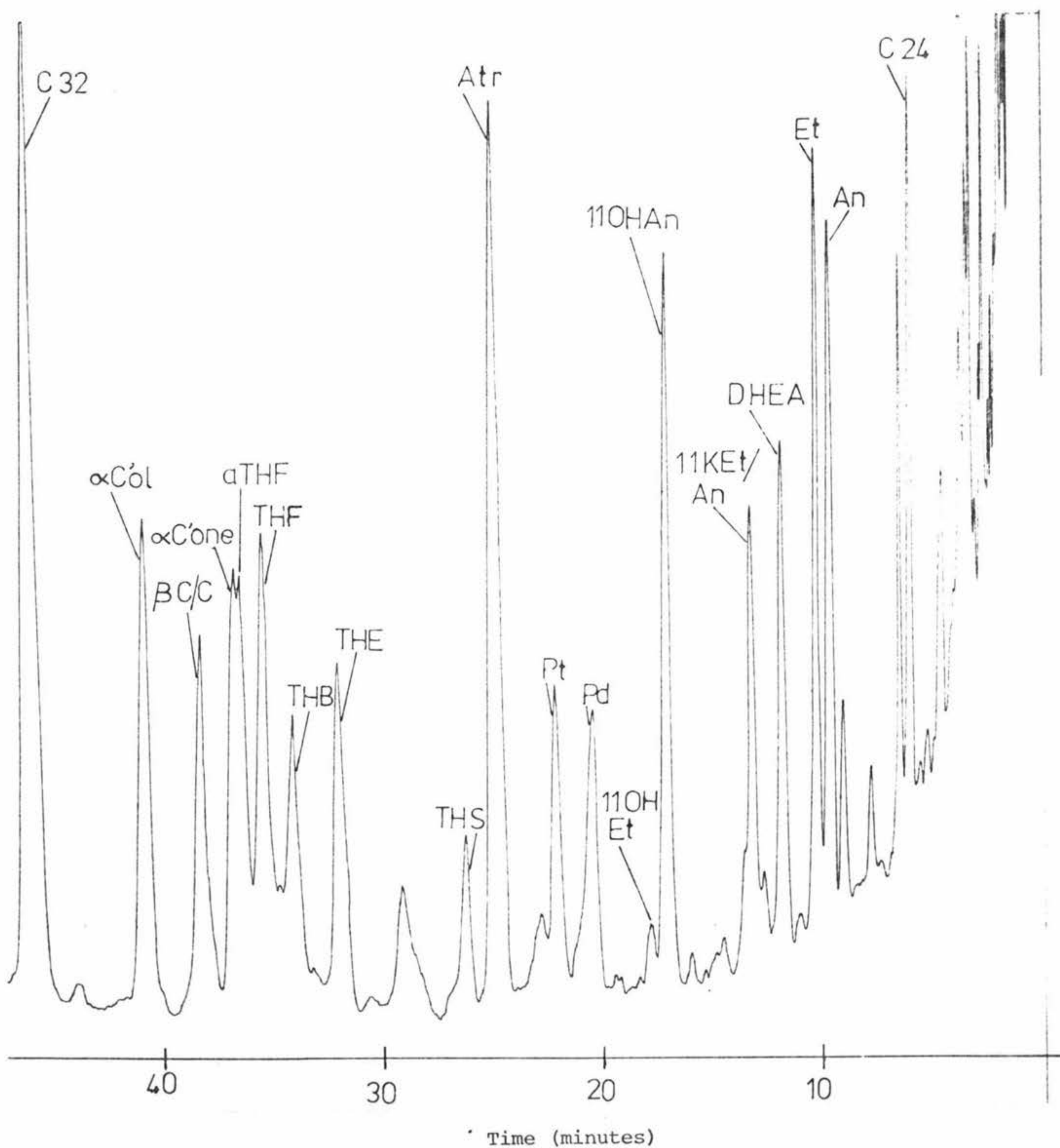


Time (minutes)

1.5 μl sample, 27 meter column

Figure 3ii(b)

GLC Profile of Subject 1, Day 1 Spiked with Synthetic Steroids



Time (minutes)

1.5 µl sample, 27 meter column

Table 3iii

Average Daily Excretion Rates

Steroid	Average Excretion (mg/day)	*Range %	Coefficient of Variation %	**From Literature (mg/day)
An	2.11	19	10	2.6
Et	3.24	14	8	2.5
DHEA	1.50	23	13	0.72
11KEt/An	0.70	24	16	0.84
11OHAn	3.79	31	14	0.39
11OHEt	1.44	22	13	0.46
Pd	2.17	31	24	0.46
Pt	1.09	52	27	1.60
Atr	o.48	44	23	-
THS	0.57	51	24	-
THE	2.60	25	15	3.10
THB	1.25	24	16	0.22
THF	0.83	35	19	3.10
aTHF	0.56	18	3	2.50
βC/C	1.10	22	11	-
αC'ol	1.33	39	18	-

* Calculated by dividing the difference between the largest variable and the average excretion, by the average excretion.

** Reproduced from Makin (1975)

Ratio of Steroids

The comparison of ratios of some urinary steroids gives an indication of the relative activity of the enzyme systems involved in the formation of steroid hormone metabolites (Table 3iv).

Table 3iv

Steroid Ratios

	Steroids	Enzymic Route	Ratio	10 Day Collection
(1)	An / Et	Reduction at C5	$3\alpha,5\alpha$ / $3\alpha,5\beta$	0.7
(2)	aTHF / THF	Reduction at C5	$3\alpha,5\alpha$ / $3\alpha,5\beta$	0.7
(3)	THF / THE	Reduction at C11	11OH / 11oxo	0.3
(4)	THF+aTHF / THE	Reduction at C11	11OH / 11oxo	0.5
(5)	αC'one / THE	Reduction at C20	20OH / 20oxo	-
(6)	αC'ol / THF+aTHF	Reduction at C20	20OH / 20oxo	1.0
(7)	αC'ol / αC'one	Reduction at C11	11OH / 11oxo	-
(8)	11OHAn+11OHEt / 11KAn+11KEt	Reduction at C11	11OH / 11oxo	7.5
(9)	An+Et / DHEA	Reduction at C5	C5 reduced / C5 not reduced	3.6
(10)	Pt / Pd	Hydroxylation C17	$3\alpha,17\alpha,20\alpha OH$ / $3\alpha,20\alpha OH$	0.5

Conclusion

Many of the excretion rates vary quite considerably when compared to values in the literature. Although the excretion follows a definite pattern in man, a great diversity of factors affect these excretion rates. No data was given on age or body build in Makin (1975), which are only two of the many factors that can affect the rates.

The aim of this experiment was to assess the combined biological plus analytical variation of excretion rates in a single individual. In general the daily variation is up to 35% (with the exception of Pt, Atr, THS and αC'ol). Many of the small peaks such as Atr, THS and αC'ol are often indistinct from baseline noise, and their integrated measurement of area is therefore liable to be much less accurate. Also in the case of αC'ol (and other late eluting steroids), the baseline drift can slightly alter the areas determined.

The coefficient of variation (CV) ranges from 3% to 27%, and a mean CV of 16%. The highest CV values are characteristic of the steroid metabolites with the highest range variation. Beale and Tyrrell (1974) in measuring some steroid metabolites over a 24 hour period found the CV ranged from 6.1% to 27.3%, which is in agreement with the CV for the steroid metabolites of Subject 1.

This experiment provides data for basal levels, and gives an indication of the range that can be concluded to be the "norm" of steroid metabolites excreted by this subject.

(2) Diurnal Rhythm

In this experiment the diurnal variation of excretion of steroid metabolites was investigated to provide an index for basal levels.

Protocol

A series of samples were taken from two adult males. Subject 1 was the same subject used for the ten consecutive day collection, the protocol of collection is shown in Table 3v and 10 ml samples of urine were processed for GLC undiluted (except that a 15 ml aliquot was taken for Subject 1, sample 2, owing to the large volume).

Table 3v

Urine Volumes

Sample	Time	Subject 1 (Urine Vol-mls)	Subject 2 (Urine Vol-mls)
1	7am - 9am	157	51
2	9am - 11am	498	119
3	11am - 1pm	169	76
4	1pm - 3pm	117	141
5	3pm - 5pm	106	92
6	5pm - 7pm	168	80
7	7pm - 7am	490	520

Subject 2 urine was analysed on a 27 meter column with loss of resolution of THB, THF, aTHF and αC'one. Subject 1 urine was analysed on a 40 meter column, with full resolution. Subject 1 was used in later experiments, so this serves as a control.

Results

The excretion rates are presented in Table 3vi, and illustrated graphically in Fig. 3iii. The corresponding chromatograms are illustrated in Fig 3iv and the steroid ratios are presented in Table 3vii.

Table 3vi

Subject 1 — mg excreted

Steroid	7am 9am	9am 11am	11am 1pm	1pm 3pm	3pm 5pm	5pm 7pm	7pm 7am	7pm-7am 6
An	.120	.165	.180	.125	.076	.091	.585	.098
Et	.130	.190	.195	.125	.076	.088	.685	.114
DHEA	.110	.255	.190	.135	.130	.150	1.245	.208
11KEt/An	.028	.055	.041	.029	.017	.018	.165	.028
11OHAn	.135	.210	.190	.120	.074	.080	.575	.096
11OHEt	.110	.175	.160	.083	.065	.067	.475	.079
Pd	.029	.090	.087	.039	.045	.051	.375	.063
Pt	.060	.165	.140	.092	.075	.087	.710	.118
Atr	.020	.043	.046	.016	.018	.015	.140	.023
THS	.025	.030	-	.014	.016	.022	.175	.028
THE	.120	.160	.155	.086	.056	.084	.655	.109
THB	.145	.240	.210	.145	.105	.130	.965	.161
THF	.054	.072	.053	.030	.029	.035	.260	.043
aTHF	.053	.062	.060	.043	.035	.047	.345	.058
αC'one	.037	.074	.069	.030	.028	.036	.300	.050
βC/C	.026	.065	.055	.037	.040	.048	.365	.061
αC'ol	.058	.093	.082	.060	.045	.053	.465	.078

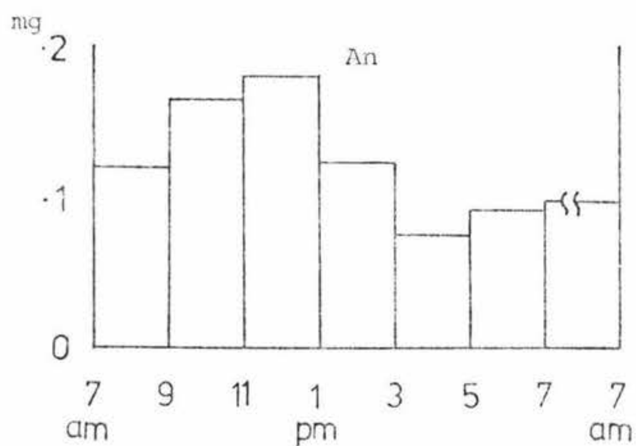
Subject 2

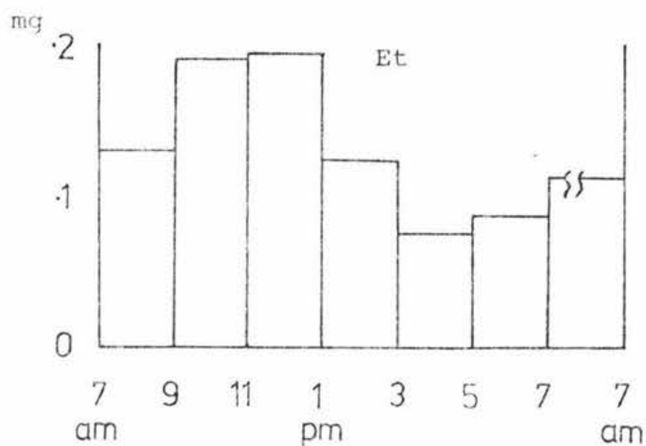
Steroid	7am 9am	9am 11am	11am 1pm	1pm 3pm	3pm 5pm	5pm 7pm	7pm 7am	7pm-7am 6
An	.165	.260	.175	.225	.170	.115	.680	.113
Et	.155	.250	.175	.270	.180	.110	.690	.115
DHEA	.039	.064	.037	.057	.041	.027	.205	.034
11KAn/Et	.035	.062	.041	.064	.038	.027	.190	.032
11OHAn	.205	.340	.195	.335	.170	.135	.795	.133
11OHEt	.058	.089	.066	.106	.080	.055	.365	.061
Pd	.052	.071	.049	.075	.063	.042	.250	.042
Pt	.022	.086	.035	.043	.025	.023	.235	.039
Atr	.024	.054	.034	.046	.034	.019	.150	.025
THS	.021	.042	.031	.070	.043	.030	.275	.046
THE	.026	.310	.044	.077	.024	.012	.255	.043
βC/C	.027	.088	.058	.047	.031	.030	.265	.045
αC'ol	.020	.055	.065	.037	.069	.031	.285	.049

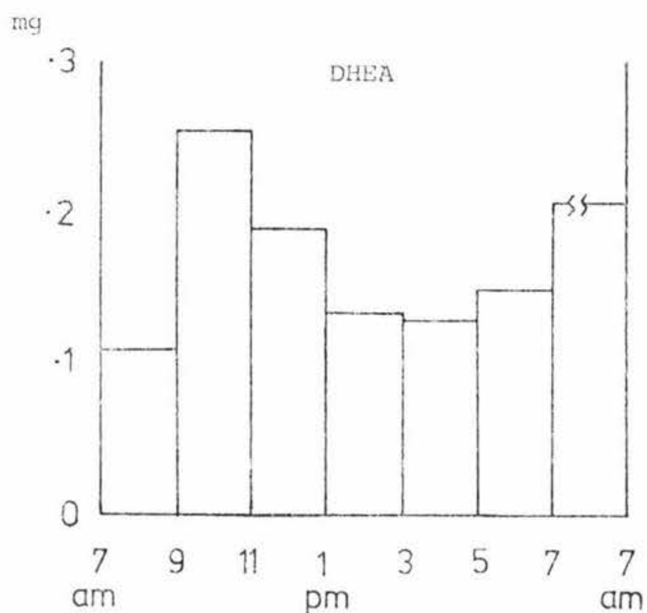
Figure 3iii

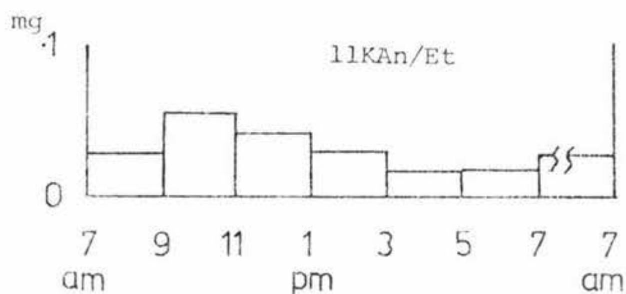
Subject 1

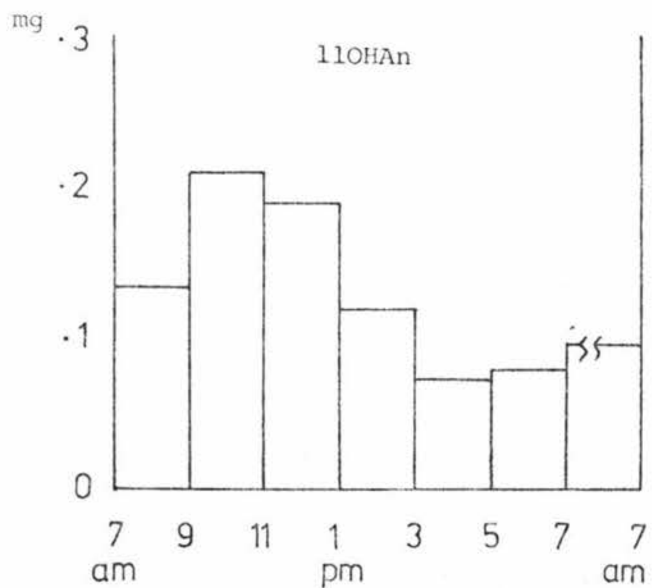
Steroid Excretion Rates











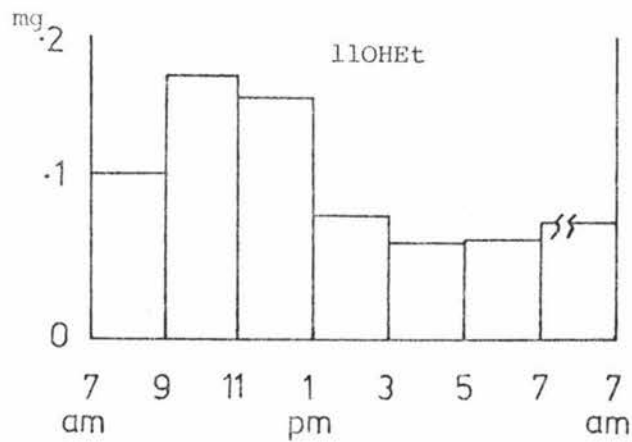
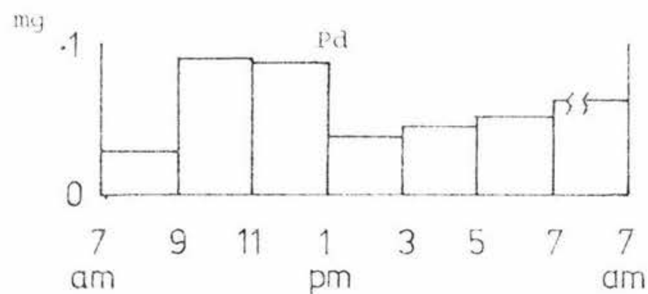
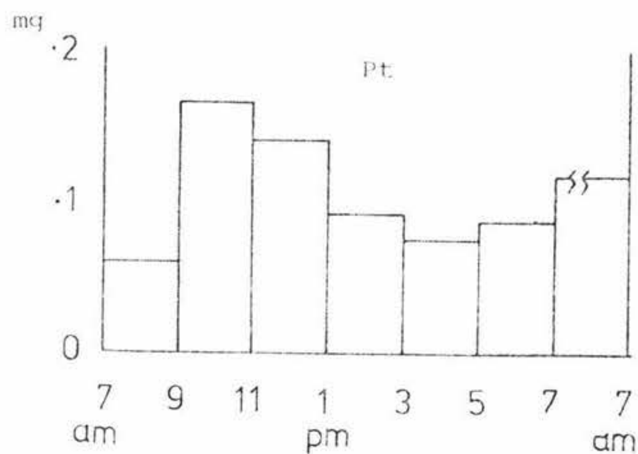
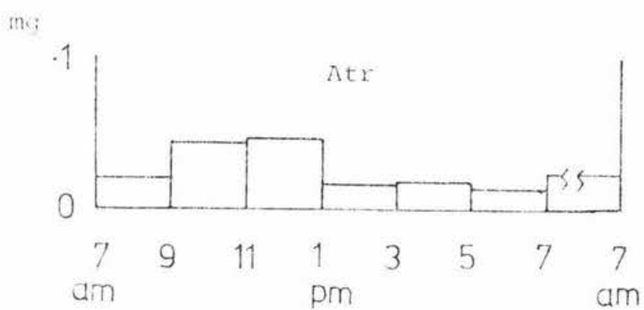


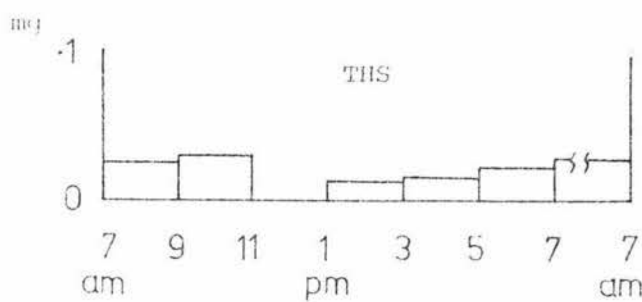
Figure 3iii (cntd.)

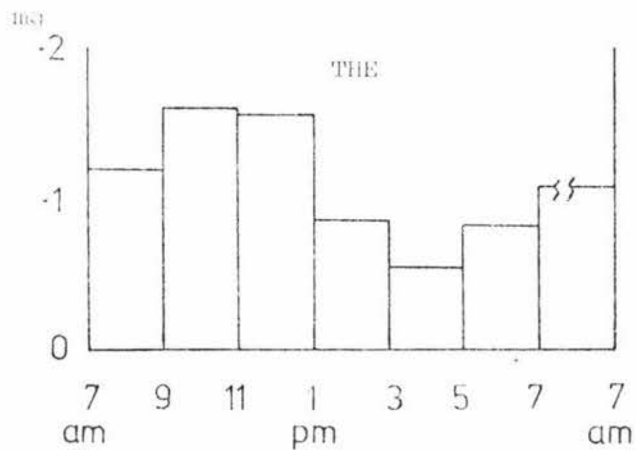
Subject 1

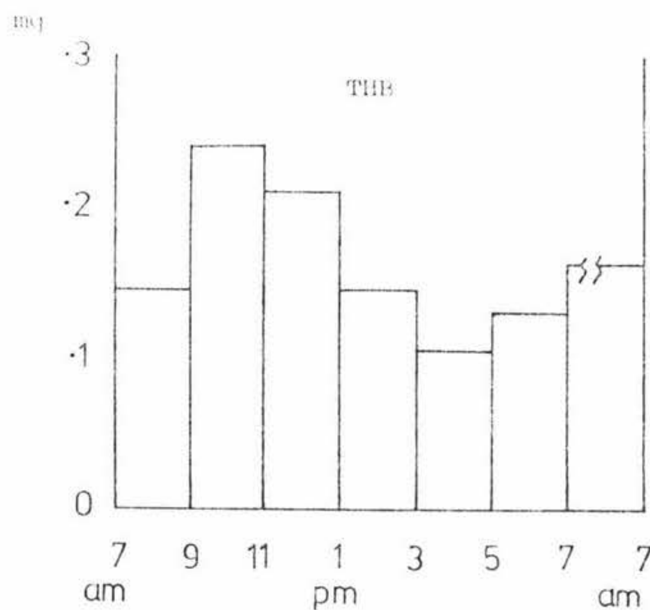


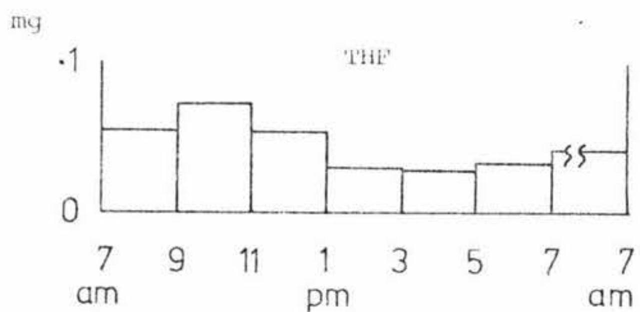












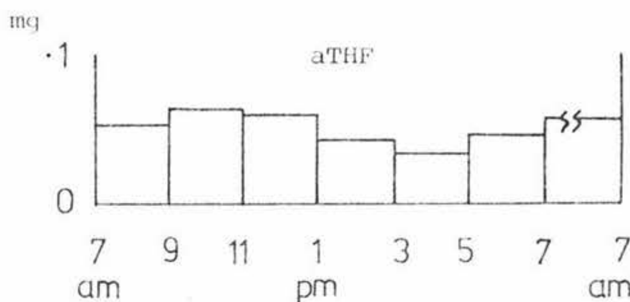
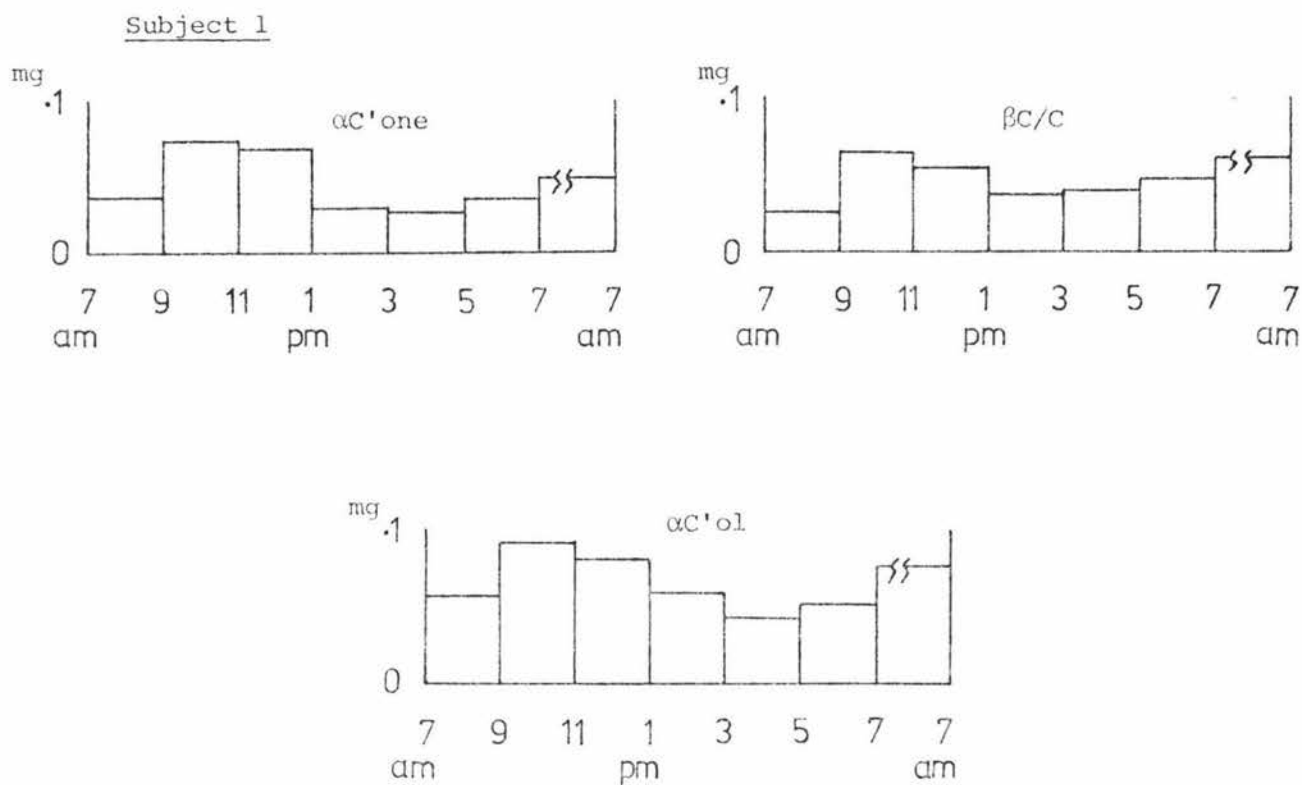


Figure 3iii (cntd.)

Subject 1



Subject 2

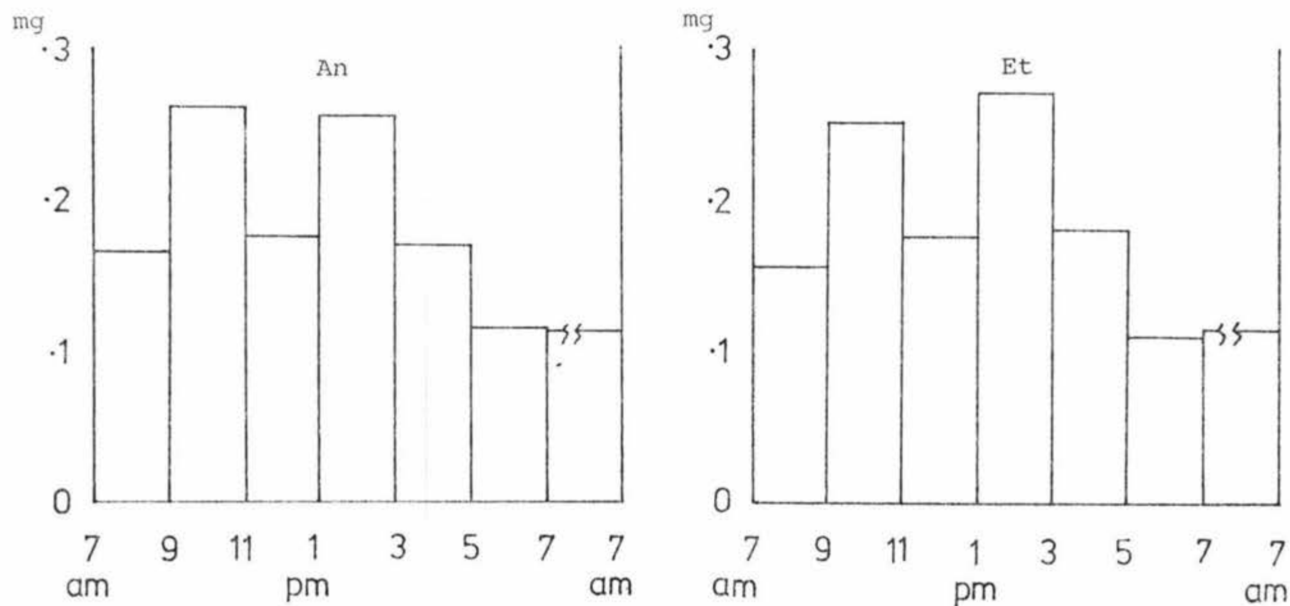
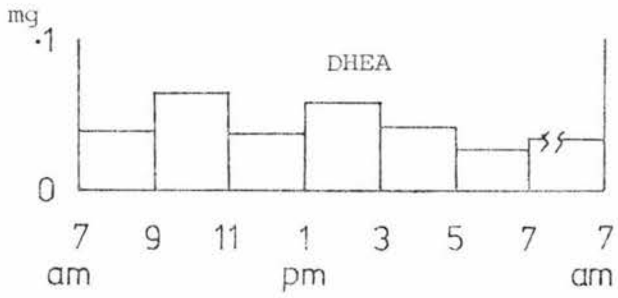
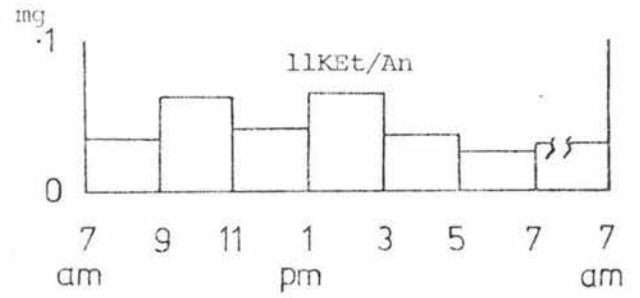
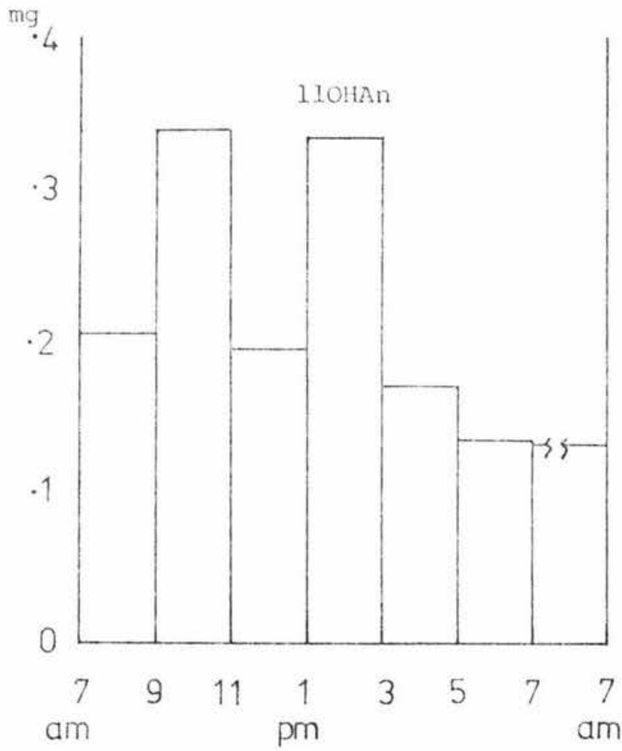


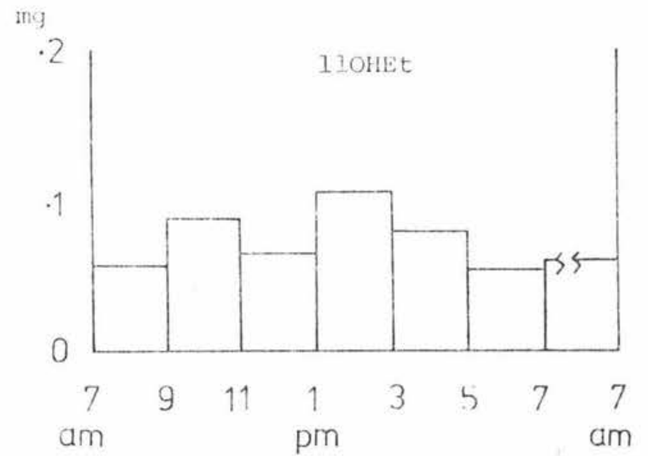
Figure 3iii (cntd.)

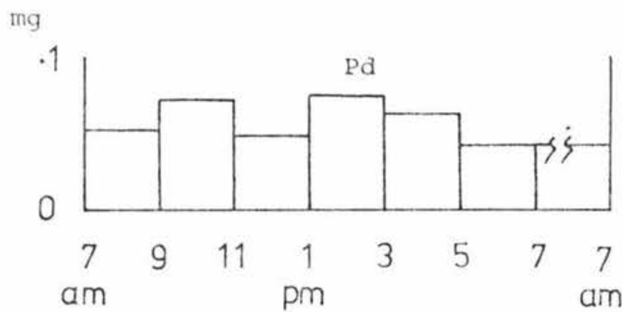
Subject 2











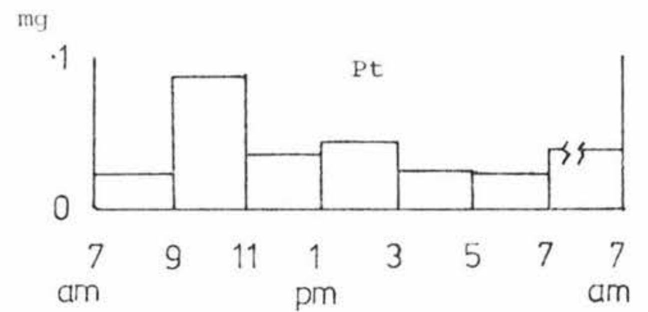


Figure 3iii (cntd.)

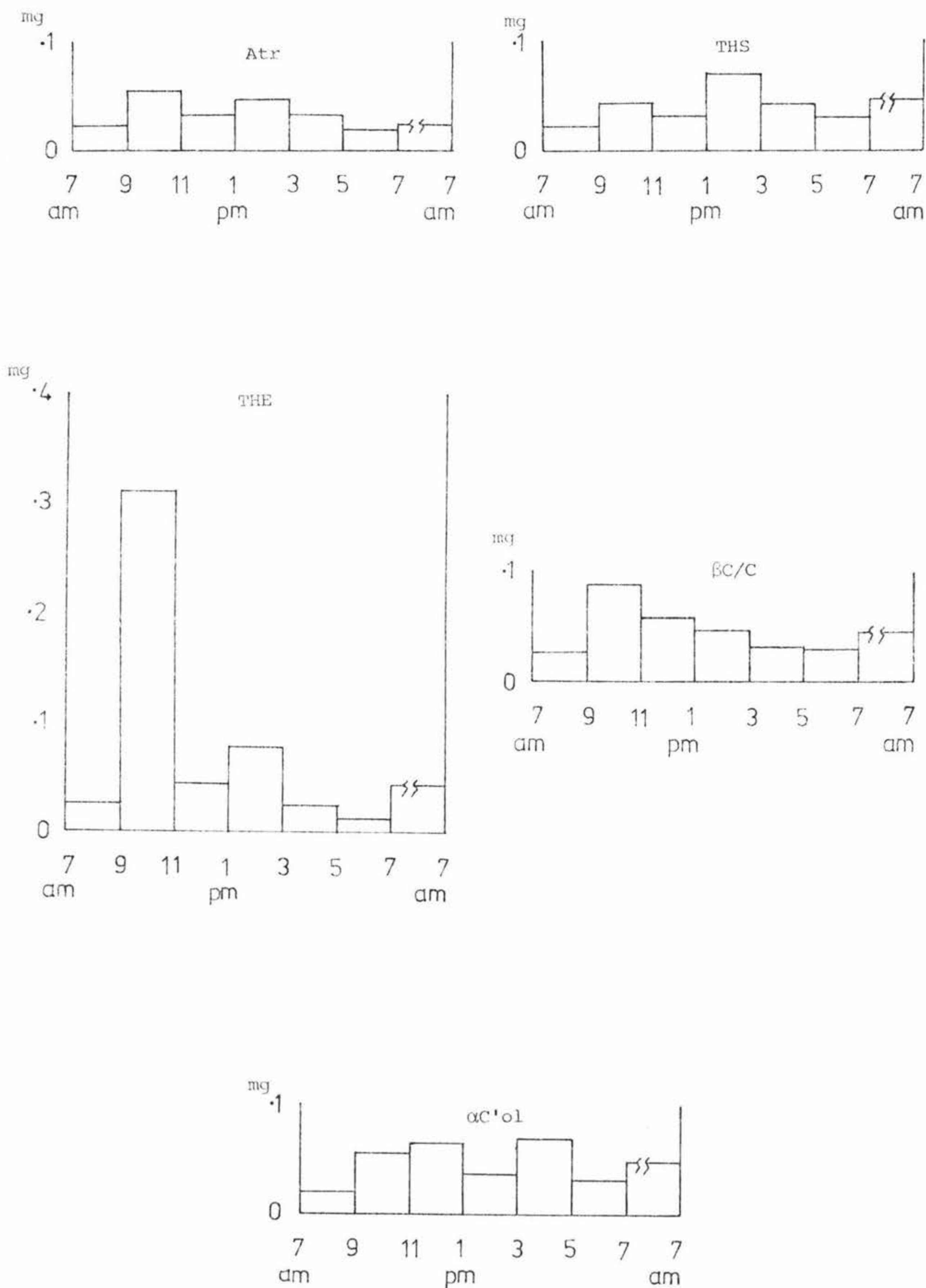
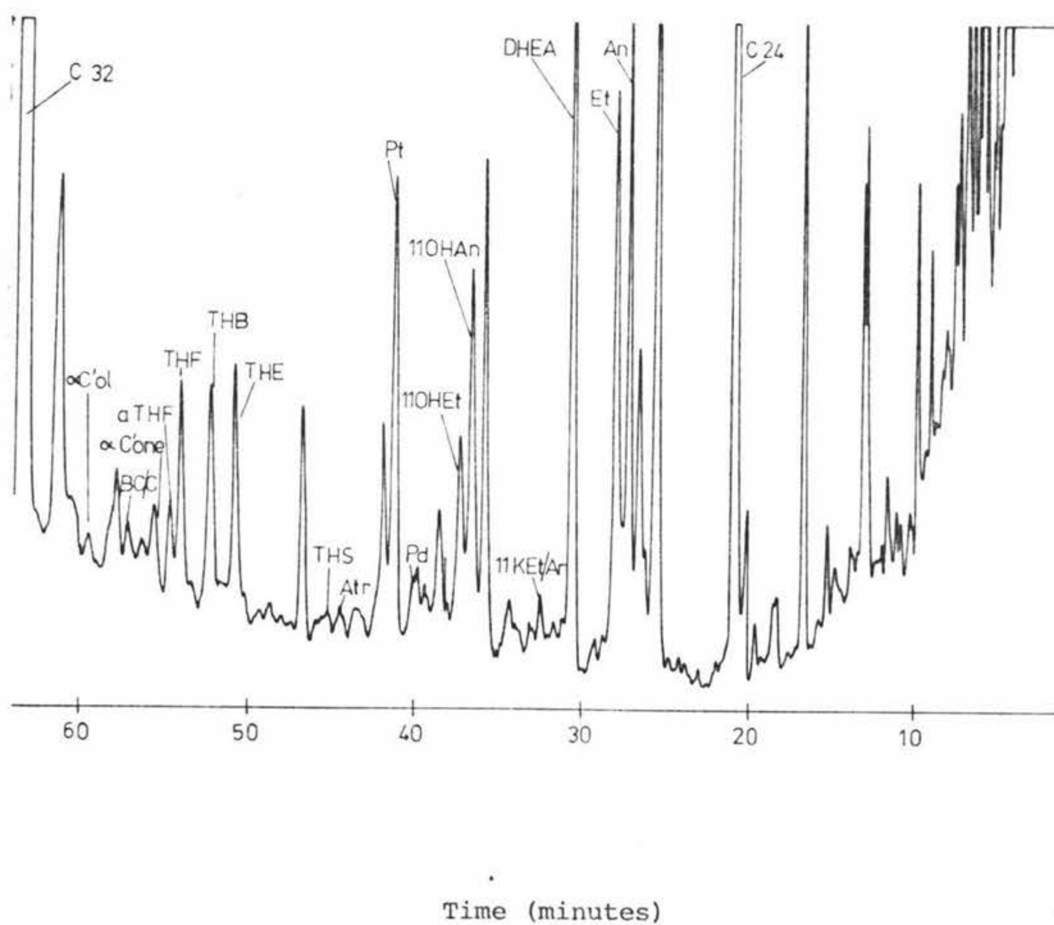


Figure 3iv(a)

GLC Profile of Subject 1, Diurnal Rhythm Experiment, 7am to 9am

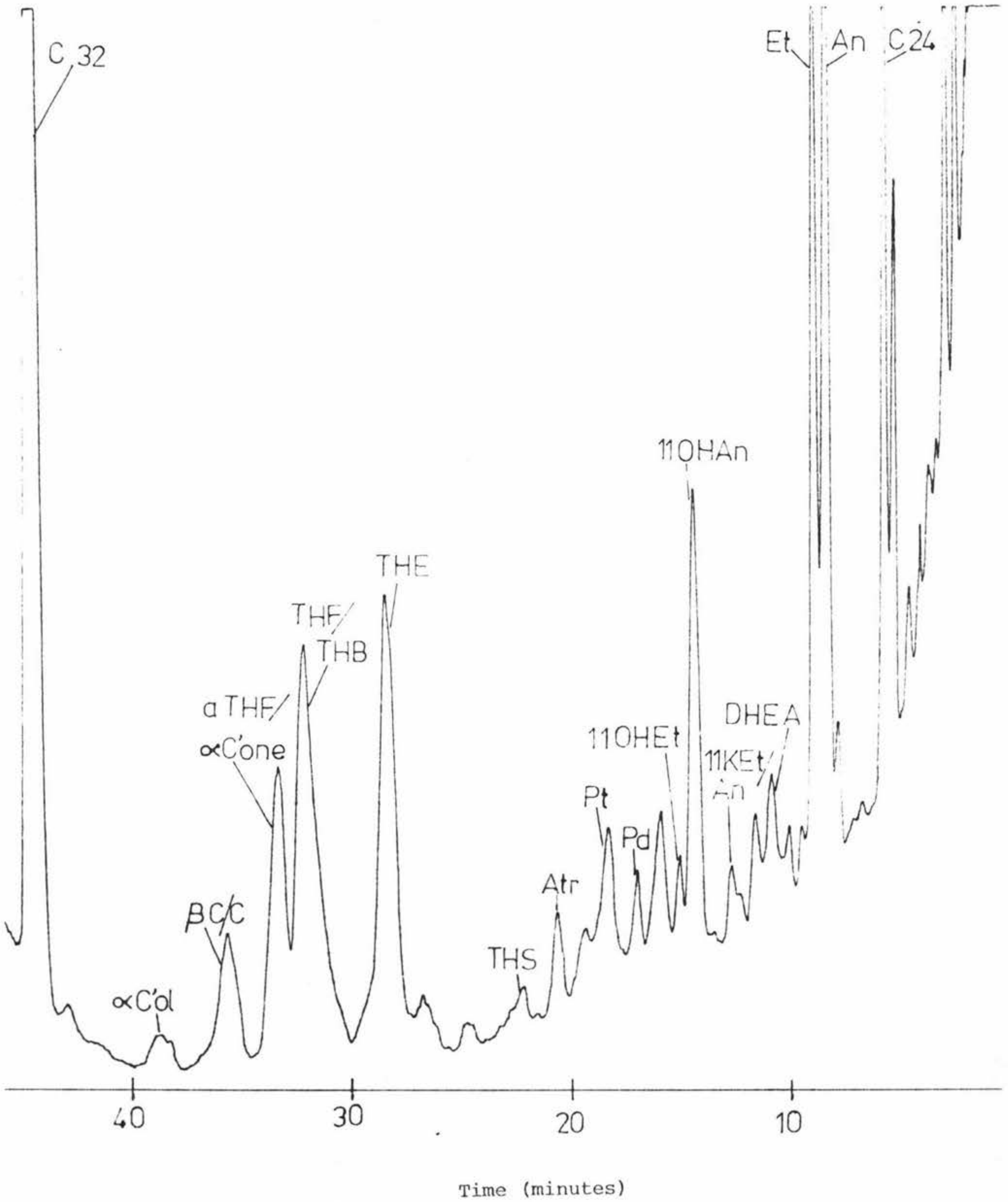


Time (minutes)

1.3 µl, sample, 40 meter column

Figure 3iv(b)

GLC Profile of Subject 2, Diurnal Rhythm Experiment, 9am to 11am



Time (minutes)

1.5 µl sample, 27 meter column

Table 3vii

Subject 1

	Steroids	Time 7am–9am	9am–11am	11am–1pm	1pm–3pm	3pm–5pm	5pm–7pm	7pm–7am
(1)	An / Et	0.9	0.9	0.9	1.0	1.0	1.0	0.9
(2)	aTHF / THF	1.0	0.8	1.1	1.4	1.3	1.3	1.4
(3)	THF / THE	0.5	0.4	0.4	0.5	0.5	0.4	0.4
(4)	THF+aTHF / THE	0.9	0.8	0.7	0.9	1.1	1.0	0.9
(5)	αC'one / THE	0.3	0.5	0.4	0.4	0.5	0.4	0.5
(6)	αC'ol / THF+aTHF	0.5	0.7	0.6	0.8	0.7	0.7	0.8
(7)	αC'ol / αC'one	1.6	1.3	1.2	2.0	1.6	1.5	1.6
(8)	11OHAn+11OHEt / 11KAn+11KEt	8.8	7.0	8.5	7.0	8.2	8.2	6.3
(9)	An+Et / DHEA	2.3	1.4	1.5	1.9	1.2	1.2	0.6
(10)	Pt / Pd	2.1	1.8	1.6	2.4	1.7	1.7	1.9

Subject 2

	Steroids	7am–9am	9am–11am	11am–1pm	1pm–3pm	3pm–5pm	5pm–7pm	7pm–7am
(1)	An / Et	1.1	1.0	1.0	0.9	0.9	1.0	1.0
(8)	11OHAn+11OHEt / 11KAn+11KEt	7.5	6.0	6.4	6.9	6.6	7.0	6.0
(9)	An+Et / DHEA	8.2	8.0	9.5	9.2	8.5	8.3	6.7
(10)	Pt / Pd	0.4	1.2	0.7	0.6	0.4	0.6	0.9

Conclusion

Subject 1 showed the highest excretion rate per 2 hours for every steroid measured between 9am and 1pm. All the neutral steroids decrease in excretion rate between 1pm and 5pm, followed by a slight increase, building up to a high between 9am and 1pm the following day. The evening collection was always collected as a single specimen, so no data on rates of excretion during the night are available, instead the rate is expressed for a 2 hour period by dividing the excretion rate by six.

Subject 2 followed the same pattern, but for almost every steroid there are two major peaks of excretion, one between 9am and 11am, and the other between 1pm and 3pm.

The level of excretion of DHEA, 11OHEt, Pt, THS and THE were markedly different compared with Subject 1, although following the same excretion pattern (a high mid-morning to mid-afternoon, preceeded and followed by a decline in excretion levels). The overall level of excretion of DHEA was about three times higher in Subject 1, and in Subject 2 a "burst" of THE excretion occurred between 9am and 11am, with the level over twice that excreted in Subject 1 in the corresponding period.

The ratios of $\frac{\text{An}}{\text{Et}}$ and $\frac{\text{11OHAn+11OHEt}}{\text{11KAn+11KEt}}$ compare well between Subjects 1 and 2. However the $\frac{\text{An+Et}}{\text{DHEA}}$ and the $\frac{\text{Pt}}{\text{Pd}}$ ratios vary quite considerably due to the high levels of excretion of DHEA and Pt respectively in Subject 1.

The pattern of excretion observed the 24 hours generally agrees with the work of Toccofondi et al (1970), Cavalleri et al (1974) and Beale and Tyrrell (1974), which concludes that the cortisol metabolites, as well as An, Et and DHEA, had highest values of excretion in the morning and a second smaller maximum in the afternoon.

(3) Cold Room Stress

The aim of this experiment was to expose the subject to a stressful situation (cold temperature). The subject was wearing normal indoor clothing so as not to counteract the effect of the cold. The subject began shivering uncontrollably at 10am and continued shivering for one hour after leaving the cold room.

Protocol

Subject 1 was subjected to cold stress by spending 2 hours (9am to 11am) in a cold room at $4^{o}C$. Urine samples were collected at 2 hour intervals as shown in Table 3viii and were analysed undiluted.

Table 3viii

Urine Volumes

Sample	Time	Volume (mls)
1	7am to 9am	215
2	9am to 11am	472
3	11am to 1pm	148
4	1pm to 3pm	130
5	3pm to 5pm	105
6	5pm to 7pm	108
7	7pm to 7am	397

Results

The excretion rates are presented in Table 3ix and illustrated graphically in Fig. 3v. Chromatograms are illustrated in Fig. 3vi and the steroid ratios are presented in Table 3x.

Table 3ix

mg excreted

Steroid	Time 7am 9am	9am 11am	11am 1pm	1pm 3pm	3pm 5pm	5pm 7pm	7pm 7am	7pm-7am 6
An	.220	.155	.145	.120	.115	.115	.530	.088
Et	.290	.190	.185	.145	.115	.115	.550	.091
DHEA	.270	.270	.180	.140	.097	.105	.370	.062
11KAn/Et	.073	.046	.049	.038	.020	.021	.084	.014
11OHAn	.295	.205	.160	.140	.120	.115	.520	.087
11OHEt	.225	.150	.140	.125	.096	.090	.425	.071
Pd	.160	.096	.089	.069	.036	.042	.225	.038
Pt	.155	.160	.087	.088	.071	.063	.385	.064
Atr	.066	.046	.033	.028	.016	.016	.076	.013
THE	.455	.215	.170	.205	.110	.110	.495	.083
THB	.300	.220	.145	.135	.080	.084	.330	.055
THF	.190	.110	.077	.081	.068	.047	.260	.043
aTHF	.180	.110	.079	.100	.072	.066	.295	.049
αC'one	.130	.079	.054	.057	.040	.036	.190	.032
βC/C	.155	.115	.064	.074	.053	.050	.185	.031
αC'ol	.195	.135	.082	.100	.079	.069	.290	.048

Figure 3v

Steroid Excretion Rates

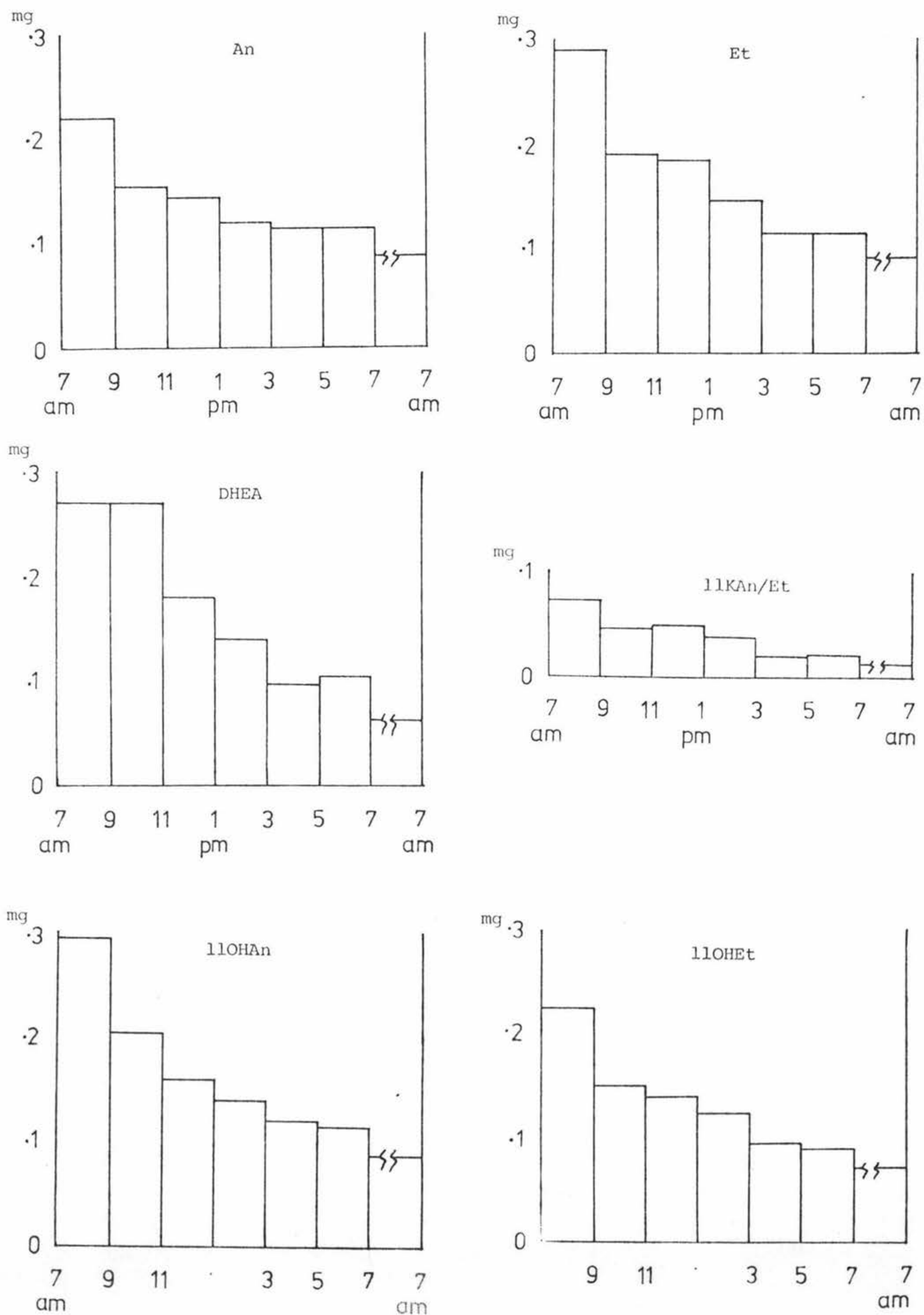
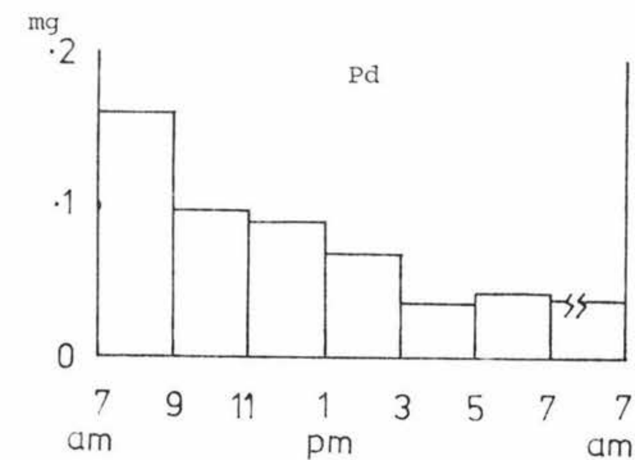
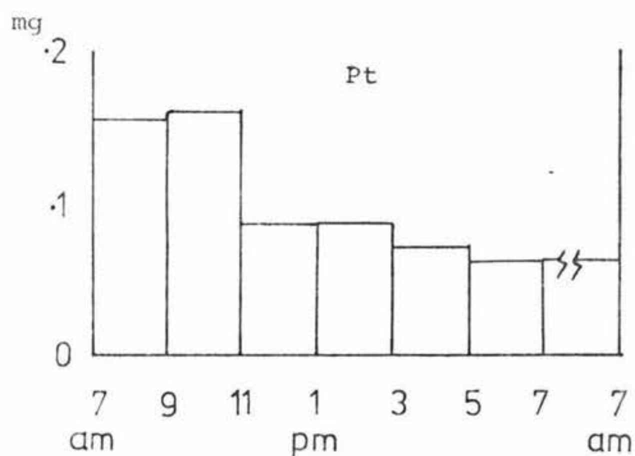
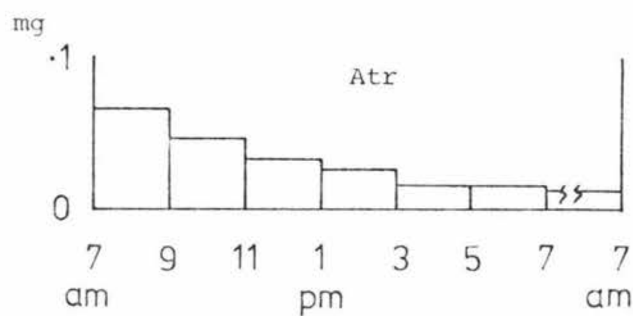
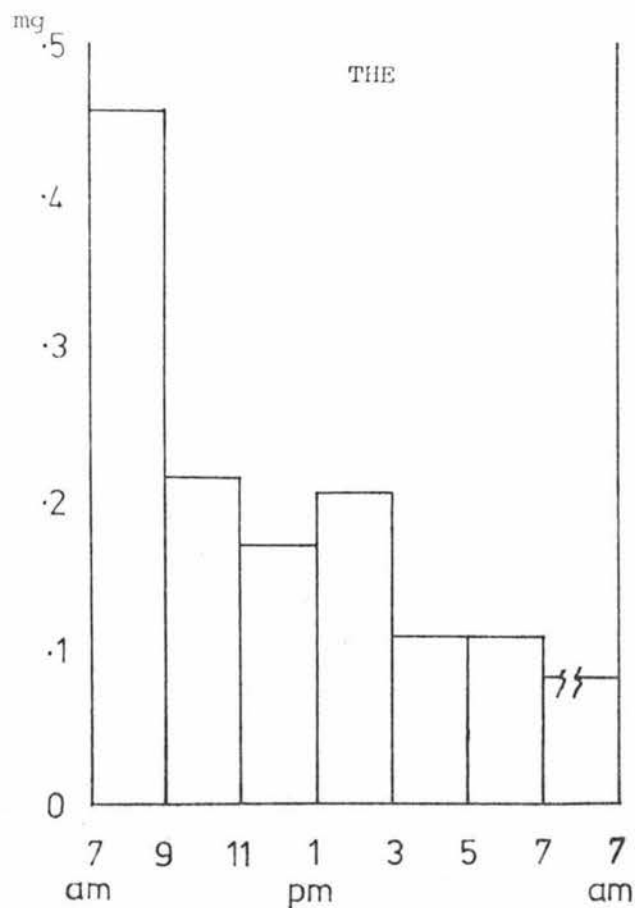


Figure 3v (cntd.)









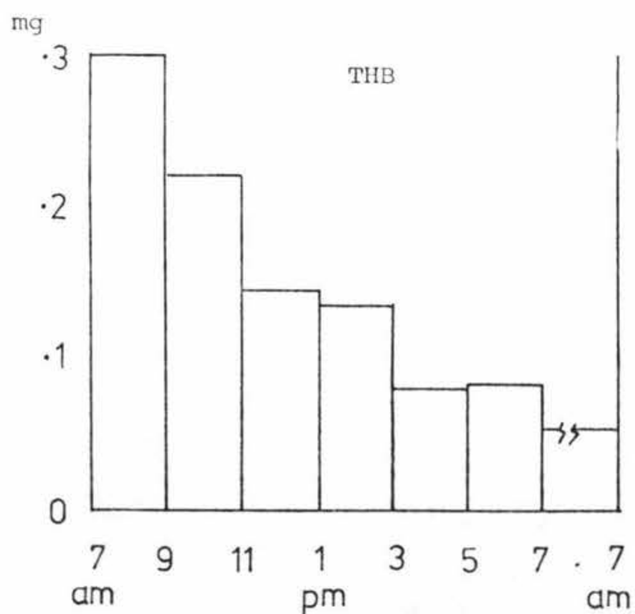
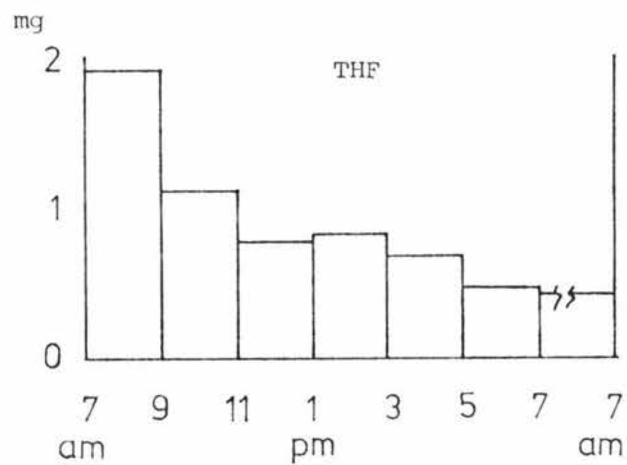
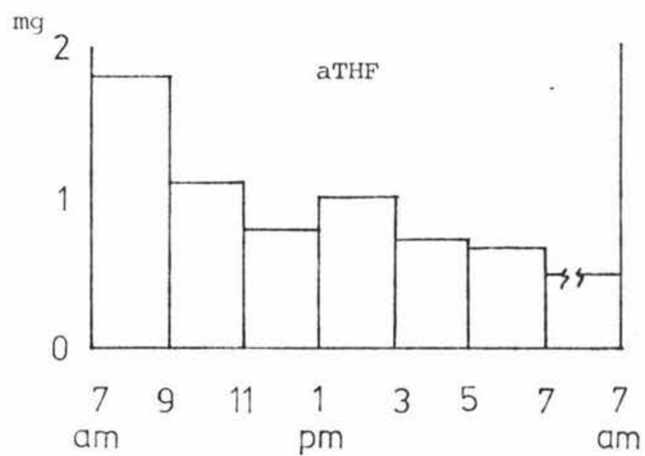
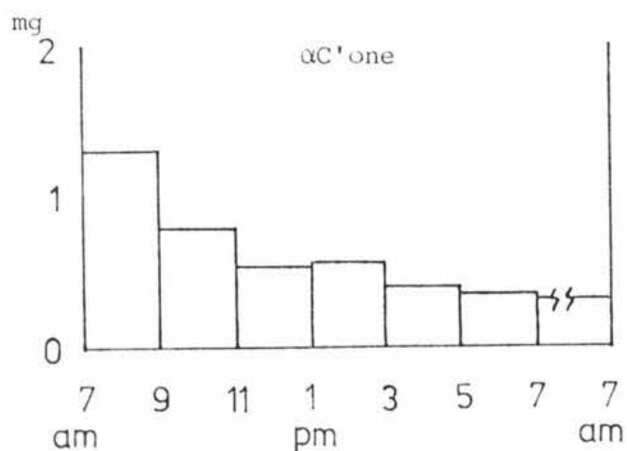
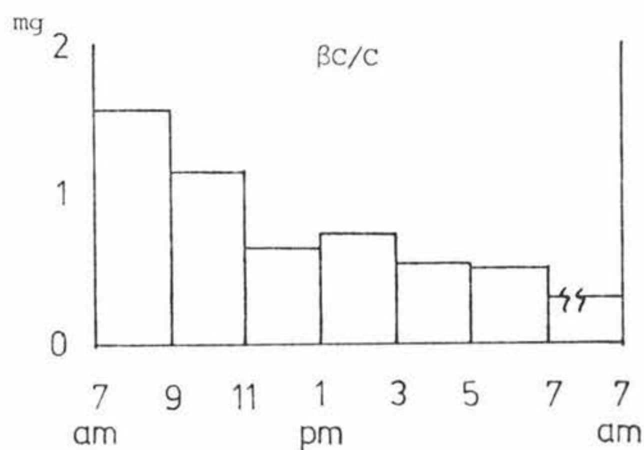


Figure 3v (cntd.)









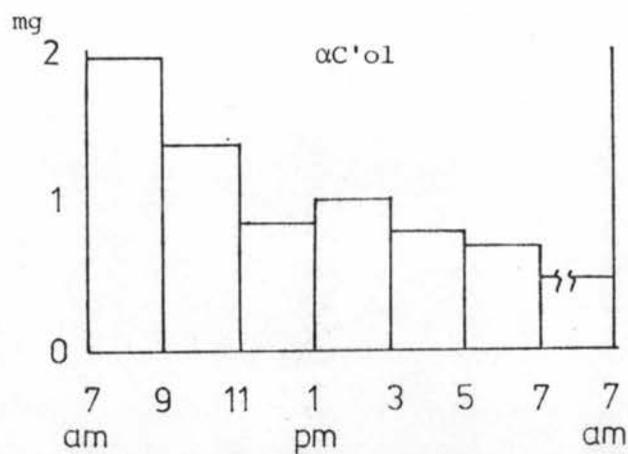
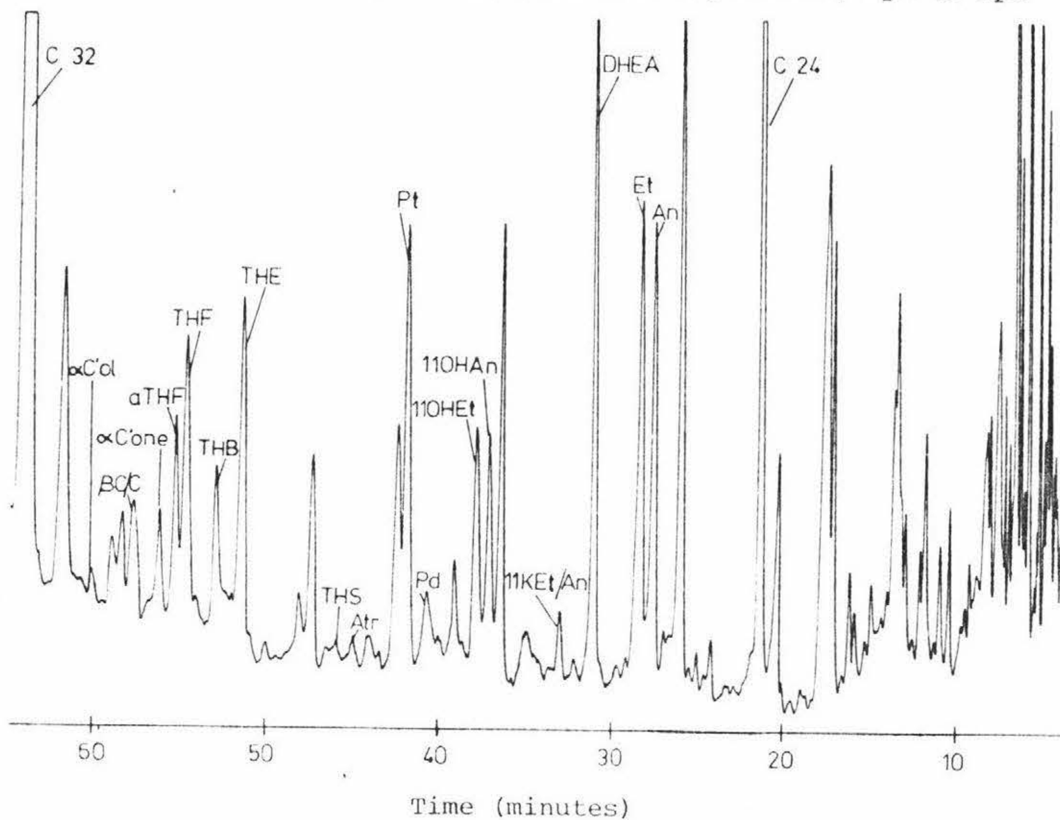


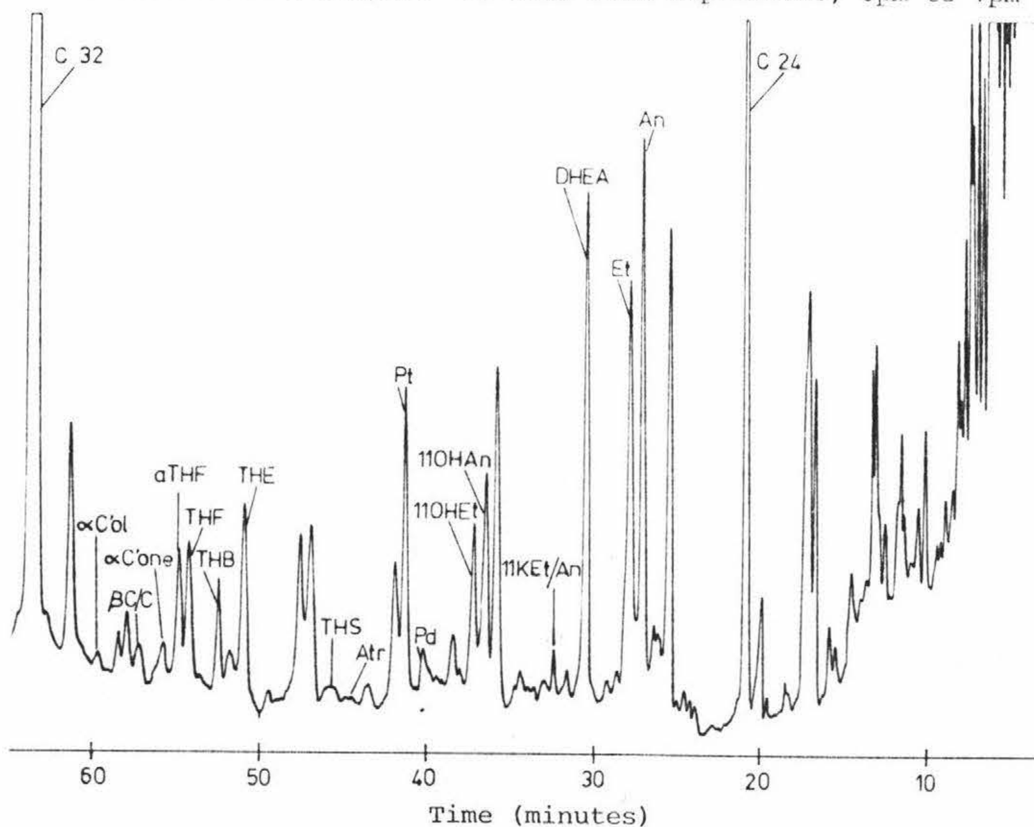
Figure 3vi

GLC Profile of Subject 1, Cold Room Experiment, 1pm to 3pm



1.2 µl sample, 40 meter column

GLC Profile of Subject 1, Cold Room Experiment, 5pm to 7pm



1.0 µl sample, 40 meter column

Table 3x

Steroid Ratios

Steroids	7am 9am	9am 11am	11am 1pm	1pm 3pm	3pm 5pm	5pm 7pm	7pm 7am
			Time				
(1) An / Et	0.8	0.8	0.8	0.8	1.0	1.0	1.0
(2) aTHF / THF	0.9	1.0	1.0	1.3	1.1	1.4	1.1
(3) THF / THE	0.4	0.5	0.5	0.4	0.6	0.4	0.5
(4) THF+aTHF / THE	0.8	1.0	0.9	0.9	1.3	1.0	1.1
(5) αC'one / THE	0.3	0.4	0.3	0.3	0.3	0.4	0.3
(6) αC'ol / THF+aTHF	0.6	0.6	0.6	0.6	0.6	0.6	0.5
(7) αC'ol / αC'one	1.5	1.7	1.5	1.8	2.0	1.9	1.5
(8) 11OHEt+11OHAn / 11KEt+11KAn	7.1	7.7	6.1	7.0	10.8	9.8	11.3
(9) An+Et / DHEA	1.9	1.3	1.8	1.9	2.4	2.2	2.9
(10) Pt / Pd	1.0	1.7	1.0	1.3	2.0	1.5	1.7

Conclusion

The pattern of excretion levels differs from the control experiment of the diurnal rhythm. There is a decrease in the excretion levels from the first collection (7am to 9am), showing no peaks at mid-morning to mid-afternoon. The high excretion rate of the 7am to 9am collection may have been due to the psychological stress imposed by the experiment, although the cold exposure itself did not appear to impose any noticeable changes of excretion of any metabolites.

Comparison of the steroid ratios show little or no change in all the steroid metabolites quantitated, except for the 7pm to 7am collection ratio for $\frac{An+Et}{DHEA}$, where a five fold difference exists, due to a much lower excretion rate of DHEA. Despite the large increase in excretion of the 7am to 9am collection, most of the excretion data for the rest of the collection periods is comparable to the levels observed in the basal diurnal rhythm experiment in the same subject.

By comparing the ratio of metabolites from adrenal origin to metabolites from testicular origin, any stress imposed on the subject should become apparent by an increase in the adrenal:testicular ratio. For this calculation it was assumed that An, Et and DHEA represent steroids of testicular origin; and cortols, cortolones, tetrahydro products, 11KAn, 11KEt, 11OHAn and 11OHEt represent steroids of adrenal origin (principally cortisol). From experiments 1 and 2 the adrenal:testicular ratio varies from 1.7 to 2.1. The ratio increased to 2.8 and 2.6 for the 7am to 9am and the 1pm to 3pm collection respectively, implying a stress effect despite the subject being stressed only between 9am and midday (cold room and shivering).

The observation of Kuhl et al (1952) that "cold stress" shows a marked increase in the excretion of total 17-oxosteroids is not validated in this experiment. Vestergaard (1978) suggests that only severe conditions will activate the adrenal cortex to a marked degree, so if this is the case, 2 hours at $4^{o}C$ was not imposing sufficient stress to alter excretion rates of the steroids.

(4) Alcohol Loading

The effect of ethanol on the steroid profile of a non-alcoholic subject was examined by collecting urine samples at 2 hour intervals before, during and after alcohol loading.

Protocol

Subject 1 consumed ethanol (vodka) to maintain a blood alcohol level of approximately 50 mg% for three hours. Breath levels were measured using an Alcolmeter AE-D1 (Lion Laboratories, Cardiff, Scotland). Protocol is shown in Table 3xi.

Table 3xi

Alcohol Loading Experiment Protocol

Time	Alcohol Consumed	"Blood Alcohol" (mg%)	Urine volume (mls)
7.00am - 9.00am	-	-	81
9.00am - 9.30am	90 mls vodka	-	-
9.50am	-	33	-
9.53am	18 mls vodka	-	-
10.10am	-	52	-
10.30am	18 mls vodka	53	-
10.50am	-	52	-
11.00am	18 mls vodka	-	470
11.25am	-	53	-
11.57am	-	46	-
12.00pm	18 mls vodka	-	-
12.20pm	-	53	-
12.55pm	-	46	-
1.00pm	-	-	229
1.15pm	-	40	-
1.36pm	-	31	-
2.03pm	-	23	-
2.38pm	-	13	-
3.00pm	.-	-	86
3.00pm - 5.50pm	-	-	131
5.50pm - 7.00pm	-	-	60
7.00pm - 7.00am	-	-	745

Results

The steroid excretion rates are presented in Table 3xii, and illustrated graphically in Fig. 3vii. Chromatograms are illustrated in Fig. 3viii, and the steroid ratios are presented in Table 3xiii.

Table 3xii

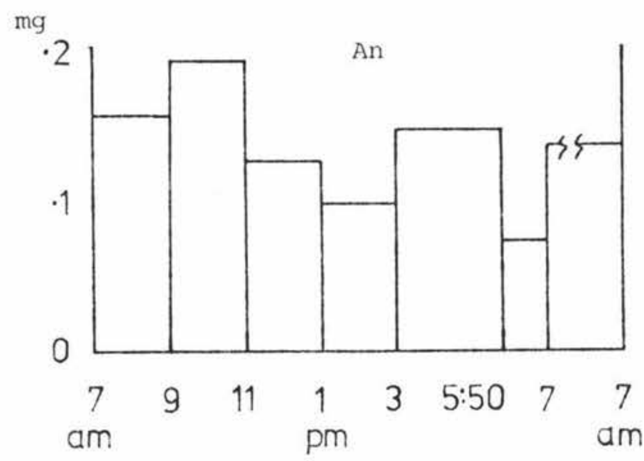
mg excreted

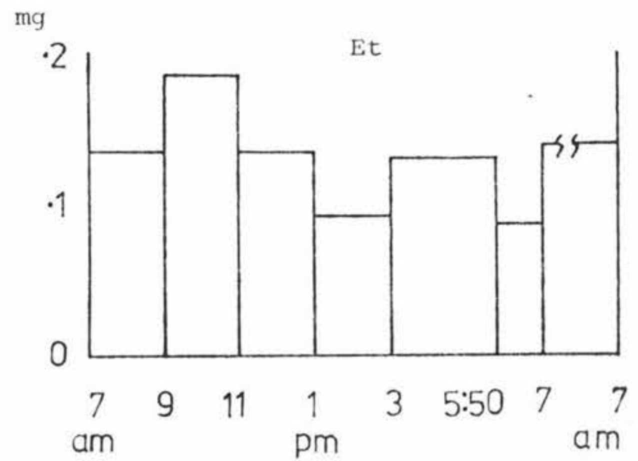
Time

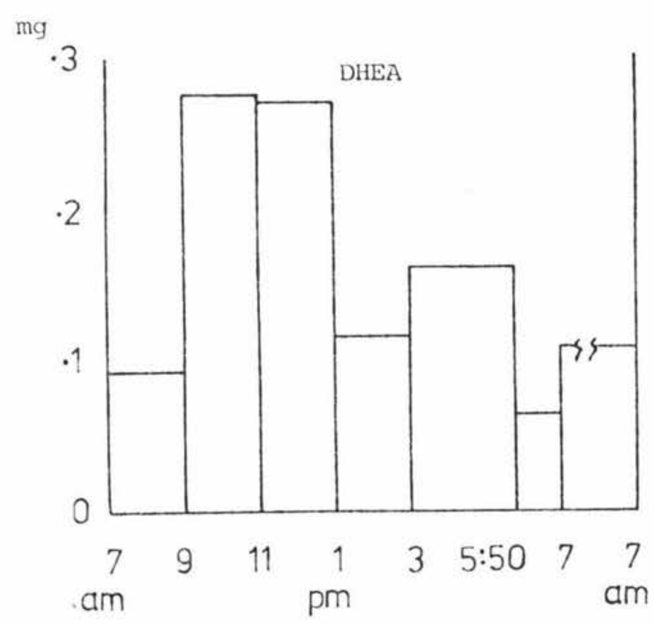
Steroid	7am 9am	9am 11am	11am 1pm	1pm 3pm	3pm 5pm	5pm 7pm	7pm 7am	7pm-7am / 6
An	.155	.190	.125	.098	.145	.072	.805	.134
Et	.135	.185	.135	.093	.130	.088	.870	.145
DHEA	.091	.275	.270	.115	.160	.062	.655	.109
11KEt/An	.017	.028	.022	.015	.025	.015	.068	.011
11OHAn	.155	.215	.150	.115	.160	.065	.790	.132
11OHEt	.062	.115	.170	.072	.115	.054	.500	.083
Pd	.039	.100	.065	.038	.062	.026	.260	.044
Pt	.052	.110	.145	.073	.160	.036	.410	.068
Atr	.014	.047	.029	.014	.023	-	.099	.017
THE	.165	.155	.120	.110	.195	.062	.815	.136
THB	.071	.190	.084	.225	.125	.060	.660	.110
THF	.094	.079	.061	.052	.098	.032	.400	.067
aTHF	.076	.097	.081	.070	.115	.034	.420	.070
αC'one	.062	.067	.047	.051	.070	.028	.280	.047
βC/C	.043	.089	.057	.036	.082	.023	.335	.056
αC'ol	.069	.100	.072	.054	.095	.031	.445	.074

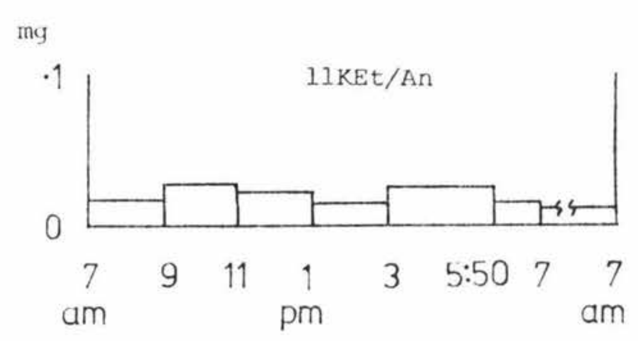
Figure 3vii

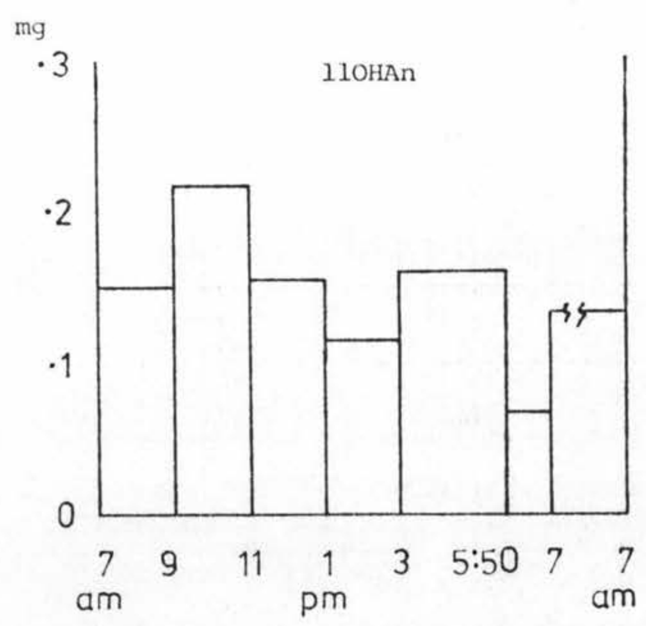
Steroid Excretion Rates











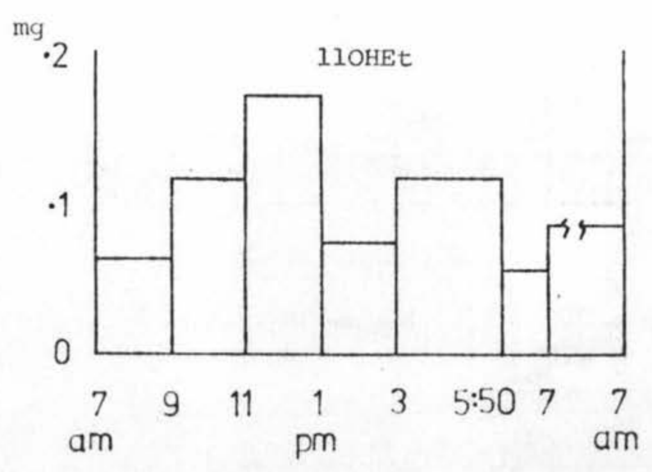
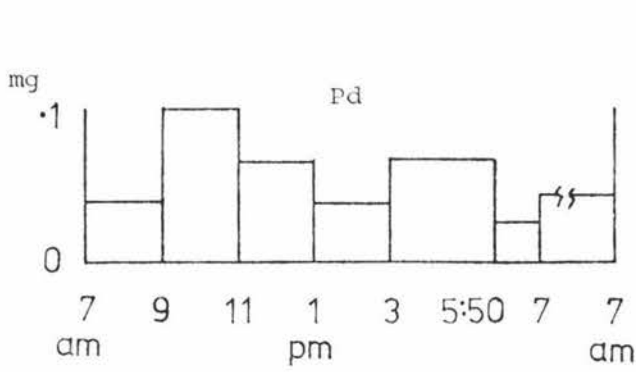
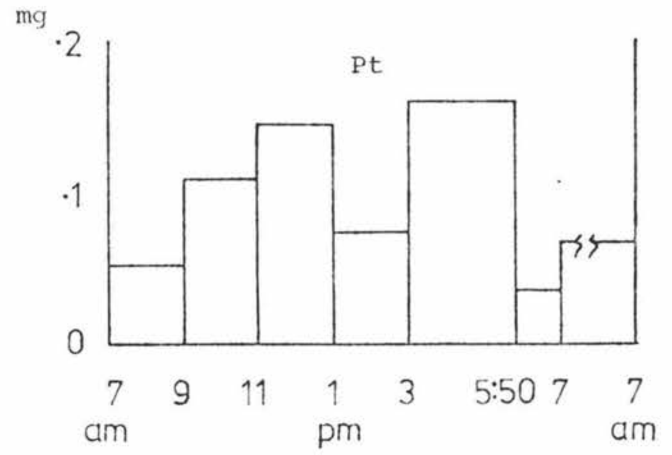
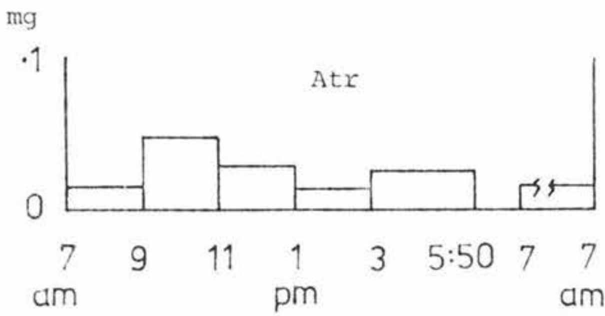
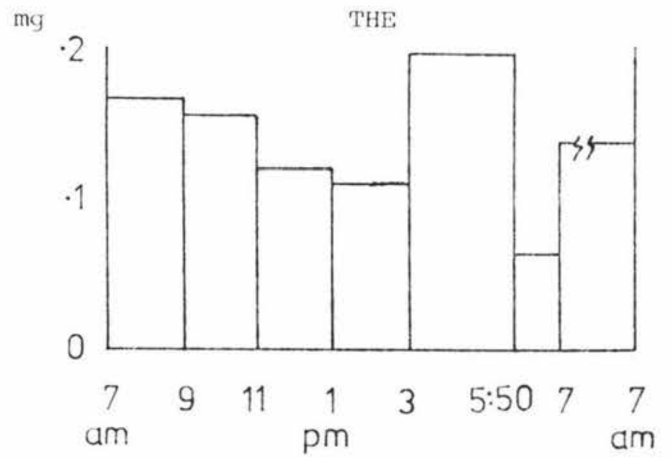


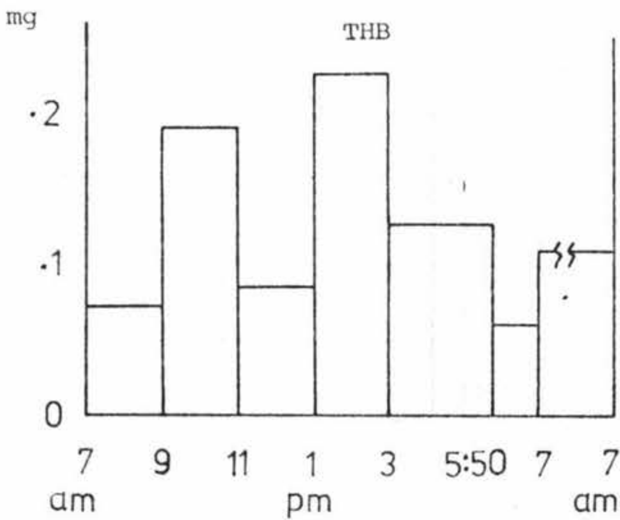
Figure 3vii (cntd.)











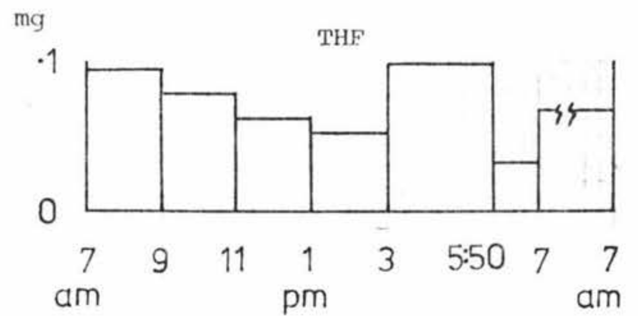
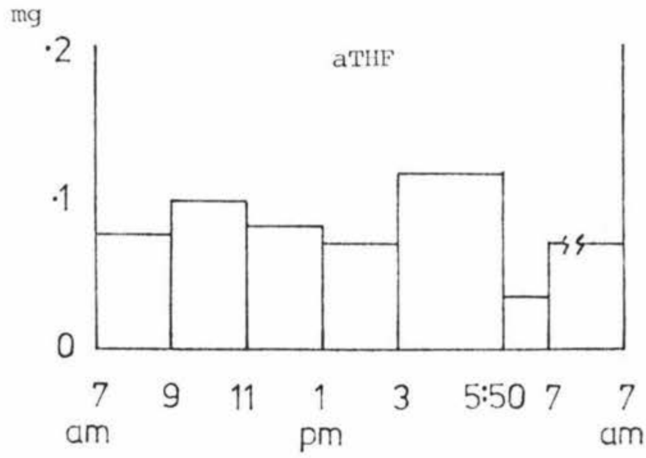
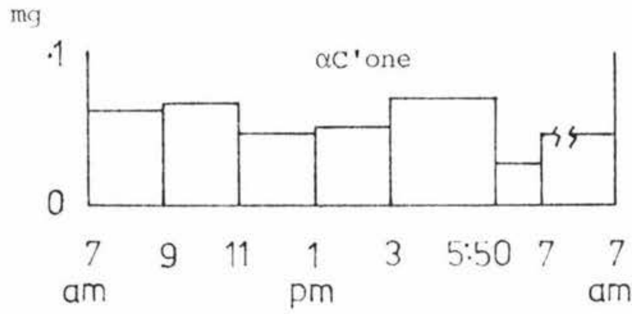
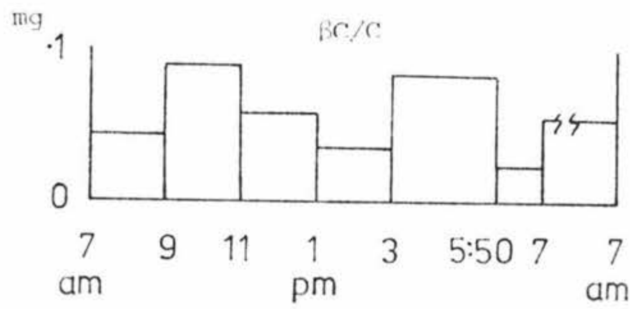


Figure 3vii (cntd.)







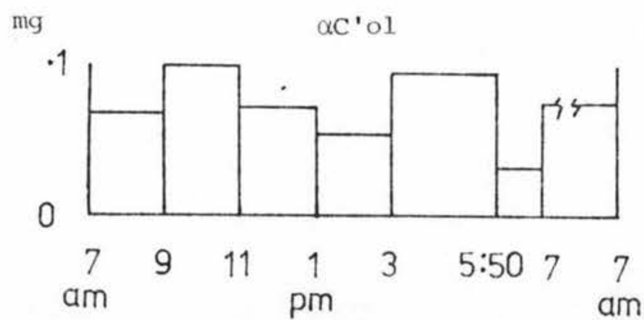
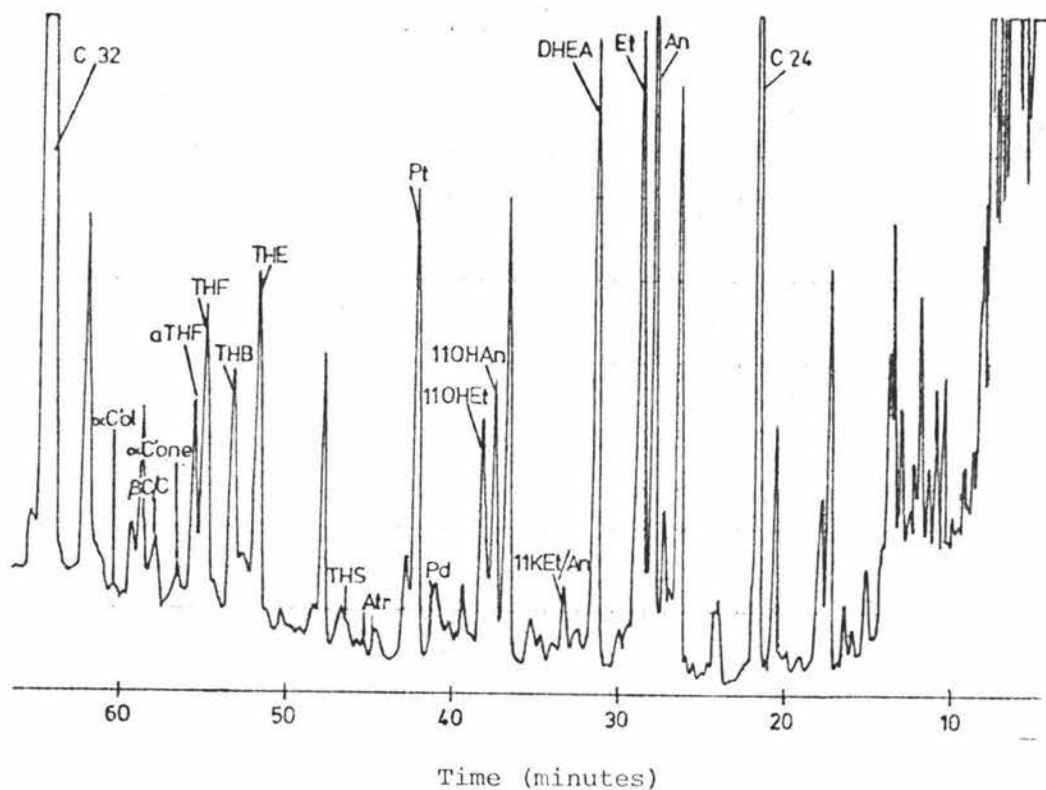


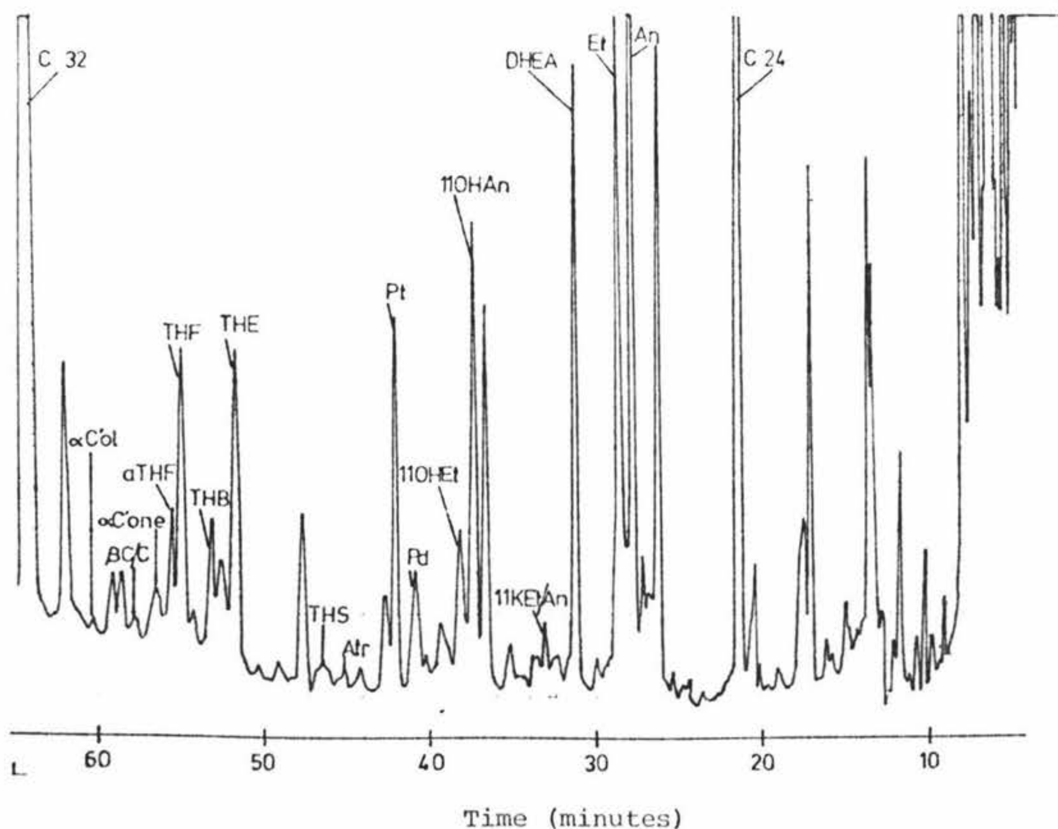
Figure 3viii

GLC Profile of Subject 1, Alcohol Loading Experiment, 7am to 9am



1.4 µl sample, 40 meter column

GLC Profile of Subject 1, Alcohol Loading Experiment, 5.50pm to 7pm



1.3 µl sample, 40 meter column

Table 3xiii

Steroid Ratios

	Steroids	Time						
		7am 9am	9am 11am	11am 1pm	1pm 3pm	3pm 5.50pm	5.50pm 7pm	7pm 7am
(1)	An / Et	1.2	1.0	0.9	1.1	1.1	0.8	0.9
(2)	aTHF / THF	0.8	1.3	1.3	1.4	1.1	1.1	1.0
(3)	THF / THE	0.6	0.5	0.5	0.5	0.5	0.5	0.5
(4)	THF+aTHF / THE	1.0	1.1	1.2	1.1	1.1	1.1	1.0
(5)	αC'one / THE	0.4	0.4	0.4	0.5	0.4	0.5	0.4
(6)	αC'ol / THF+aTHF	0.4	0.6	0.5	0.4	0.5	0.5	0.5
(7)	αC'ol / αC'one	1.1	0.7	1.5	1.1	1.4	1.1	1.6
(8)	11OHEt+11OHAn / 11KEt+11KAn	12.8	11.8	14.6	12.5	11.0	8.0	19.5
(9)	An+Et / DHEA	3.2	1.4	1.0	1.7	1.7	2.2	2.6
(10)	Pt / Pd	1.3	1.1	2.2	1.9	2.6	1.4	1.5

Conclusion

Overall the pattern of excretion and the steroid ratios are similar to the control diurnal experiment, but there is a distinct increase in the excretion of steroid metabolites between 3pm and 5.50pm in the alcohol loading experiment compared with the basal levels of all steroids measured. The 7pm to 7am levels of excretion were increased also for An, Et, 11OHAn, THE and THF from 20 to 30%, but this variation is possible within the daily experimental variation.

DHEA showed a similar excretion pattern in alcohol loading compared to the basal levels from 7am to 5.50pm, but the level of excretion was almost 50% less than the control from 5.50pm to 7am, implying increased levels of reduction of the C5 double bond, supported by an increase in the $\frac{An+Et}{DHEA}$ ratio, but this may not be due to any influences exerted by ethanol loading as a similar ratio exists for the 7am to 9am collection.

THB shows a significantly different excretion pattern, with a decrease between 11am and 1pm, and an increase between 1pm and 5.50pm, compared with basal levels. No significant changes in the adrenal:testicular ratio were observed.

11KEt and 11KAn showed the same basic pattern of excretion (except for 3pm to 5.50pm), but the values were about half that of the basal level. Consequently the $\frac{11OHAn+11OHEt}{11KAn+11KEt}$ ratio was increased two fold during alcohol loading, implying increased reduction at the C11 keto function.

(5) Dexamethasone Administration

Administering dexamethasone causes a blockage in the excretion of 17 hydroxycorticoids, and to a lesser extent 17 ketosteroids, which will be evident from the steroid profiles.

Protocol

Subject 1 was administered dexamethasone, 0.5 mg per 6 hours (2 mg/day) on day 2 and 3 of the four day experiment. Protocol is shown in Table 3xiv.

Table 3xiv

Dexamethasone Administration Protocol

Day	Time	Dexamethasone (mg)	Urine Volume (mls)
1	7am - 7am	-	940
2	7am	0.5	-
	1pm	0.5	-
	7pm	0.5	-
3	1am	0.5	-
	7am - 7am	-	1872
	7am	0.5	-
	1pm	0.5	-
	7pm	0.5	-
4	1am	0.5	-
	7am - 7am	-	1525
5	7am - 7am	-	805

Results

The excretion rates are presented in Table 3xv and illustrated graphically in Fig. 3ix. Chromatograms are shown in Fig. 3x and the steroid ratios are presented in Table 3xvi.

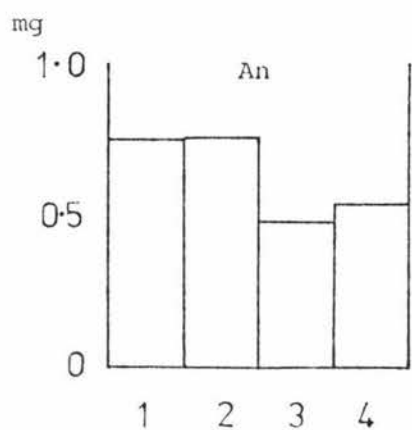
Table 3xv

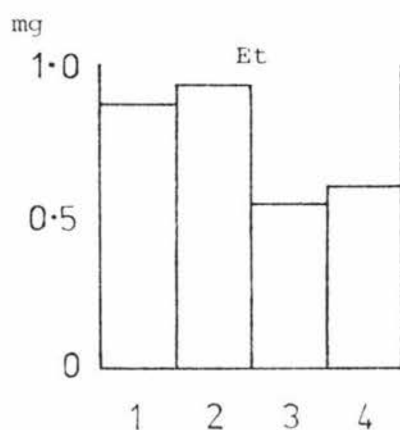
mg excreted/day

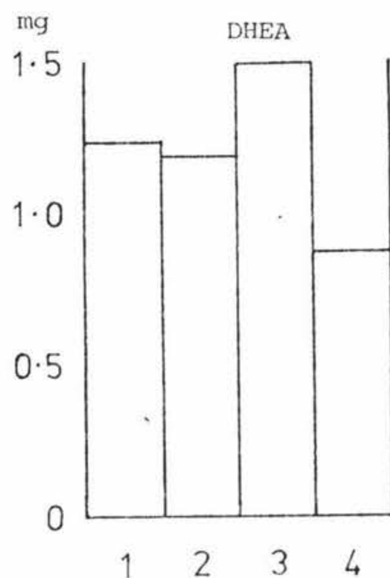
Steroid	Day 1	2	3	4
An	0.75	0.76	0.48	0.54
Et	0.87	0.93	0.54	0.60
DHEA	1.23	1.19	1.49	0.87
11KEt/An	0.19	0.15	0.025	0.036
11OHAn	0.85	0.97	0.42	0.46
11OHEt	0.71	0.44	0.11	0.17
Pd	0.45	0.52	0.26	0.19
Pt	0.72	1.31	0.87	0.46
Atr	0.17	0.13	0.064	0.073
THS	0.23	0.30	0.16	0.11
THE	0.95	0.75	0.27	0.16
THB	1.11	1.60	0.99	0.64
THF	0.55	0.41	0.12	0.099
aTHF	0.64	0.53	0.19	0.15
αC'one	0.48	0.42	0.24	0.15
βC/C	0.60	0.48	0.23	0.17
αC'ol	0.72	0.69	0.33	0.21

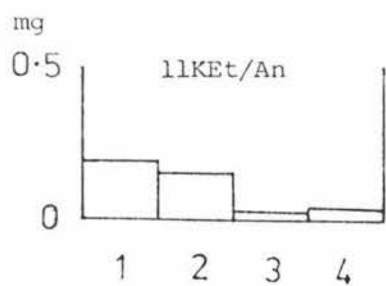
Figure 3ix

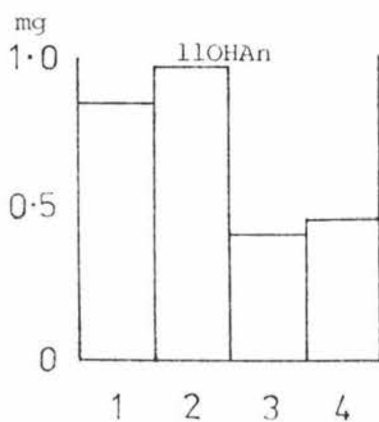
Steroid Excretion Rates

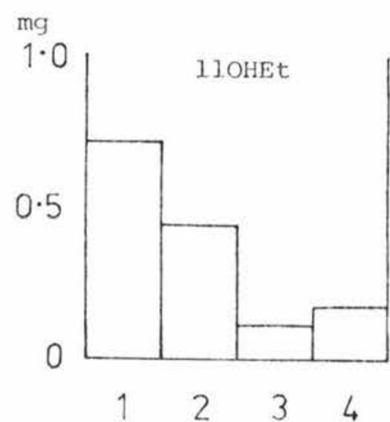


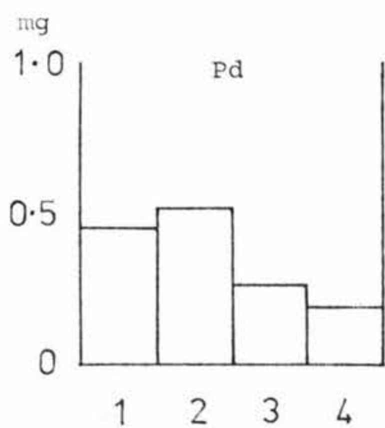


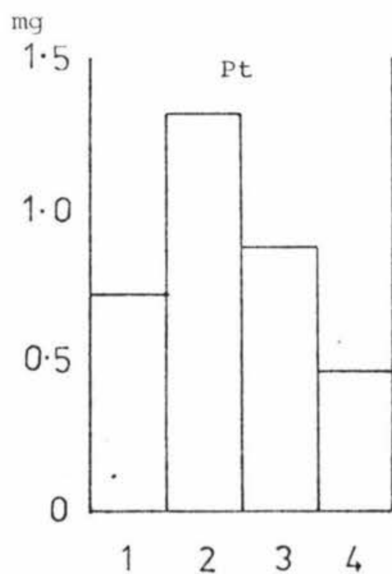












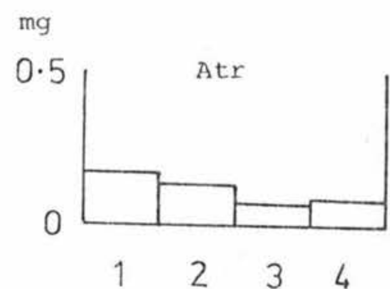
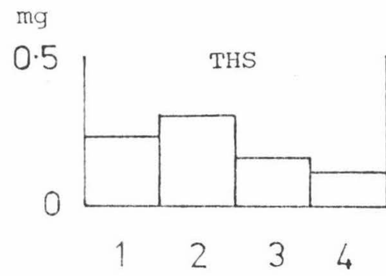
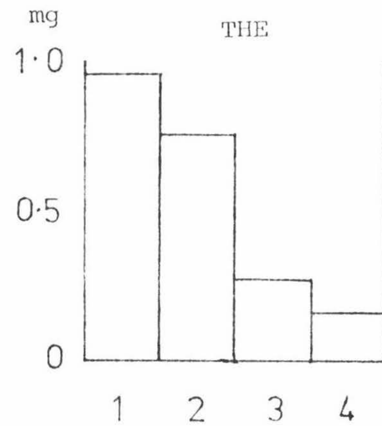
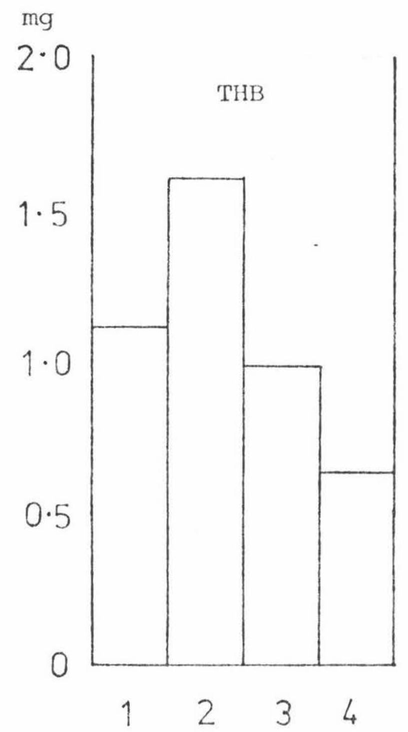
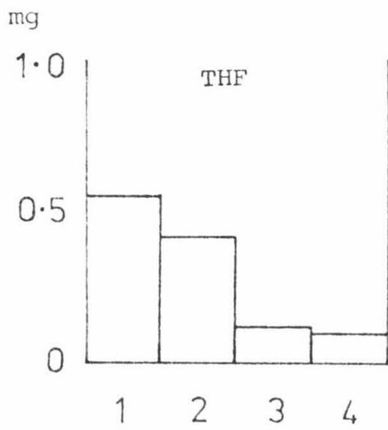


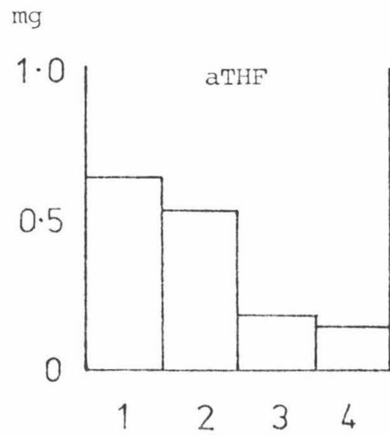
Figure 3ix (cntd.)

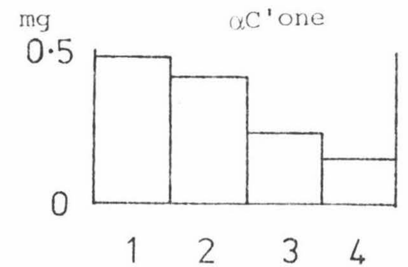


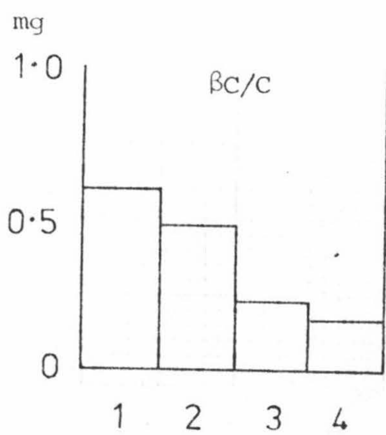












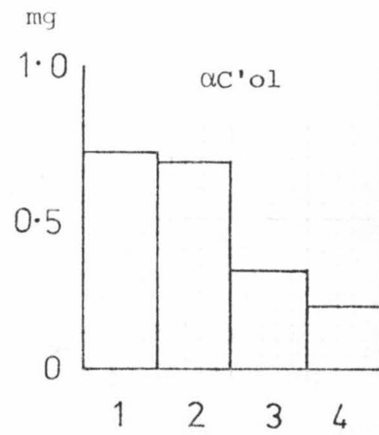
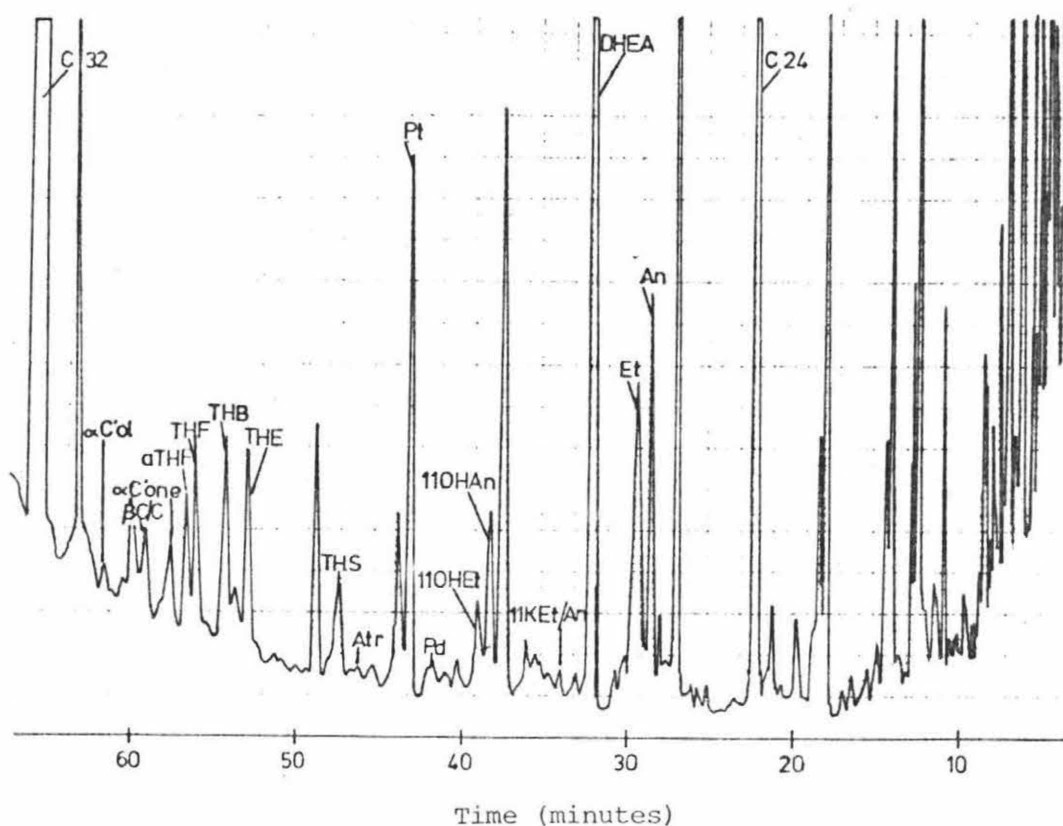


Figure 3x

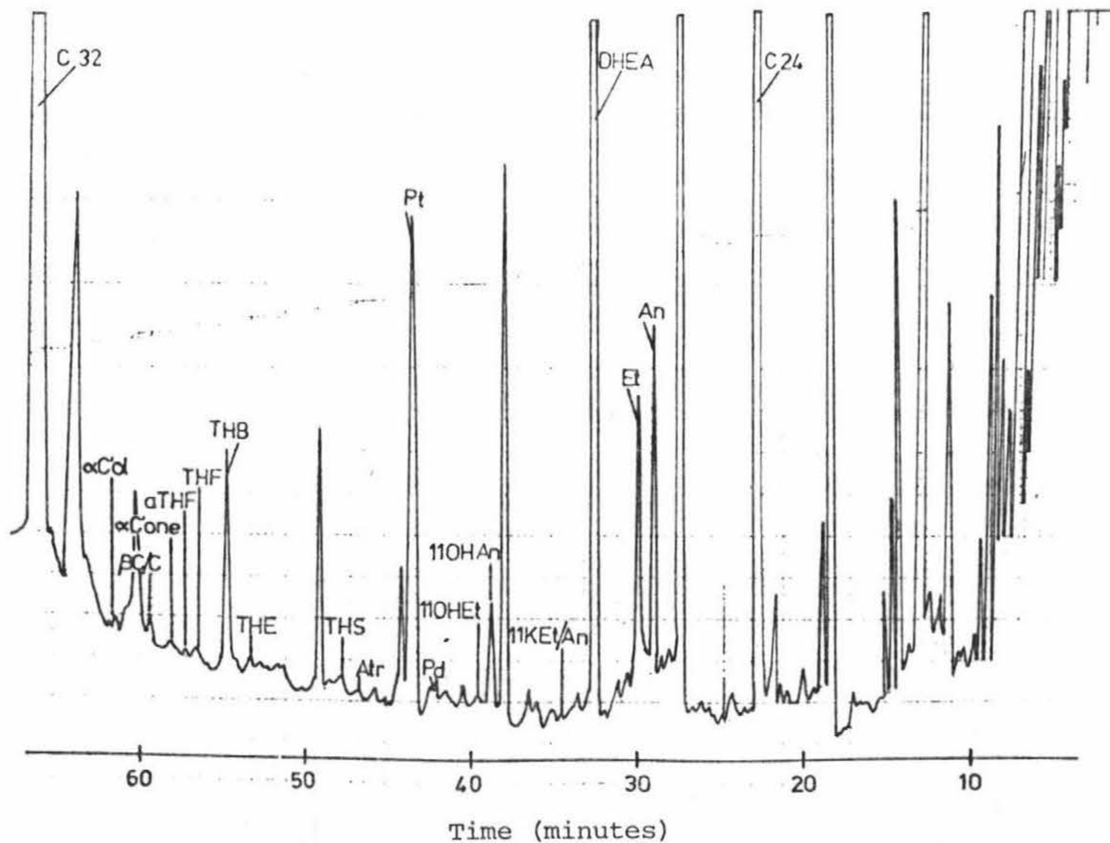
GLC Profile of Subject 1, Dexamethasone Administration Experiment, Day 1



Time (minutes)

1.5 µl sample, 40 meter column

GLC Profile of Subject 1, Dexamethasone Administration Experiment, Day 4



Time (minutes)

1.4 µl sample, 40 meter column

Table 3xvi

Steroid Ratios

	Steroids	Day 1	2	3	4
(1)	An / Et	0.9	0.8	0.9	0.9
(2)	aTHF / THF	1.1	1.3	1.7	1.4
(3)	THF / THE	0.6	0.6	0.4	0.6
(4)	THF+aTHF / THE	1.3	1.3	1.2	1.6
(5)	αC'one / THE	0.5	0.6	0.9	0.9
(6)	αC'ol / THF+aTHF	0.6	0.7	1.1	0.9
(7)	αC'ol / αC'one	1.5	1.6	1.4	1.4
(8)	11OHAn+11OHEt / 11KAn+11KEt	8.2	9.4	21.2	17.5
(9)	An+Et / DHEA	1.3	1.4	0.7	1.3
(10)	Pt / Pd	1.6	2.5	3.3	2.4

Conclusion

Dexamethasone at a dosage of 0.5 mg every six hours for eight doses induces almost complete suppression of 17 hydroxycorticoid excretion by blocking corticotropin releasing hormone from the hypothalamus (Liddle, 1960). An increase of cortisol (or analog) initiates a sequence of events which ultimately restrains further secretion of cortisol. The urinary 17 ketosteroid excretion rate does not fall during suppression of ACTH as readily as does 17 hydroxycorticoid excretion.

The excretion of 17 hydroxysteroids decreased three fold for THS, to six fold for THE, from the first day to the fourth day. The excretion of 17 ketosteroids decreased one and a half fold for Et to five fold for 11KEt/An, from the first day to the third day.

The 17 hydroxysteroids appear to be more sensitive to suppression by dexamethasone than the 17 ketosteroids, in both the degree of suppression and the duration of effect. For all the 17 ketosteroids, with the exception of DHEA, the levels on the third day were the lowest, with a slight increase of excretion on the fourth day. The excretion level on Day 1 and 2 was similar except for 11OHEt, where the level on Day 2 was 40% less.

DHEA levels were similar throughout the four day experiment. Consequently the $\frac{\text{An+Et}}{\text{DHEA}}$ ratio decreased on Day 3, but was consistent for Day 1, 2 and 4. The $\frac{\text{11OHAn+11OHEt}}{\text{11KAn+11KEt}}$ ratio increased about two and a half fold on Day 3, and to a lesser extent on Day 4, indicating increased reduction at the C11 oxo function.

All the 17 hydroxysteroid excretion rates decreased over Days 2, 3 and 4, with the exception of Atr which increased marginally on Day 4. This may have been due to the base-line noise which produces a decreased viability of the measurement of steroids with small peak areas.

The $\frac{\alpha\text{C'one}}{\text{THE}}$ and $\frac{\alpha\text{C'ol}}{\text{THF+aTHF}}$ ratios did increase almost two fold suggesting an increase in the reduction at the C20 oxo function on Day 3. An increased $\frac{\text{Pt}}{\text{Pd}}$ ratio also supports this increased reduction.

The adrenal:testicular ratio drops from 2.4 on Day 1 to 1.2 on Days 3 and 4, implying a 50% decrease in adrenal excretion of steroids by administration of dexamethasone (assuming excretion of steroids from testicular origin was constant).

The absolute excretion values of the steroid metabolites are noticeably lower than the control ten consecutive day collection values. This can be attributed to the β-Glucuronidase enzyme used for

the hydrolysis of the steroid conjugates in this experiment and also in Experiment 7. The delivery of this enzyme was delayed, and a decrease in the activity occurred due to improper storage of the enzyme.

Because of the differing rates of enzymic hydrolysis of steroid conjugates the absolute excretion values provide invalid results. However if the hydrolysis rates are assumed constant then the ratio of steroids still provides useful information.

Despite the loss of some activity of the β-Glucuronidase, the general trends of the results reinforces the findings of Liddle (1960), that dexamethasone decreases 17 hydroxycorticoid excretion, and to a lesser extent 17 ketosteroid excretion

(6) Alcoholic Alcohol Loading

In this experiment a more dramatic degree of alcohol loading was used. The subject was a 29 year old male with a history of alcohol abuse over the previous ten years.

Protocol

The subject provided urine collected over a three day period. On Day 1 and 3 no alcohol was consumed, on Day 2 a 26 ounce bottle of Scotch was consumed. Very little alcohol was consumed on the day before the three day programme commenced. Protocol is illustrated in Table 3xvii. The subject was taking prescribed ephedrine hydrochloride for asthma. Breath levels were measured on an Alcolmeter AE-Dl (Lion Laboratories, Cardiff, Scotland).

Table 3xvii

Day	Time	"Blood Alcohol" (mg%)	Urine Volume (mls)
1	-	-	510
2	12.10pm	102	-
	1.10pm	121	-
	2.20pm	140	-
	-	-	2135
3	-	-	700

Results

The excretion rates are presented in Table 3xviii and illustrated graphically in Fig. 3xi. Chromatograms are shown in Fig. 3xii and the steroid ratios are presented in Table 3xix.

Table 3xviii

mg excreted/day

Steroid	Day 1	Day 2	Day 3
An	1.05	1.70	1.10
Et	1.77	3.26	1.86
DHEA	0.50	1.62	0.87
11KEt/An	0.14	0.27	0.17
11OHAn	0.92	1.64	1.09
11OHEt	0.57	1.16	0.74
Pd	0.18	0.52	0.38
Pt	0.43	1.19	0.46
Atr	0.33	0.89	0.31
THS	0.19	0.49	0.26
THE	0.81	1.74	0.79
THB	1.06	2.52	1.33
THF	0.47	1.03	0.37
aTHF	0.29	0.70	0.43
αC'one	0.36	0.69	0.34
βC/C	0.35	0.76	0.47
αC'ol	0.68	0.96	0.75

Figure 3xi

Steroid Excretion Rates

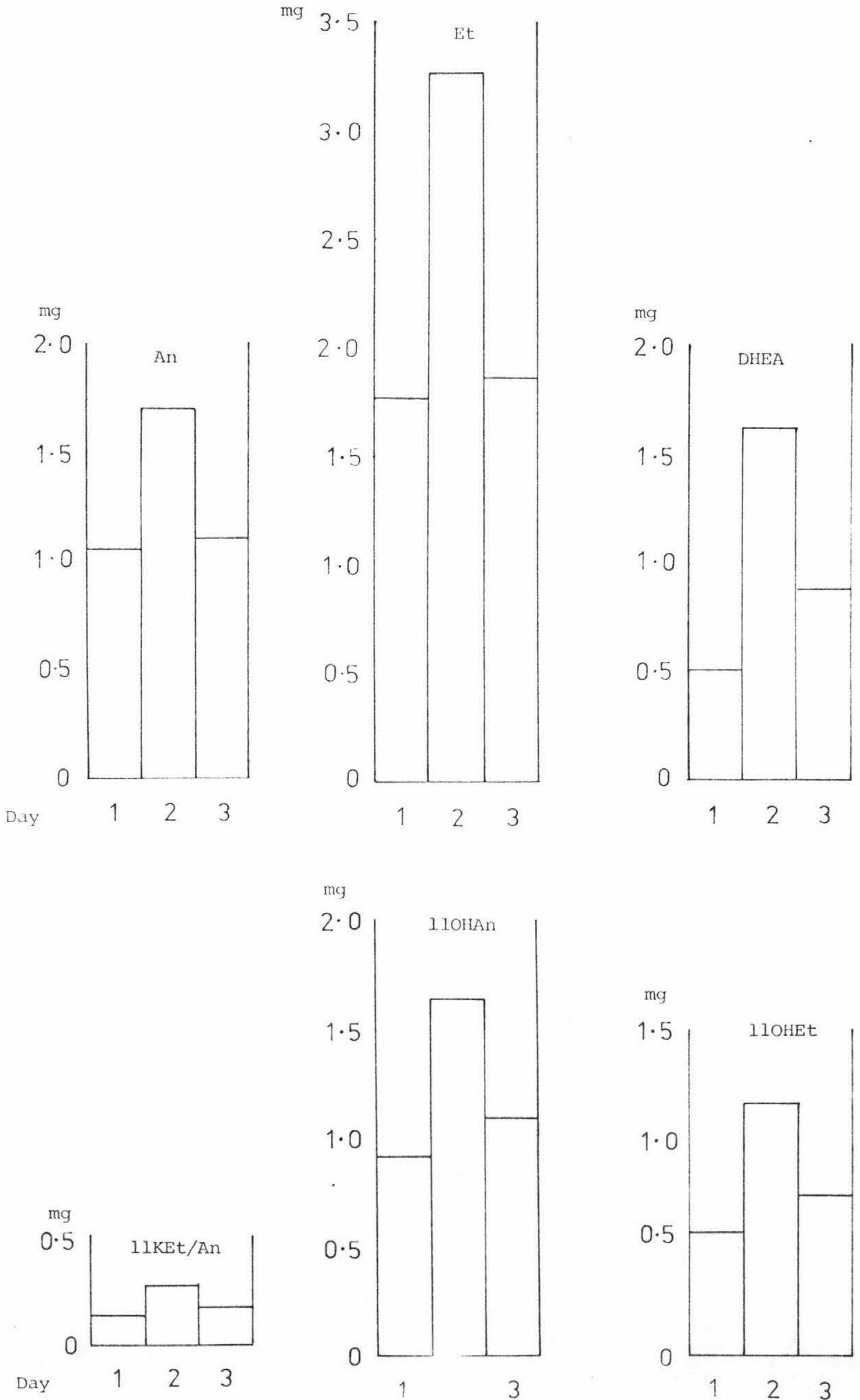


Figure 3xi (cntd.)

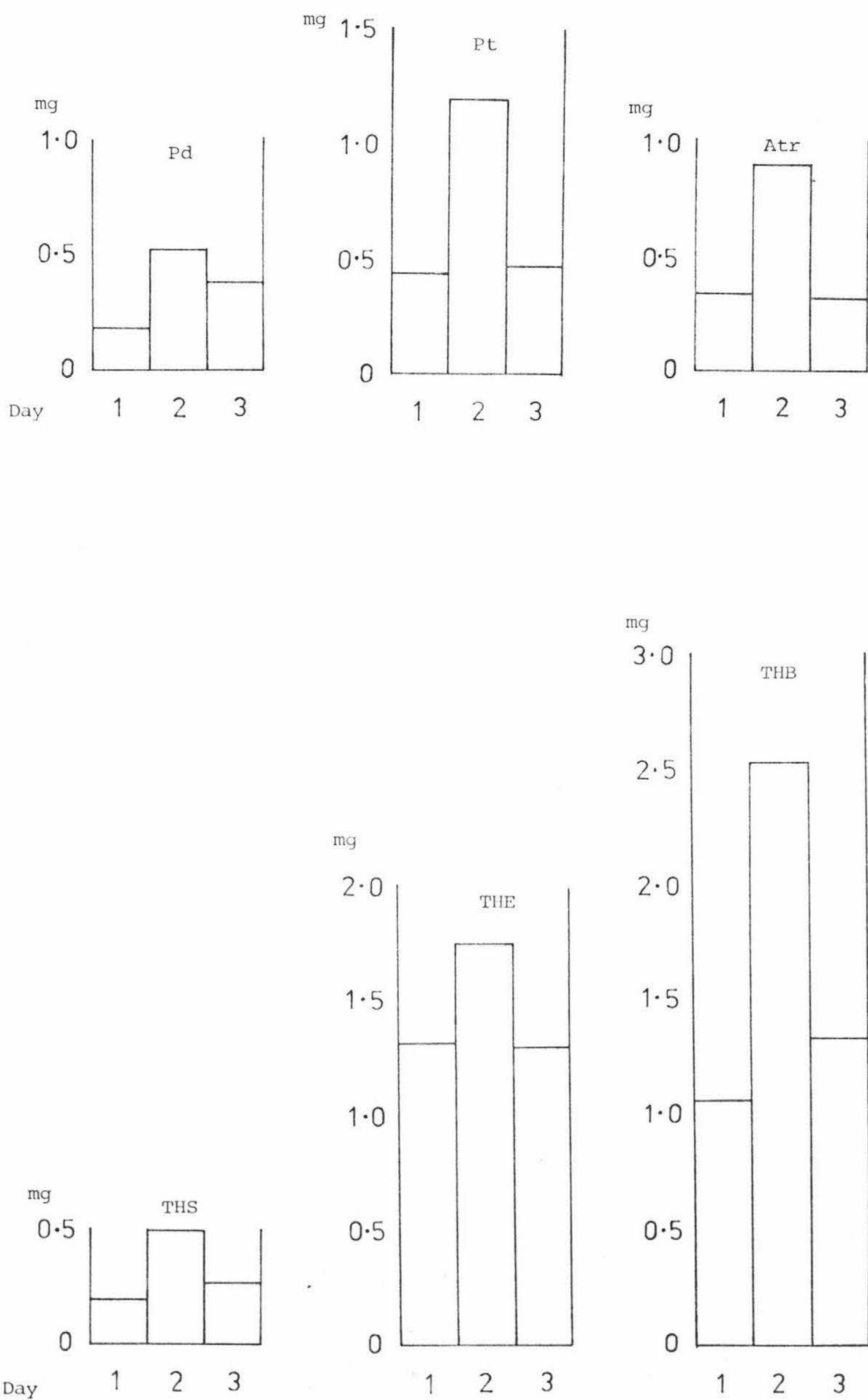
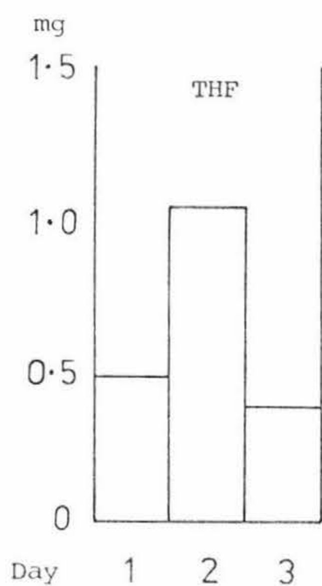
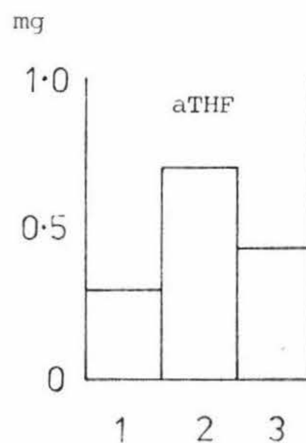
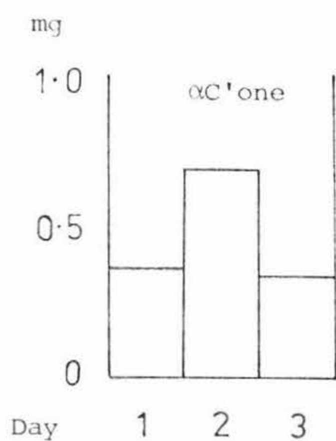
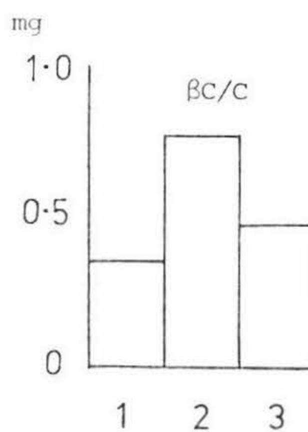


Figure 3xi (cntd.)









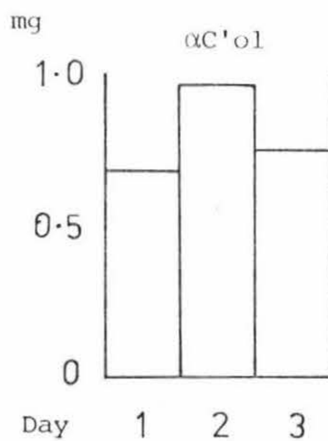
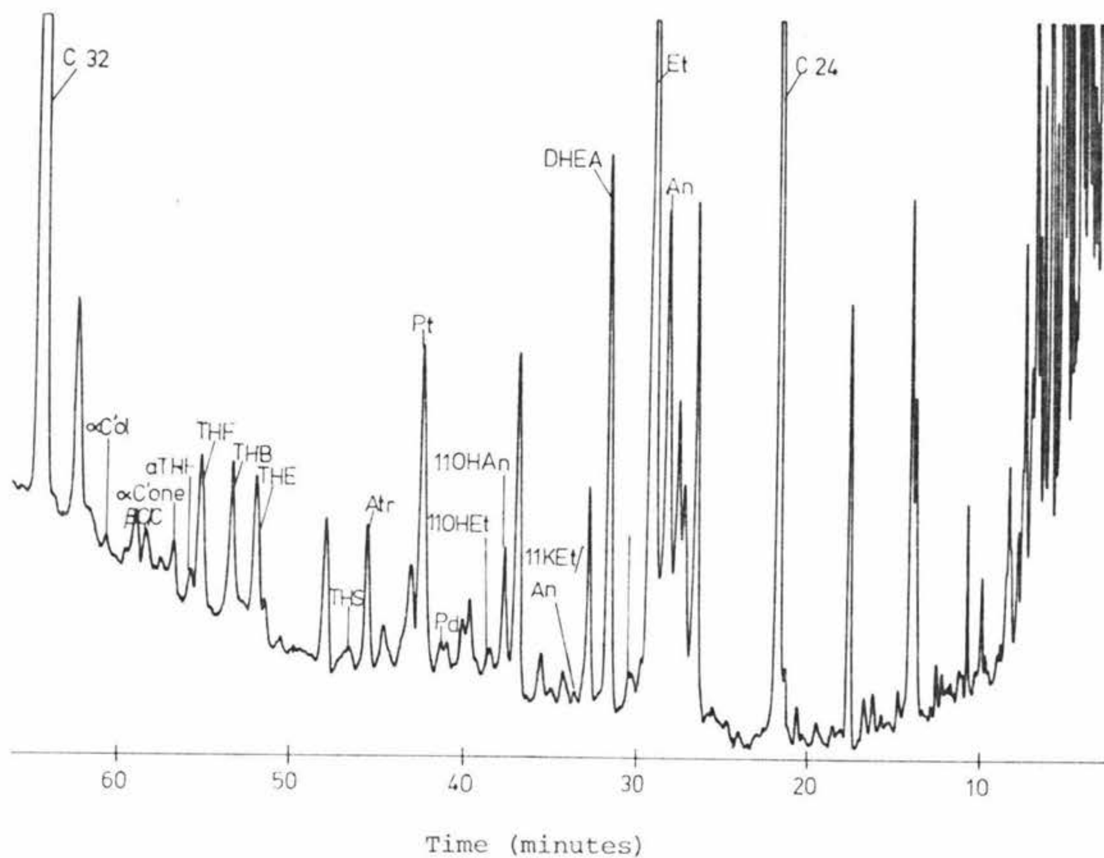


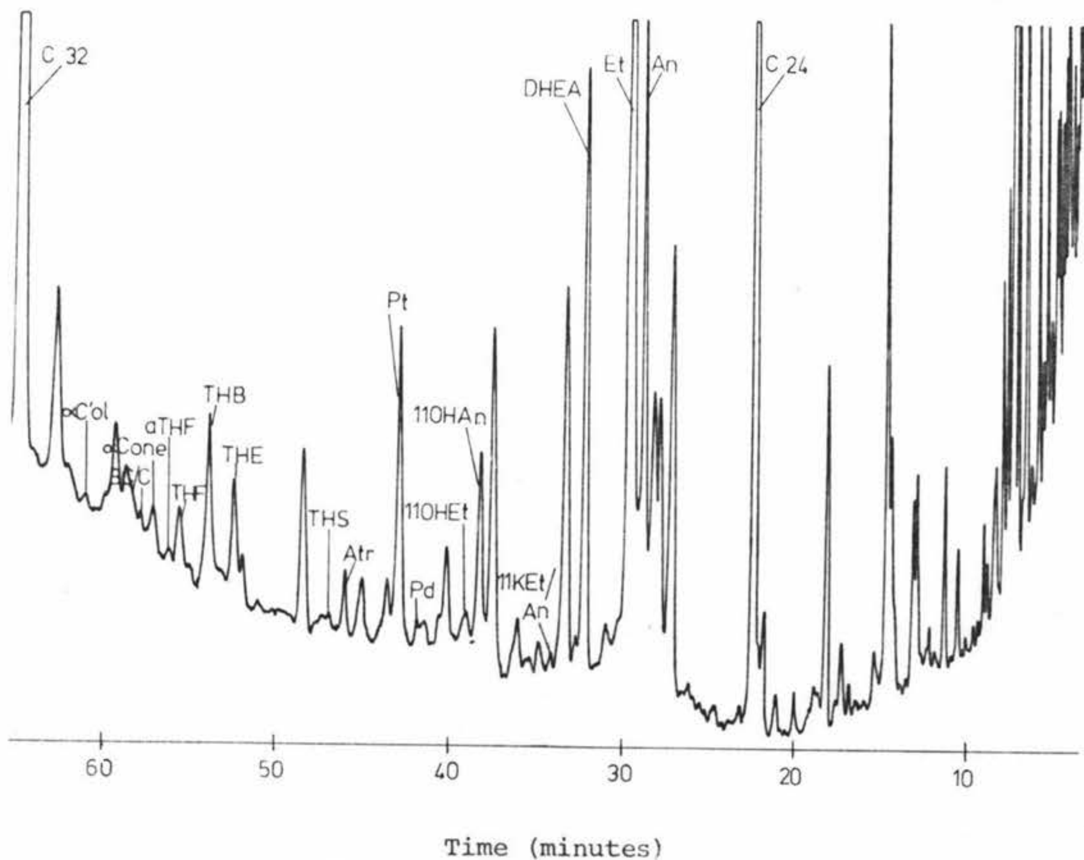
Figure 3xii

GLC Profile of Ethanol Loading with Alcoholic Subject, Day 2



1.0 µl sample, 40 meter column

GLC Profile of Ethanol Loading with Alcoholic Subject, Day 3



1.1 µl, 40 meter column

Table 3xix

Steroid Ratios

Steroids	Day 1	Day 2	Day 3
(1) An / Et	0.6	0.5	0.6
(2) aTHF / THF	0.6	0.7	1.1
(3) THF / THE	0.6	0.6	0.5
(4) THF+aTHF / THE	0.9	1.0	1.0
(5) αC'one / THE	0.4	0.4	0.4
(6) αC'ol / THF+aTHF	0.9	0.6	0.9
(7) αC'ol / αC'one	1.9	1.[illegible]	2.2
(8) 11OHAn+11OHEt / 11KAn+11KEt	10.6	10.4	10.7
(9) An+Et / DHEA	5.6	3.1	3.4
(10) Pt / Pd	2.4	2.2	1.2

Conclusion

The excretion rate of all steroids was increased on the day of alcohol loading (Day 2), compared to Day 1 and 3. Increased rates of excretion for Day 2 varied, a 30% increase with αC'ol, and at the other extreme a 160% increase in Pt. This increase, reflecting an increased secretion of ACTH, may have been due to stress imposed on the subject, despite the apparent relaxed state of the subject on Day 2. The subject did however experience a slight stress on Day 3, due to the total lack of alcohol, which may account for the slightly higher levels of excretion on Day 3 (compared to Day 1) of all steroids except Atr, THE, THF and αC'one, which were either the same or slightly less. However the adrenal:testicular ratio remained constant at 1.7 for the three days of the experiment.

There are no dramatic changes in the steroid ratios, but the $\frac{An+Et}{DHEA}$ ratio is almost two fold lower on the second and third day, implying decreased reduction of the C5 double bond.

The $\frac{aTHF}{THF}$ ratio increased on the third day, but was almost the same on Days 1 and 2. This change was of the same magnitude of the change in the reduction of the C5 double bond, and implies a favouring of the 5α to the 5β in A ring reduction.

(7) Alcoholic Urine Samples from the Detoxification Unit

Three twenty-four hour samples from patients admitted to the Palmerston North Detoxification Unit were analysed.

Patient A

A 46 year old European alcoholic male, admitted to hospital with delirium tremens. The patient was diagnosed as having peripheral neuropathy, pancreatitis, respiratory infection, gout, Wernickes encephalopathy and ostioarthritic hip. The patient had a firm enlarged liver, and definite signs of liver damage - elevated bilirubin, LDH, HBD, serum amylase and CPK. The patient had been off alcohol for four days when the 24 hour collection was taken. The patient had been on a high alcohol intake for at least six years, and had a daily intake of at least 80 grams of alcohol per day (rum and beer). The patient was being administered Hemineurin, vitamins, Amoxycillin and Phenothiazine at the time of collection.

Patient B

A 35 year old Maori/Italian alcoholic female with diabetes mellitus. The patient had mild liver damage indicated by an elevated bilirubin, other liver function tests normal. The patient had been on a high alcohol intake for five years, 1 bottle of vodka and 1½ flagons of sherry on alternate days. Prior to this she had a valium dependence and 3 years heroin addiction eighteen years ago. The patient had been off alcohol four days at the time of the urine collection, and was been administered Hemineurin.

Patient C

A 44 year old European alcoholic male with alcoholic cerebellar degeneration and liver damage indicated by elevated bilirubin. The patient had been drinking alcohol for many years, consuming 1 bottle of vodka a day.

Results

The excretion rates are presented in Table 3xx and illustrated graphically in Fig. 3xiii. Chromatograms are shown in Fig.3xiv and the steroid ratios are presented in Table 3xxi.

Table 3xx

Steroid	mg excreted/day		
	Patient A	Patient B	Patient C
An	0.42	0.61	0.36
Et	0.41	0.56	0.74
DHEA	0.72	1.51	1.51
11KEt/An	0.051	0.11	0.094
11OHAn	0.74	1.25	0.69
11OHEt	nd	nd	nd
Pd	0.23	0.40	0.38
Pt	0.40	0.86	0.95
Atr	0.11	0.20	0.24
THS	0.17	0.19	0.16
THE	0.66	0.88	0.56
THB	0.62	1.16	1.34
THF	0.66	0.45	0.49
aTHF	0.34	0.52	0.19
αC'one	0.58	0.51	0.51
βC/C	0.37	0.34	0.31
αC'ol	0.75	0.50	0.52

Figure 3xiii

Steroid Excretion Rates

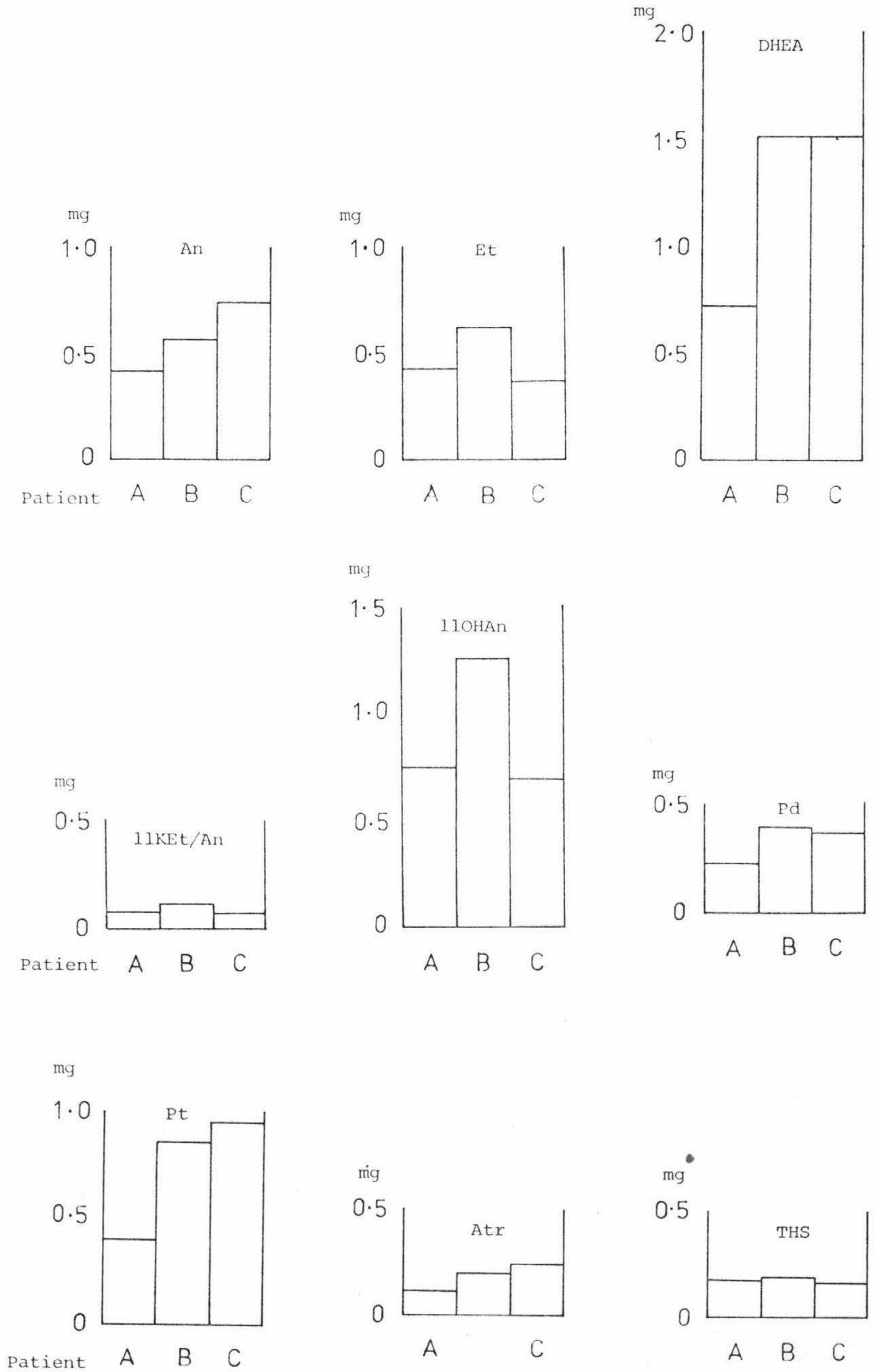


Figure 3x111 (cntd.)

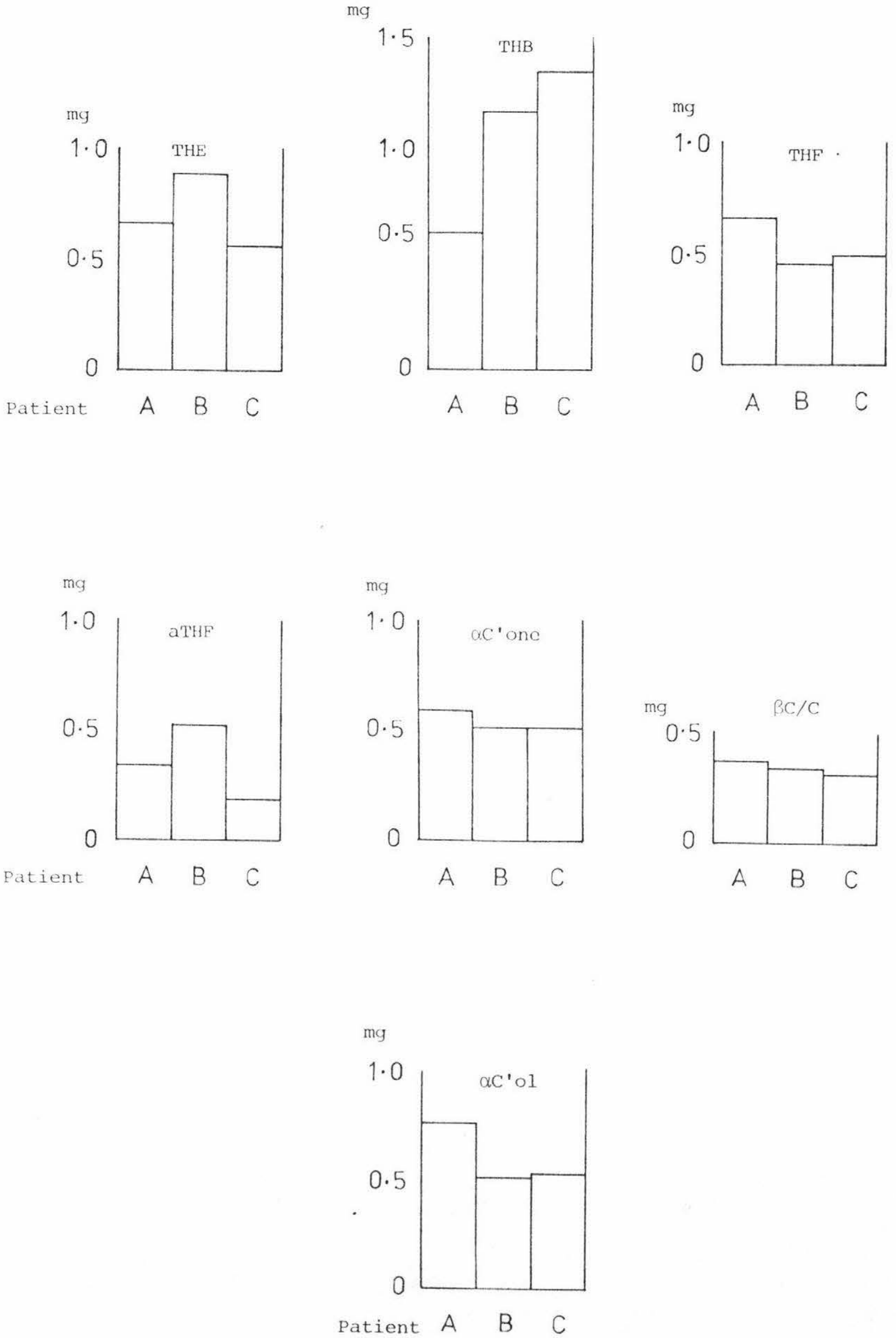
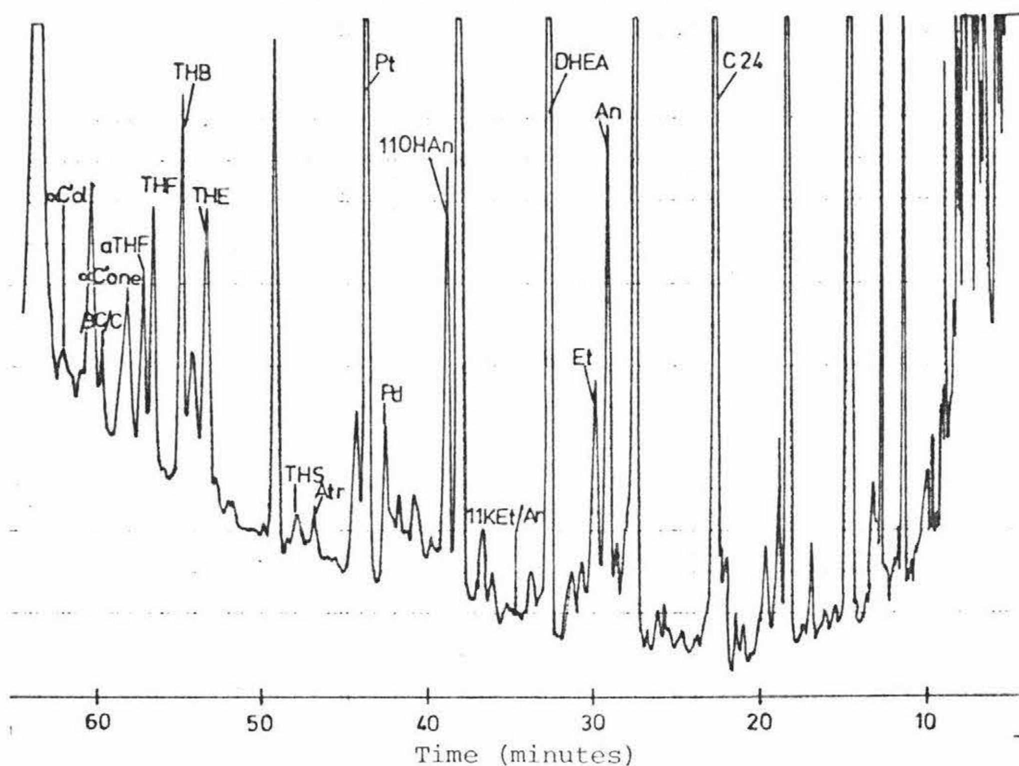


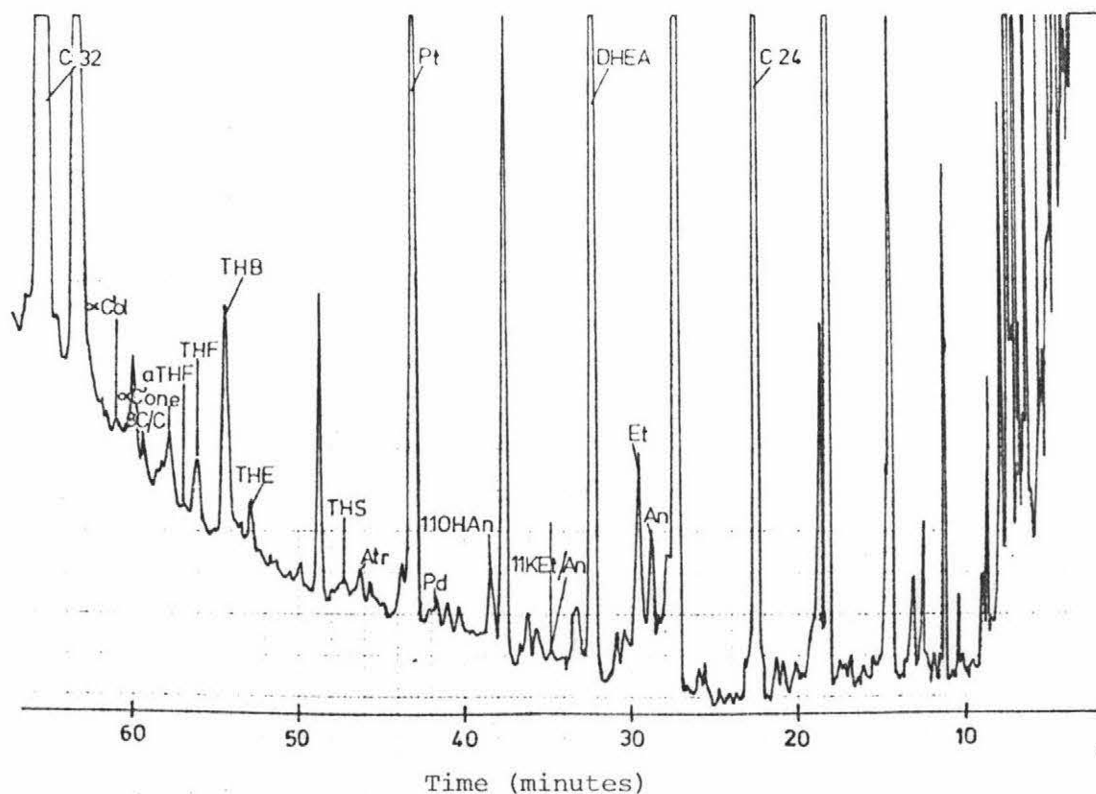
Figure 3xiv

GLC Profile of Alcoholic Patient A



1.5 µl sample, 40 meter column

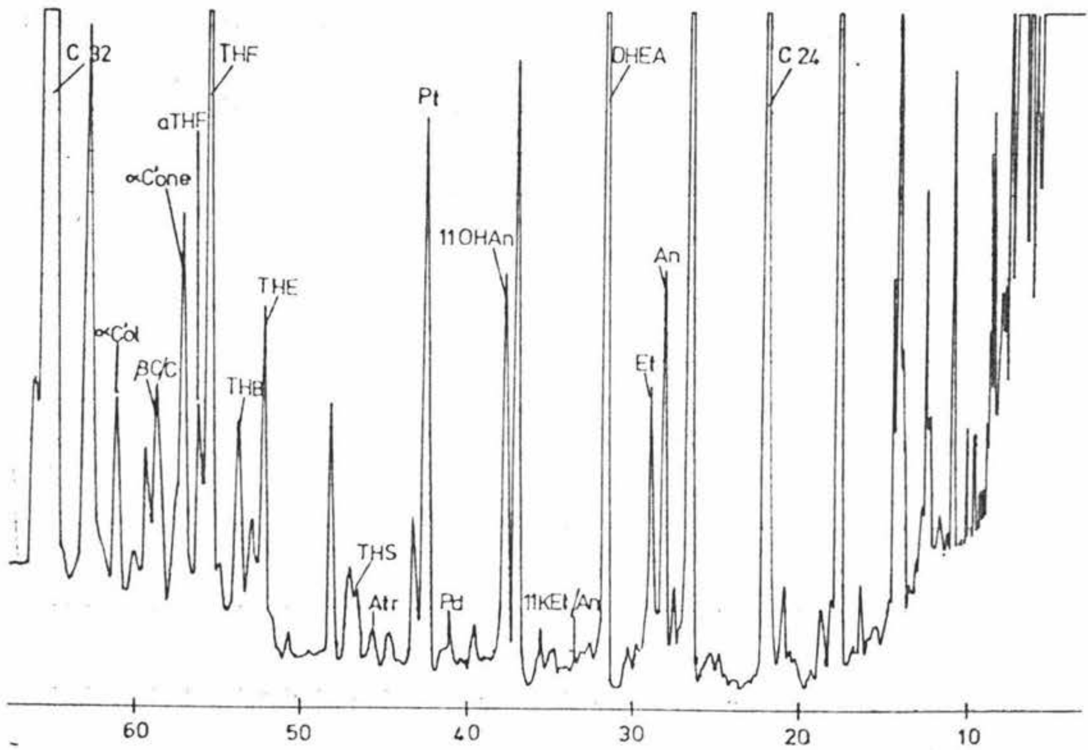
GLC Profile of Alcoholic Patient B



1.5 µl sample, 40 meter column

Figure 3xiv (cntd.)

GLC Profile of Alcoholic Patient C



Time (minutes)

1.5 μl sample, 40 meter column

Table 3xxi

Steroid Ratios

Steroids	Patient A	Patient B	Patient C
(1) An / Et	1.0	1.1	0.5
(2) aTHF / THF	0.5	1.1	0.4
(3) THF / THE	1.0	0.5	0.9
(4) THF+aTHF / THE	1.5	1.1	1.2
(5) αC'one / THE	0.9	0.6	0.9
(6) αC'ol / THF+aTHF	0.8	0.5	0.8
(7) αC'ol / αC'one	1.3	1.0	1.0
(8) 11OHAn+11OHEt / 11KAn+11KEt	14.5	11.4	7.3
(9) An+Et / DHEA	1.2	0.8	0.7
(10) Pt / Pd	1.7	2.2	2.5

Conclusion

No basal levels were available for these three patients, so conclusions can only be drawn from comparing the levels of excretion with other subjects. Absolute value comparison is of little use due to the large numbers of parameters governing excretion levels, but comparison of the steroid ratios may be more valuable in indicating any changes in steroid excretion.

One of the most striking observations is the non-detectable amounts of 11OHEt, which in previous experiments was atleast 50% of the value of 11OHAn, implying an increase in the 5α reduction compared to 5β.

The steroid ratio varies for each of the three patients, showing no general trend in changes of reduction in the catabolic processes.

Patient A showed a higher level of excretion of THF and a lower level of THB excertion than Patient B and C. The chromatograms of Patient A and B suggest an increased production of cortisol, due to increased levels of the tetrahydro and cortol/cortolone metabolites. This is probably due to the stress of hospitalization, and may be devoid of any relationship with altered or impaired liver function due to alcohol.

The adrenal:testicular ratio for Patient B and C was in the normal range, but was 75% higher for Patient A, supporting the conclusion that Patient A was stressed.

The enzyme used for the hydrolysis of steroid conjugates of all three alcoholics had a decreased activity. By comparing the levels of steroid metabolites measured in Patient C using the low activity enzyme, it is evident that hydrolysis with the "normal" enzyme was 126% to 310% as effective as the low activity enzyme (Table 3xxii).

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Table 3xxii

β-Glucuronidase Activity

Steroid	"Normal" β-Glucuronidase (mg/day)	"Low Activity" β-Glucuronidase (mg/day)	$\frac{N\beta G}{LA\beta G} \times \frac{100}{1}$ (%)
An	0.92	0.36	256
Et	1.43	0.74	193
DHEA	1.90	1.51	126
11KEt/An	0.24	0.094	255
11OHAn	1.13	0.69	164
Pd	0.72	0.38	189
Atr	0.55	0.24	189
THE	1.36	0.56	241
THB	2.00	1.34	149
THF	1.00	0.49	204
αC'one	0.73	0.51	143
βC/C	0.73	0.31	236
αC'ol	1.62	0.52	310

NβG = "Normal" β-Glucuronidase

LAβG = "Low Activity" β-Glucuronidase

CHAPTER 4

DISCUSSION

This work, using established methods for analysis of urinary steroid profiles by GLC, was performed in an attempt to illustrate changes in steroid metabolism induced by exogenous and endogenous factors.

Initial work involved determining basal data for the excretion of steroid metabolites in the subject used for most of the experimental work. This was necessary due to the variation in the excretion of steroid metabolites both during the day and also on consecutive days.

Ten Day Collection (Experiment 1)

Experiment 1 formed the basis of data on the daily basal excretion rates of steroid metabolites in a "normal" adult male volunteer. Some of the steroids (An, Et, THE and 11KEt/An) compared well with the data of excretion rates published in Makin, 1975 (Chapter 3, Page 46). However a number of values from this experimental work did not compare well with the published values. In particular 11OHAn, Pd, THB, THF and aTHF were markedly different, with 11OHAn showing the largest difference of almost ten times Makin's value.

A comprehensive presentation of excretion data for common neutral steroids from a total of 330 healthy subjects of differing nationalities and different age and sex has been published by Vestergaard (1978). The data illustrates the range encountered in the measurement of excretion values of steroid metabolites.

The urine of 33 American males was analysed by Vestergaard and the results of this are shown in Table 4i, together with the results of daily excretion rates obtained in this experimental work.

Comparison of the excretion data of this experimental work to the range from Vestergaard's work gives a good evaluation for most of the steroids. The 11OHAn excretion values for Subject 1 lies outside the range in Table 4i, and is over three times the value given in Vestergaard's work as the mean. The value quoted by Makin is about one third of the value quoted by Vestergaard, giving an indication of the variability encountered.

The values of THF and aTHF for Subject 1 are low compared to the

Table 4i

Average Excretion Rates

Steroid	Vestergaard's Mean (mg/day)	Vestergaard's Range (mg/day)	Subject 1 Mean (mg/day)
An	3.88	1.26 - 10.80	2.11
Et	3.04	1.34 - 8.15	3.24
DHEA	1.36	0.80 - 5.45	1.50
11KAn	0.57	0.23 - 1.41	{ 0.70
11KEt	0.76	0.20 - 2.73	
11OHAn	1.16	0.39 - 2.95	3.79
11OHEt	0.63	0.14 - 1.50	1.44
THE	3.87	2.16 - 7.39	2.60
THF	1.80	0.89 - 3.00	0.83
aTHF	1.37	0.39 - 2.56	0.56
αC'one	0.20	0.05 - 0.41	-
βC'one	2.22	1.03 - 3.03	{ 1.10
βC'ol	0.72	0.27 - 1.07	
αC'ol	0.40	0.17 - 0.78	1.33

published data, and the THF value is not in the range for THF in the work of Vesterdaard.

The mean value for THF and aTHF in Vestergaard is almost half that published in Makin. Some of the mean values of Vestergaard, Rubin and Comitas and Beale et al (Vestergaard, 1978) all quoted values for aTHF excretion below the value obtained for Subject 1.

Subject 1 may have an abnormally active system for side chain cleavage of cortisol metabolites resulting in low levels of THF/aTHF and increasesd production of 11OHAn.

The value of βC/C for Subject 1 is less than the sum of the two lower limits of the range in Table 4i, but the value of αC'ol is above

the range presented.

No data on Pd or THB excretion is published in Vestergaard, but the values for Subject 1 are appreciably higher than the value quoted by Makin.

So whilst some of the excretion data values appear to be out of the "normal" range, sufficient evidence of variability of data is presented to validate the results of the basal excretion rates presented here.

The reduction rate of the 3-oxo-4-ene group of the steroid metabolites is governed by the enzymes in the liver. The relative activities of these enzymes is reflected by the ratios of metabolites which undergo these reductions. The normal ratio for An:Et (5α:5β) is about 2:1, and for aTHF:THF (5α:5β) is about 1:1 (Pfaffenberger and Horning, 1977). The ratio of An:Et for the ten day collection is 0.7, considerably lower than the value expected. This may be due to not utilising solvolysis after enzyme hydrolysis. The androsterone sulphate present in considerable amounts in urine is not split by the commonly used glucuronidase/sulphatase preparations (Roy, 1956), but solvolysis releases this androsterone, which would increase the An:Et ratio. The value of the aTHF:THF ratio for the ten day collection is 0.7, close to the value of 1.0 expected.

Pfaffenberger and Horning (1977) calculated the ratio for the cortisol:cortisone metabolites, defined as:-

(αC'one + THE + βC'one):(THF + aTHF + αC'ol).

For males the value ranged from 1.00 to 2.25 with an average of 1.74. For Subject 1 the ratio is 1.36, but this has been calculated as (THE + βC/C):(THF + aTHF + αC'ol) as no value for αC'one was obtained and βC/C coelute. From the published data however, the value of αC'one is low, and the value of βC'one represents most of the value of βC/C, so that the ratio would only be slightly altered by substitution of the same values used by Pfaffenberger and Horning, and would still be within the 1.00 to 2.25 range.

Diurnal Rhythm (Experiment 2)

Experiment 2 formed the basis of data on basal excretion of steroid metabolites during a 24 hour period. Since Pincus first described 24 hour periodicity in the excretion of total 17-ketosteroids in 1943, and excretion of reduced corticosteroids in 1948, a considerable amount of work has been done with such group specific assays.

Migeon et al (1956) suggested that the diurnal variation in plasma levels and urinary excretion is a basic physiological phenomenon. They found that changes in urinary output followed plasma levels by about the same lag interval as when cortisol was injected intravenously, implying that the cycle is primarily related to the production of adrenal steroids, rather than alterations in their metabolism.

Martin and Hellman (1964) stated that normal circadian variation can not be accounted for by a simple negative feedback mechanism where fluctuations in blood level cortisol regulate ACTH secretion. They suggested changes in diurnal variation are due to changes in rate of ACTH secretion in response to a centrally located control mechanism. When metopirone (an 11β-hydroxylase inhibitor) was administered, the plasma cortisol concentration was lowered, resulting in increased adrenal response when ACTH was administered without altering periodicity. The results of this experiment suggested ACTH secretion occurs as a periodic event independent of plasma cortisol concentration, but cortisol concentration regulates the magnitude of ACTH discharge. Data supports the concept of circadian variation in ACTH secretion, presumably the result of inherent rhythmicity in the neural centres concerned with pituitary regulation.

Vestergaard and Leverett (1957) found a biphasic excretion pattern for total 17-ketosteroids when 2 hour sampling was utilised. Of the 200 collection days, 179 days showed this biphasic pattern. The following observations were made:

(1) There were great individual differences in excretion variability, some persons had a tendency to constant pattern formation, others showed great variability from period to period.

(2) In persons with most stable excretion patterns, a number of factors claimed to influence 17-ketosteroid excretion were found to have no verifiable influence on excretion (eg. climatic, nutritional and emotional factors).

(3) In persons with a more labile excretion pattern, one of the

factors most frequently correlated with increased 17-ketosteroid excretion seemed to be emotional tension.

(4) The possibility of the 17-ketosteroid excretion pattern being caused by changes in renal glomerular filtration rate is proposed, however in the majority of people examined there has been little variation in creatinine excretion, implying the ketosteroid variability is extra-renal.

There have been a few studies of 24 hour periodicity in excretion of individual urinary steroids.

Toccofondi et al (1970) found a circadian rhythm for DHEA, An and Et in fourteen "normal" subjects (age, 21 to 34 years old) with peak excretion between 6am and 2pm. The excretion pattern from this experiment agrees with these findings, which are also supported by the work of Cavalleri et al (1974).

Molino et al (1973) compared two-hour samples taken between 8am and 10am. They found significant differences between the samples for most 17-oxosteroids, except 11KEt and 11OHEt. The present work confirms the increased excretion of steroid metabolites in mid-morning, but not the exceptional behaviour of 11OHEt. The 11OHEt mid-morning excretion does increase in both of the subjects analysed, and was of the same magnitude as the increase in excretion of 11OHAn for the corresponding times. The data on change in excretion rate of 11KEt is uncertain since 11KEt and 11KAn coelute on the GLC column, but the excretion of these two metabolites show a significant increase over the initial two 2-hour urine collections.

Molino et al (1973) found no significant increases in THS and Pt excretion in morning urines. Again this experimental work does not agree with this observation: for THS the highest excretion value recorded for the 7am to 7pm period for Subject 1 was the 9am to 11am collection, where most steroid metabolites peaked in excretion rate. However the overnight excretion rate was similar to the value for the 9am to 11am collection. In Subject 2 a biphasic pattern of excretion was evident with a peak value at 1pm to 3pm, and the value for the overnight collection was similar to the 9am to 11am collection.

When urinary THS excretion was artificially elevated by administration of the 11β-hyroxylase inhibitor, metyrapone, to eight male volunteers (Touitou et al, 1977), a rhythm in urinary THS with a peak at 2.30pm became apparent, although not seen in the control data before metyrapone. This peak time for THS coincided with that for

other 17-hydroxycorticosteroids.

Molino et al therefore may have misinterpreted their findings about THS due to insufficient sampling times. Alternatively it is possible that 2-hour samples at midnight may have higher excretion rates than the rest of the night, and may account for the assumption that THS and Pt do not exhibit a circadian variation.

In this experimental work the same basic pattern appears to exist for Pt as with THS, except the changes are not so subtle.

In Subject 1 the Pt excretion rate peaks at 9am to 11am with 0.16 mg/2 hours, compared to 0.118 mg/2 hours for the 7pm to 7am collection. This 36% increase is not as large as the difference observed with some of the steroids. Subject 2 does not show the same similarity with the 9am to 11am excretion rate being over twice that of the 7pm to 7am collection. However it should be emphasized that the overnight collection is a 12 hour collection expressed as mg/2 hours. With the collection protocol in these experiments, such pooling of night samples was necessary since waking each 2 hours during the night may have imposed a stress effect that resulted in increased steroid metabolite excretion.

Kobberling and Muhlen (1974) showed a circadian rhythm for free cortisol in the urine with a peak at 7am to 8am slowly falling off upto 11am and a smaller maximum in the afternoon, most evident between 2pm and 4pm.

Beale and Tyrrell (1974) when measuring the excretion rates of some steroid metabolites over one hour, three hour and twenty-four hour periods found significant variations. Each metabolite had its own individual pattern of variability differing from other metabolites and differing in each subject. For the metabolites measured, two maxima were observed, one at about 9am and the other at about 2.30pm, with a minimum value just after noon.

This observation is similar to the pattern of excretion of the cortisol metabolites in this experimental work. Table 4ii presents a comparison of the values obtained in this experimental work and those obtained from Beale and Tyrrell's data, from their one hour sampling. Although absolute values vary, particularly THF, the excretion values do follow a similar basic pattern.

In Subjects 1 and 2, most of the peaks for the cortisol and cortisone metabolite excretion occurred between 9am and 11am. Both subjects demonstrated a second peak later in the day. Subject 2 fits

Table 4ii

Comparison of Excretion Rates

mg/2 hours

Steroid	Subject	7am 9am	9am 11am	11am 1pm	1pm 3pm	3pm 5pm
THE	Beale & Tyrrell	.188	.176		.139	.127
	1	.120	.160	.155	.086	.056
THF	Beale & Tyrrell	.071	.151	.093	.136	.097
	1	.054	.072	.053	.030	.029
aTHF	Beale & Tyrrell	.064	.060	.045	.086	.055
	1	.053	.062	.060	.043	.035

the previously reported pattern, but Subject 1's second peak occurs later in the afternoon.

Cold Room Stress (Experiment 3)

Experiment 3 involved exposing Subject 1 to a stressful situation, an environment of $4^{\circ}C$ for two hours. The subject did experience some discomfort from this exposure which resulted in uncontrollable shivering after being in the cold room for one hour, and continued for the next two hours. With this effect on the body, one would have expected an increased rate of cortisol secretion in response to the cold stress imposed. However the results of this experiment do not support this.

The pattern of excretion during the day did change, each steroid measured, except Pt, the 7am to 9am excretion value was greater than that for any two hour period in the control experiment, and Pt was only 0.005 mg/2 hours higher in the control experiment.

Unlike the control, the excretion data showed no predominant peaks mid-morning or mid-afternoon, but steadily decreased for most steroids as the day progressed.

This suggests a change in the pattern in secretion of ACTH, reflected by the pattern of steroid metabolite excretion. The high excretion value for the 7am to 9am collection was unexpected. This collection was

completed before the subject entered the cold room (9am) and is therefore not related to the physical cold experienced. Vestergaard and Leverett (1957) did comment that emotional tension tended to produce the greatest change in 17-ketosteroid excretion and the factors commonly claimed to influence 17-ketosteroid excretion (including climatic conditions) often did not show such a tendency. However the emotional tension imposed upon the subject before entering the cold room did not appear to be of the magnitude reflected in the steroid excretion rates.

No literature could be cited on the effect of cold on individual steroids and the literature on total assays is somewhat conflicting. Kuhl (1952) exposed subjects to cold water ($9^{o}C$) for eight minutes and observed a marked increase in the excretion of 17-ketosteroids. This protocol was similar to the present experimental situation, although the latter did not produce a marked 17-ketosteroid excretion increase.

Later work, as reviewed by Collins and Weiner (1968) gave ambiguous results with regard to steroid excretion after cold stress. Vestergaard (1978) suggests only severe conditions will activate the adrenal cortex to a marked degree.

Bush (1962) pointed out that it is doubtful whether any of the physical stimuli which are commonly supposed to be stresses are effective in causing increased secretion of ACTH at all. Severe exercise, cold and fasting produce little or no effect on the secretion and metabolism of cortisol in man unless they are part of a situation that provokes emotion. However strong emotion in the absence of any recognisable physical stimuli or stress regularly causes maximal increases in the excretion rate of cortisol.

Budd and Warhoft (1969) did find the excretion rates of 17-hydroxycorticosteroids and 17-ketosteroids were significantly greater in four men in Antarctica compared to the excretion levels from when they were in Australia. These conditions were much more dramatic than the experimental situation here.

The levels of excretion after the high 7am to 9am collection were of a comparable level for the control period, although they did not drop as low as the excretion values for the control in the afternoon.

The results of this experiment were unexpected in view of the high steroid excretion rates before the cold stress period and the failure of the cold to increase these. More observations are needed to

quantify the response to cold stress due to the apparently minimal response of the adrenal.

Dexamethasone Administration (Experiment 5)

In Experiment 5, dexamethasone, the 16α-methylated analog of Δ^1-9α-fluorocortisol was employed as a suppressive agent of ACTH release, and hence of adrenal steroidogenesis.

No data could be cited on the effect of dexamethasone on individual steroid metabolites, and the effect on total assays is sparce. Liddle (1960) reported that 17-ketosteroid excretion does not fall during suppression of ACTH as readily as does 17-hydroxycorticoid excretion. This experimental work supports this observation, however there is an overlap in the degree of suppression of these two steroid groups. The 17-hydroxysteroids were more sensitive to dexamethasone than the 17-oxosteroids, showing greater duration and degree of suppression.

All the steroids measured, with the exception of DHEA, in this experiment showed decreased excretion by the second day after the initial dexamethasone administration. This was accompanied by a 50% decrease in the adrenal:testicular ratio, implying a decrease in adrenal activity, assuming excretion of metabolites from the testicular origin was constant.

Alcohol Loading (Experiments 4, 6 and 7)

Experiments 4, 6 and 7 investigate the effects of ethanol on steroid metabolism at varying levels of loading in different subjects.

Experiment 4 utilises Subject 1 and a minimal level of ethanol loading, up to a maximum of 53 mg%. The subject maintained a blood alcohol level of approximately 50 mg% for three hours. The only major change in the pattern of excretion during ethanol loading, was an increased rate of excretion in the 3pm to 5.50pm collection. However it is difficult to accurately compare this with the 3pm to 5pm control collection due to the different collection protocol. By comparing a four hour collection, calculated by summing the excretion rates between 3pm and 7pm, one can observe a significant increase in the excretion of some steroids (Table 4iii).

Table 4iii

Steroid Excretion Rates - Comparison of Loaded State to Control

Steroid	Control 3pm-7pm (mg/4 hours)	Alcohol Loading 3pm-7pm (mg/4 hours)	*Increase (%)
An	0.167	0.217	+30
Et	0.164	0.218	+33
DHEA	0.280	0.222	-20
11KEt/An	0.035	0.040	+14
11OHAn	0.154	0.225	+46
11OHEt	0.132	0.169	+28
Pd	0.096	0.088	-8
Pt	0.162	0.196	+21
THE	0.140	0.257	+84
THB	0.235	0.185	-21
THF	0.064	0.130	+103
aTHF	0.082	0.149	+82
αC'one	0.064	0.098	+53
βC/C	0.088	0.105	+20
αC'ol	0.098	0.126	+29

$$* = \frac{(\text{Alcohol Loading mg/4 hours}) - (\text{Control mg/4 hours})}{(\text{Control mg/4 hours})} \times \frac{100}{1}$$

Table 4iii shows the tetrahydro products of cortisol and cortisone are excreted in much greater quantities than any other metabolite compared to the control. The next most significant increases are αC'one and 11OHAn, also products of cortisol. All other metabolites either increased less than 35% or decreased.

Table 4iv shows the percentage increase in the 7am to 11am and 11am to 3pm four hour periods are not of the magnitude seen in Table 4iii.

Table 4iv

Steroid Excretion Rates - Comparison of Loaded State to Control

Steroid	7am-11am Increase (%)	11am-3pm Increase (%)
11OHAn	+7	-15
THE	+14	-3
THF	+37	+36
aTHF	+50	+47
αC'one	+16	+1

The present increases in cortisol metabolites may at least partially result from increased cortisol secretion from the adrenal after ethanol loading. Mendelson et al (1971) concluded from studies with alcoholic subjects that ethanol induces an increased secretion of cortisol, and this response to ethanol is probably mediated via neural pituitary mechanisms.

Studies in "normal" humans maintained on ethanol showed a marked increase in hepatic testosterone A ring reductase (Lieber, 1977). There was a two to five fold increase in activity of the A ring reductase system, and it is suggested that other 3-oxo-4-ene steroids may undergo increased A ring reduction resulting from ethanol loading. The increase in some of the steroid metabolites (Table 4iii) due to ethanol appears to be selective, possibly due to an alteration in steroid metabolism in the liver. In particular the increase of THE, THF and aTHF excretion may be due to an increased activity of the A ring reductase system. THE is the precursor of αC'one, which is also increased significantly in excretion rate. This may be related to the known increase that occurs in the $NADH:NAD^+$ ratio in the liver during ethanol oxidation (Forsander et al, 1958).

It is possible that steroids which do not undergo A ring reduction are not metabolised at the same rate when the $NADH:NAD^+$ ratio is increased. DHEA is one such steroid, and the excretion rate decreased 20% during the 3pm to 7pm period.

THB and Pd also showed a decreased rate of excretion, even though they have both under gone A ring reduction, it is worth noting that they are from a different metabolic route than any of the other steroids measured. Pd is a direct catabolic metabolite of progesterone and THB is the catabolic metabolite of corticosterone (derived from progesterone via DOC or 11βOH progesterone).

To test the theory that increased A ring reduction occurs in a state of ethanol loading, the plasma cortisol concentration would need to be measured in a number of subjects. If the plasma cortisol concentration remained the same in the control and during ethanol loading, and the increments in cortisol metabolites after ethanol loading still occurred, then this would provide a basis for the hypothesis of increased A ring reduction associated with ethanol loading.

Cronholm et al (1971) found that during ethanol loading the 17β-hydroxysteroids are increased with a simultaneous decrease in corresponding ketosteroids. Admirand et al (1970) also reported that ingestion of relatively small quantities of ethanol is associated with a four to five fold increase in 17β-hydroxysteroid sulphates in human plasma. No evidence to support these findings was observed in this experimental work.

Experiment 6 involved a more dramatic degree of ethanol loading than Subject 1. The subject in this experiment had a history of alcohol abuse over the previous ten years.

In this experiment the subject consumed a 26 ounce bottle of Scotch in approximately fifteen hours (10am to 1am) with a maximum recorded blood alcohol of 140 mg%.

The excretion rate of all steroids measured increased on Day 2 (ethanol loading) of the three day experiment, but were of similar values on Days 1 and 3.

No indication of selective reduction was observed in these results. The increase in excretion on Day 2 of THE, THF aTHF, 11OHAn and αC'one was no more distinctive than any other steroid excretion rate. However in Subject 1, this selective reduction appeared to occur only a few hours after ethanol loading. The 24 hour samples collected may have masked a transient effect. If a number of collections were made within the 24 hour period, the selective reduction may have been evident.

The increased excretion rate on Day 2 may have in part been a stress effect, but since all the metabolites increased, not just the cortisol metabolites, the increase cannot be solely due to stress.

Experiment 7 involved the collection of 24 hour urine samples from patients at the Palmerston North Detoxification Unit, in advanced stages of ethanol intoxication.

Little information can be derived from the analysis of these urines as no basal data is available for these patients. Also the enzyme used had a lower activity than the normal enzyme rendering the absolute values useless.

The chromatograms and the adrenal:testicular ratio of Patient A suggests that this patient was under some stress, probably due to hospitalization. The chromatogram of Patient B also suggests this, but the adrenal:testicular ratio shows no significant change from the norm.

The apparent absence of 11OHEt in the urine collections was the most unusual facet of these patients' urine collections. The amount of 11OHAn relative to other steroids appears to be higher than that in Subject 1 and in the subject for Experiment 6. However this potential shift in steroid metabolism is not conclusive. Complete hydrolysis and basal data would be required to determine if the lack of 11OHEt was reflected by increased 11OHAn concentration.

This experimental work established the daily variation in one subject, and the diurnal variation in two subjects of the common neutral urinary steroid metabolites.

It provides the only known data on the effect of dexamethasone on individual steroid metabolites, although they followed the expected pattern. No meaningful information could be derived from the cold exposure experiment, the significance of the results of this experiment would require a more thorough investigation.

One of the ethanol loading experiments did indicate that changes in hepatic A ring reductase activity occurs. The only other observation resulting from ethanol intoxication was the absence of 11OHEt in the profiles of hospitalized alcoholics.

Changes in the excretion of urinary steroids must be carefully interpreted due to the large individual variation which is possible.

Conclusive results would require a large sampling range and carefully controlled conditions to detect these changes from the individual variation.

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