FACTORS AFFECTING THE EXCRETION OF URINARY STEROID METABOLITES IN MAN

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

Evan Edward Olsen
1980

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.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. R. M. Greenway for his continual advice, assistance and encouragement, and for the many samples he provided. Dr. N. A. Smidt for her medical advice, and together with the staff of the Detoxification Unit at Palmerston North Hospital are gratefully acknowledged for collection of urine samples. I am indebted to Mr. D. J. Brigden for his assistance in compiling of this manuscript. Lastly for Christine, whose encouragement throughout this project was invaluable.

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 (THE)*, cortols and cortolones,	G 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

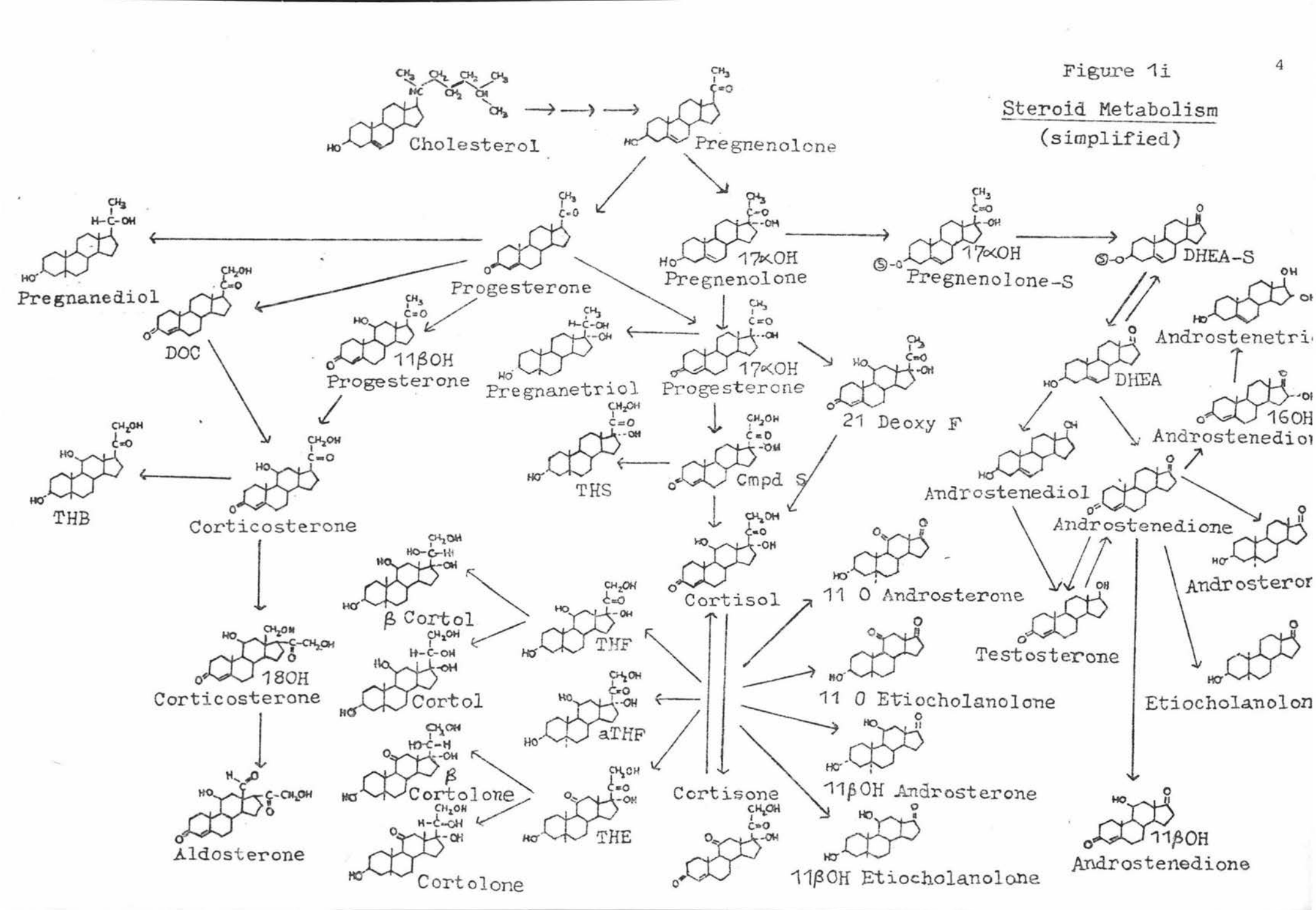
G = glucuronide; S = sulphate.

Reproduced from Makin (1975)

Figure 1i

Steroid Metabolism

(simplified)



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).