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RIJKSUNIVERSITEIT TE GRONINGEN

POLAR CORTISOL METABOLITES IN THE URINE OF NEWBORN INFANTS

PROEFSCHRIFT

TER VERKRIJGING VAN HET DOCTORAAT IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE RIJKSUNIVERSITEIT TE GRONINGEN
OP GEZAG VAN DE RECTOR MAGNIFICUS DR. M. J. JANSSEN
IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 10 JUNI 1977
DES NAMIDDAGS TE 2.45 UUR PRECIES

DOOR

HENRICUS JOHANNES GERARDUS MARIA DERKS
geboren te Bergen (L)

VRB drukkerijen bv - Groningen

PROMOTOR: DR. N. M. DRAYER
COPROMOTOR: PROF. DR. H. WIJNBERG
COREFERENT: DR. J. J. H. THIJSSEN

Dit onderzoek werd gesteund door de Stichting voor Medisch Wetenschappelijk Onderzoek (FUNGO).

STELLINGEN

I

Een vierde deel van de cortisol metabolieten van het pasgeboren kind wordt uitgescheiden in de vorm van een onbekend conjugaat.

II

6α-hydroxylatie en niet 6β-hydroxylatie speelt een kwantitatief belangrijke rol in het cortisol metabolisme in de pasgeborene.

Daniilescu-Goldinberg D. & Giroud C.J.P.: J. Clin. Endocr. Metab. 38 (1974) 64
Dit proefschrift

III

De argumenten die door Setchell et al gebruikt zijn voor het vaststellen van de ruimtelijke configuratie van de 6-hydroxy-groep in de door hen geïdentificeerde corticosteroïden zijn zeer aanvechtbaar.

Setchell K.D.R., Gontscharow N.P., Axelson M. & Sjövall J.: J. steroid Biochem. 7 (1976) 801

IV

Bij de gaschromatografische analyse van steroïden met behulp van gepakte kolommen moet ernstig rekening worden gehouden met het verschijnsel van irreversibele kolomadsorptie.

Derks H.J.G.M., Muskiet F.A.J. & Drayer N.M.: Anal. Biochem. 72 (1976) 391

V

De bepaling van polyamines in urine ten behoeve van de diagnostiek van patiënten met kanker moet met voorzichtigheid gehanteerd worden, gezien de verhoogde uitscheiding van deze stoffen bij een aantal niet-maligne ziekteprocessen.

Russell D.H., Durie B.G.M. & Salmon S.E.: The Lancet 25 (1975) 797

VI

Bij metabole studies, waarin gebruik wordt gemaakt van meervoudig gedeutereerde tracers, is het noodzakelijk te onderzoeken of natuurlijk en gedeutereerd materiaal op dezelfde wijze gemetaboliseerd worden.

VII

Aangezien de enzymatische hydrolyse van steroïdconjugaten de meest tijdrovende stap is in de klinisch chemische analyse van steroïden in lichaamsvloeistoffen, is het zinvol te onderzoeken of deze hydrolyse versneld kan worden door de toepassing van matrixgebonden enzymen.

VIII

Het nut van de bepaling van vanilamandelzuur en 3-methoxy-4-hydroxy-fenylethyleenglycol in liquor cerebrospinalis ten behoeve van de patiëntenzorg moet nog worden aangetoond.

Karoum F., Gillin J.C., McCullough D. & Wyatt R.J.: Clin. Chim. Acta 62 (1975) 451

IX

Nader onderzoek naar het verband tussen de antepartum toediening van glucocorticoïden aan aanstaande moeders en de preventie van hyaliene membranen ziekte (respiratory distress syndrome) bij pasgeborenen is zeer wenselijk in verband met de hoge mortaliteit tengevolge van deze afwijking.

Year Book of Pediatrics 1976, p. 21

X

In verband met de analytische problemen verbonden aan de bepaling van de extreem polaire neonatale cortisol metabolieten, is het voor het vaststellen van de bijnierschorsfunctie bij pasgeboren kinderen van groot belang een cortisol productiesnelheidsbepaling te ontwikkelen, waarbij gebruik wordt gemaakt van stabiele isotoopverdunning.

XI

Er wordt te weinig aandacht besteed aan de ontwikkeling van een bruikbaar vliegwiel-aandrijfaggregaat ter vervanging van de verbrandingsmotor in auto's.

Post R.F. & Post S.F.: Scientific American 229 (1973-6) 17

XII

Het ministerie van Verkeer en Waterstaat zou moeten laten onderzoeken of het mogelijk is om door middel van een systeem, dat op overeenkomstige wijze werkt als het automatisch treinbeïnvloedings-systeem, in gebruik bij de Nederlandse Spoorwegen, de gehoorzaamheid van automobilisten t.a.v. snelheidsbeperkingen en verkeerslichten af te dwingen.

Stellingen behorend bij het proefschrift van H.J.G.M. Derks.

Aan mijn vader

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De urines gebruikt in dit onderzoek waren afkomstig van babies opgenomen of ter wereld gebracht in de klinieken voor Kindergeneeskunde en Obstetrie & Gynaecologie. De ouders van deze kinderen ben ik erkentelijk voor hun toestemming. De afdelingshoofden Prof. Dr. J.H.P. Jonxis en Prof. Dr. H.J. Huisjes bedank ik voor hun medewerking. Voor het nauwkeurig verzamelen van urine monsters ben ik Zr. P.A. Schaafsma en Zr. M.L.E. Rijsouw en hun beider medewerksters (-ers) zeer erkentelijk.

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Tenslotte dank ik Mrs. W. Drayer, Frits Muskiet, Paula, Josée en Jo Simons en last but not least Elvire Derks-Simons, die allen op hun manier hebben bijgedragen aan het tot stand komen van dit proefschrift.

CONTENTS

Slightly modified versions of chapters 2, 4, 5 and 6 have been submitted for publication in The Journal of Clinical Endocrinology and Metabolism (ch. 2), Steroids (ch. 4) and The Journal of Steroid Biochemistry (chapters 5 & 6).

CHAPTER 1

INTRODUCTION.

Cortisol (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione) is the major glucocorticoid secreted by the adrenal cortex in humans. In addition to its anti-inflammatory action, cortisol has other biological actions, such as the stimulation of catabolic processes, the most important of which are the gluconeogenesis from proteins in the liver and the resorption of bone matrix. Furthermore, cortisol suppresses the secretion of pituitary growth hormone, and the presence of an excess of cortisol in children leads to growth arrest (Netter 1965).

There is a probability that cortisol has special functions in the foetus and the newborn infant. In sheep parturition commences when glucocorticoids are given to the foetal lamb, but not when such steroids are given to the pregnant ewes. The administration of glucocorticoids to pregnant ewes induces lung maturation in the foetal lamb (Liggins, 1969). In humans the data are conflicting (Liggins & Howie, 1972; Baden, Bauer & Colle, 1972). Liggins and Howie reduced the incidence of respiratory distress syndrome in the premature newborn infant from 26 to 9% by the administration of high doses of glucocorticoids to the mothers for two days prior to delivery.

Cortisol is metabolised in the human adult predominantly by A ring reduction, side chain reduction and side chain degradation (see fig. 1.1). In addition approximately one third of the cortisol metabolites are oxidised at C-11.

Quantitatively less important pathways are 6β-hydroxylation (Katz, Lipman, Frantz & Jailer, 1961; Burstein, Kimball, Klaiber & Gut, 1967; Trasher, Werk, Young Choi, Sholiton, Meyer & Olinger, 1969) and

oxidation of the C-21 hydroxy group to yield the C-21 carboxylic acids (Bradlow, Zumoff, Monder & Hellman, 1973a; Bradlow et al, 1973b; Monder, Zumoff, Bradlow & Hellman, 1975). 1β-hydroxylation has been shown to be a minor pathway of cortisol metabolism in patients suffering from Cushings syndrome (Dixon, Jones & Pennington, 1968a).

The metabolites mentioned above are excreted in the urine mainly as glucuronides (β-D-glucopyranosiduronic acid derivatives). Only small amounts of free and sulphate conjugated cortisol metabolites are found in the urine of the adult (Kornel & Saito, 1975a; Kornel & Miyabo, 1975b).

Early studies on the metabolism of cortisol in the human newborn indicated that the cortisol half life in neonatal blood is prolonged compared to that in adult blood, and that the formation of glucuronides is impaired (Bongiovanni, Eberlein, Westphall & Boggs, 1958; Holman & Migeon, 1958; Migeon, 1959; Bertrand, Gilly & Loras, 1960; Ulstrom, Colle, Burley & Gunville, 1960a). Migeon (1959) administered (4-^{14}C)cortisol to newborn infants and found that only 30% of the radioactive urinary metabolites could be accounted for. Only small amounts of radioactivity were extractable from the neonatal urine after incubation with β-glucuronidase. Similar results were obtained by Aarskog, Støa & Thorsen (1964) who found that the extent of glucuronoconjugation was even lower in premature newborn infants. 50% of the urinary radioactivity could not be accounted for in spite of the use of rigorous (ethyl acetate) extraction methods. Reynolds, Colle & Ulstrom (1962) concluded that the newborn infant has a relative insufficiency of both ring A reducing and glucuronoconjugation enzymes.

Drayer & Giroud (1965) tentatively identified the sulphates of several corticosteroids in neonatal urine hydrolysable both by solvolysis and *Helix Pomatia*

sulphatase. Klein, Chan & Giroud (1969) found that the sum of cortisol- and cortisone sulphates is comparable to the amount of tetrahydrocortisone-glucuronide excreted. In recent reports it was again confirmed that neonatal urine contains relatively large amounts of free cortisol metabolites and small amounts of glucuronides (Daniilescu-Goldinberg & Giroud, 1974). These authors reported that after radioactive cortisol was given to newborn infants, part of the radioactivity not extractable after the application of several hydrolytic methods by extensive ethyl acetate extraction could be isolated from the urine by butanol extraction and Amberlite XAD-2 chromatography. However, this material could not be identified.

With regard to the identity of the free steroids which could be isolated from neonatal urine, Ulstrom et al (1960b) identified 6β-hydroxy-cortisol in neonatal urine and postulated the presence of the ring A reduced analog of this compound. The presence of 6β-hydroxy-cortisol in neonatal urine was confirmed by Reynolds et al (1962), Cathro, Birchall, Mitchell & Forsyth (1963) and Kenny, Malveaux and Migeon (1963). Cathro et al and Kenny et al found also cortisol and cortisone, and Kenny et al showed that cortisol occurred as an unconjugated steroid. The latter authors found only extremely low amounts of tetrahydrocortisol in neonatal urine, and this contrasts sharply with the amounts found in adult urine in which this steroid is one of the predominant cortisol metabolites. This observation on neonatal urine was confirmed by several authors (Klein et al, 1969; Daniilescu-Goldinberg & Giroud, 1974; Shackleton, Honour & Taylor, 1975). Tetrahydrocortisone was generally found as a glucuronide, but its quantitative contribution to the total of cortisol metabolites was low in neonatal urine when compared to that in adult urine. 6β-hydroxy-cortisone was found by

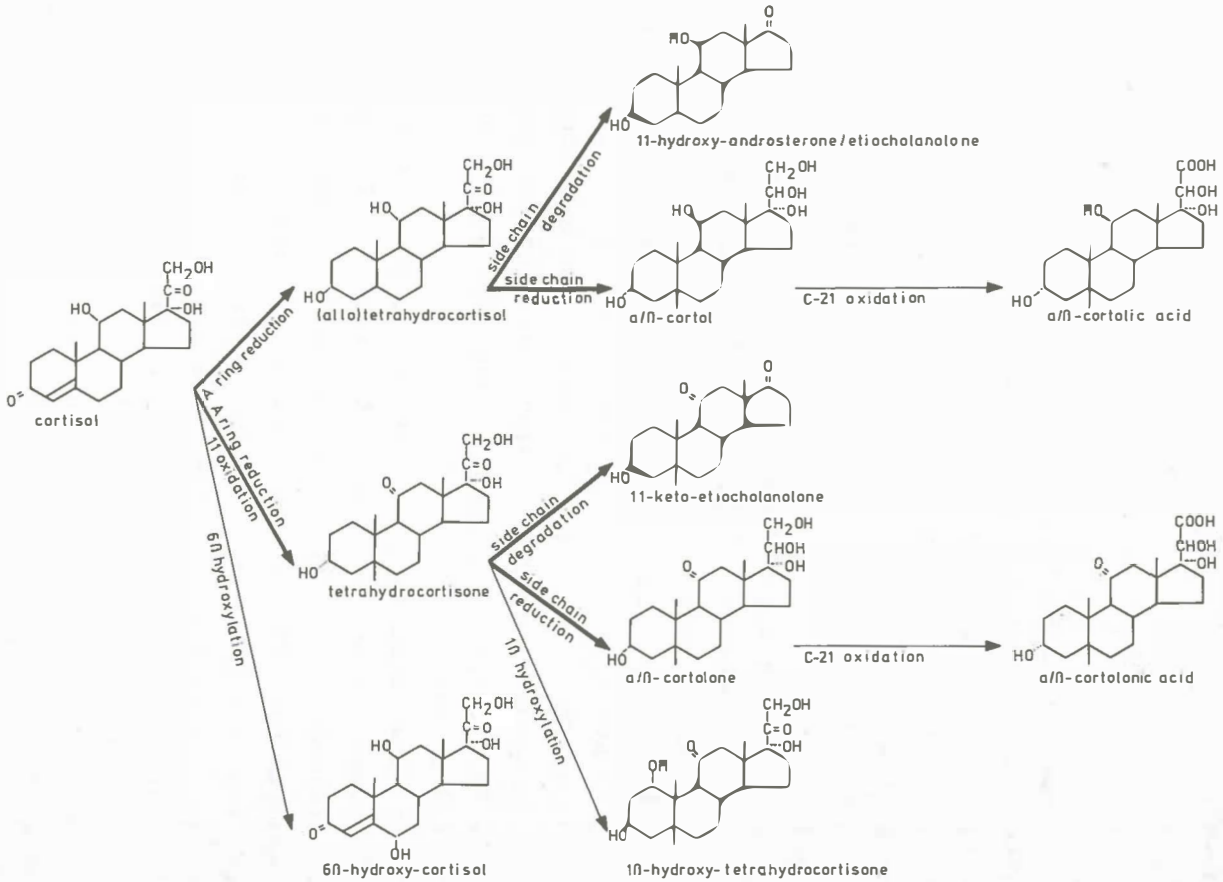
Cathro et al (1963) and Daniilescu-Goldinberg, Branchaud, Arato & Giroud (1973). The cortolones were detected by Daniilescu-Goldinberg et al (1973 and 1974) and Shackleton et al (1973 and 1975). Shackleton & Snodgrass (1974) identified 1β-hydroxy-20β-cortolone and 1β-hydroxy-tetrahydrocortisone in the urine of an infant suffering from pseudo-hypoaldosteronism. 1β-hydroxy-20β-cortolone was also found by Shackleton et al (1975) in the urine of normal and premature newborn infants.

Further, it is interesting that cortisone, rather than cortisol, is the predominant glucocorticoid present in the blood of newborn infants (Buus, Bro-Rasmussen, Trolle & Lundwall, 1966; Hillman & Giroud, 1965; Schweitzer, Branchaud & Giroud, 1969; Klein, Baden & Giroud, 1973).

Summarising it can be stated that the newborn infant excretes relatively large amounts of free cortisol metabolites, small amounts of glucuronides and substantial amounts of sulphates. Approximately half of the cortisol metabolites can not be isolated from the urine of the newborn infant using current methods.

The aim of the study described in this thesis was to elucidate the neonatal cortisol metabolism.

Fig. 1.1 (see opposite page). Metabolic pathways of cortisol in the human adult. The reaction sequence in vivo is not necessarily identical to that presented in this figure. Conjugation is not indicated. The quantitatively most important pathways are indicated by heavy lines. Minor metabolites such as the allo epimers of tetrahydrocortisone, the cortolones and the cortols are not shown.


cortisol
A ring reduction
(allo)tetrahydrocortisol
side chain degradation
11-hydroxy-androsterone/etiocholanolone
side chain reduction
α/β-cortol
C-21 oxidation
α/β-cortolic acid
A ring reduction
11 oxidation
tetrahydrocortisone
side chain degradation
11-keto-etiocholanolone
side chain reduction
α/β-cortolone
C-21 oxidation
α/β-cortolonic acid
6β hydroxylation
1β hydroxylation
6β-hydroxy-cortisol
1β-hydroxy-tetrahydrocortisone

CHAPTER 2.

THE ISOLATION OF CORTISOL METABOLITES FROM NEONATAL URINE.

2.1 Introduction.

From the literature it was evident that the methods which had been proved successful in other steroid metabolic studies were insufficient when applied to the investigation of the metabolism of cortisol in the newborn infant.

The failure of the earlier methods may be assigned as follows:

1. The human newborn excretes cortisol metabolites as conjugates which can not be hydrolysed by the known methods.
2. The unconjugated steroids or the steroids liberated by hydrolytic methods are too polar to be extracted quantitatively from neonatal urine by extraction procedures based on solvent-solvent partition.
3. The neonatal cortisol metabolites are unknown steroids.

Several authors (Ulstrom et al, 1960; Daniilescu-Goldinberg, Branchaud, Arato & Giroud, 1973; Daniilescu-Goldinberg & Giroud, 1974) provided evidence which supports possibility No. 3,and as the unidentified cortisol metabolites detected by these authors were very polar, possibility No. 2 must be seriously considered. In that case it is very well possible that there is at least a partial overlap in polarity between the unconjugated polar steroids and steroid conjugates such as glucuronides. This makes the use of the traditional methods in the elucidation of neonatal cortisol metabolism very questionable, as in these methods the separation of the free and different types of conjugated

steroids is based on selective extraction of the relatively apolar free steroids before and after the application of several hydrolytic methods.

Therefore,it was considered necessary to introduce a method which met the following requirements:

- The isolation of free or conjugated cortisol metabolites from neonatal urine should be quantitative.
- The separation of the free and the different types of conjugated steroids should be achieved without using solvent-solvent partitioning methods.

This chapter describes the merits of the adopted isolation and separation methods. These methods were tested using various hydrolytic methods.

2.2 Materials and Methods.

All analytical grade solvents, reagents and ready made thin layer chromatography plates were purchased from Merck, Darmstadt, Germany. Radioactive steroids were obtained from New England Nuclear, Dreieichenhain, Germany, and were purified by thin layer chromatography to give at least 95% purity. Non radioactive reference steroids were from Steraloids, Pawling, N.J., U.S.A., and Servachrom (Amberlite) XAD-2, particle sizes 300-1000 µ and 100-200 µ from Serva, Heidelberg, Germany. Ketodase was obtained from Warner Chilcott, Morris Plains, N.J., U.S.A. and Glusulase from Endo Laboratories, Garden City, N.Y., U.S.A. DEAE-Sephadex A-25 and LH-20 lipophilic Sephadex were from Pharmacia, Uppsala, Sweden.

(4-^{14}C)cortisol-21-sulphate, sodium salt, was prepared essentially according to the method of Griebsch and Garn (1958). From 2 g of cortisol, added to which were 4 µCi of (4-^{14}C)cortisol, specific activity 50-60

mCi/mMole, 1.54 g of the sodium salt of (4-^{14}C)cortisol-21-sulphate were prepared. Thin layer chromatography in the system benzene-acetone-water 2 : 1 : 2 (v/v/v), upper phase - methanol 128 : 55 (Dusza, Joseph & Bernstein, 1968) showed that the product was equimobile to the ammonium salt of dehydroepiandrosterone-3-sulphate (R_F = 0.40) and was slightly contaminated with starting material. 400 mg of the product were purified by Sephadex LH-20 column chromatography in the system chloroform-methanol 1 : 1, 0.01 M NaCl. The eluate was monitored with an UV detector (LDC, LUV-monitor) at 254 nm. The bulk of the product appeared in the sulphate fraction (Sjövall & Vikho, 1966). After pooling this fraction, and evaporation of the solvent in vacuo, 270 mg,m.p. 179-180°C (Griebsch & Garn; 185°C) of the sulphate were collected. The infrared spectrum (KBr) confirmed the structure, exhibiting strong absorption bands at 1250 and 1060 cm^{-1} and medium bands at 1130-1160 cm^{-1} (Bernstein, 1970).

Collection of urine.

To estimate the cortisol production rate, 0.15 µCi (3.3 x 10^5 dpm) of (1,2,6,7-^{3}H)cortisol of specific activity 80-100 Ci/mMole dissolved in 50 µl of ethanol and added to 2 ml of 5% glucose solution in water (w/v) was administered intravenously to a two days old female infant (birth weight 3110 g). This infant was admitted to the hospital suspected to be suffering from congenital adrenal hyperplasia. After fully explaining the study to both parents written parental consent was obtained. Urine was collected during 2 x 24 hours periods. The urine not needed for the determination of the cortisol production rate was used in the experiments described below.

Also the remainder of a 24 hours urine collection

of an adult male who was suspected of having adrenocortical insuffiency and received 1 μCi of tritiated cortisol to estimate his cortisol production rate, was used.

Determination of radioactivity.

Radioactivity was determined by counting duplicate 1 ml samples, to which 10 ml of scintillation cocktail (Wiegman, Woldring & Pratt, 1975) were added, in a Nuclear Chicago Mark III liquid scintillation counter. The results were corrected for quenching by the external standard method.

Amberlite XAD-2 procedure.

For routine purposes, 1.4 x 25 cm Amberlite XAD-2 columns (particle size 100-200 μ) were prepared as described by Shackleton, Sjövall & Wisén (1970). The columns were washed with 50 ml of water before use. 50-70 ml of urine or aqueous urine fractions (see below) were applied to the column, and after the sample had gone through, the column was immediately eluted with 100 ml of absolute ethanol without any washings with water (Bradlow, 1968; Shackleton et al, 1970). The ethanol eluate was collected from the precise moment that the ethanol reached the lower end of the column. The ethanol solvent front was clearly recognisable by the formation of small air bubbles in the Amberlite XAD-2 bed on the water-ethanol interphase. Duplicate 1 ml portions were taken from the sample, the aqueous eluate and the ethanol eluate, and assayed for radioactivity. The ethanol fraction was filtered and evaporated in vacuo, and the dry extract was used in further experiments.

DEAE-Sephadex ion exchange chromatography.

DEAE-Sephadex weakly basic ion exchanger was swollen in water for at least three days at room temperature. A Pharmacia K-9 column of dimensions 60 x 1 cm was packed with the DEAE-Sephadex gel and the column was run initially with 50 ml of distilled water, pumped at a flow rate of 1.05 ml/min by an LKB Varioperpex peristaltic pump. 20-50 ml of urine or aqueous urine fractions were applied and the column was eluted with a linearly increasing NaCl gradient in water, starting at zero concentration and increasing with 0.075 M NaCl/100 ml of water. The gradient mixer consisted of two interconnected cylindrical vessels (height 10 cm, diameter 8 cm), the mixing chamber of which was filled with 400 ml of distilled water and the other chamber with 400 ml of 0.6 M NaCl solution in water. The eluate was collected in fractions of 10.5 ml and samples of 1 ml were drawn from each fraction and counted. Essentially this procedure was described before by Hobkirk & Davidson (1971) for the separation of free and conjugated 17-keto-steroids.

Hydrolytic methods.

Several hydrolytic methods were applied consecutively to the different fractions obtained by DEAE-Sephadex chromatography and Amberlite XAD-2 isolation, as will be discussed below. These hydrolytic methods were carried out as follows.

Ketodase hydrolysis: The dry urine extracts were dissolved in 20 ml of 0.2 M acetate buffer pH 5.0 and 2 ml of Ketodase (10,000 units of β-glucuronidase) were added. This solution was incubated at 37^{o}C for 7 days.

Glusulase hydrolysis: The dry urine extracts were

dissolved in 20 ml of 0.2 M acetate buffer pH 4.7, and 0.2 ml of Glusulase (15,000 units of β-glucuronidase and 35,000 units of sulphatase) was added. The solution was incubated at 37°C for 5 days.

Solvolysis: The dry urine extracts were dissolved in 2 ml of methanol, and 38 ml of peroxide free tetrahydrofuran and 0.035 ml of a 70% solution of perchloric acid in water were added. The reaction mixture was allowed to stand at 37°C for 15 hours, 0.2 ml of pyridine was added and the solvents were evaporated in vacuo. The dry sample was dissolved in 20 ml of distilled water.

By Ketodase all known steroid glucuronides, except aldosterone glucuronide, are hydrolysed in excellent yields. Glusulase hydrolyses all glucuronides and also the 3-sulphates of 3β,Δ-5- and 3β,5α-steroids. This enzyme preparation contains also C-21 sulphatase. Steroid-sulphates are easily hydrolysed by solvolysis in good yields. An important disadvantage of solvolysis is that corticosteroids are partly destroyed under solvolytic conditions.

For a review of hydrolytic methods in steroid analysis see Bradlow (1970).

2.3 Experiments and Results.

Preliminary experiments with the Amberlite XAD-2 isolation procedure (see fig. 2.1) showed that washing the column with water, following the application of neonatal urine, eluted a substantial amount of radioactivity from the column. This was not the case when adult urine was processed. Adding 25% NaCl (w/v) to both the urine sample and the water used for washing the column raised the amount of radioactivity eluted in the ethanol fraction to 95.4% of the amount applied to the

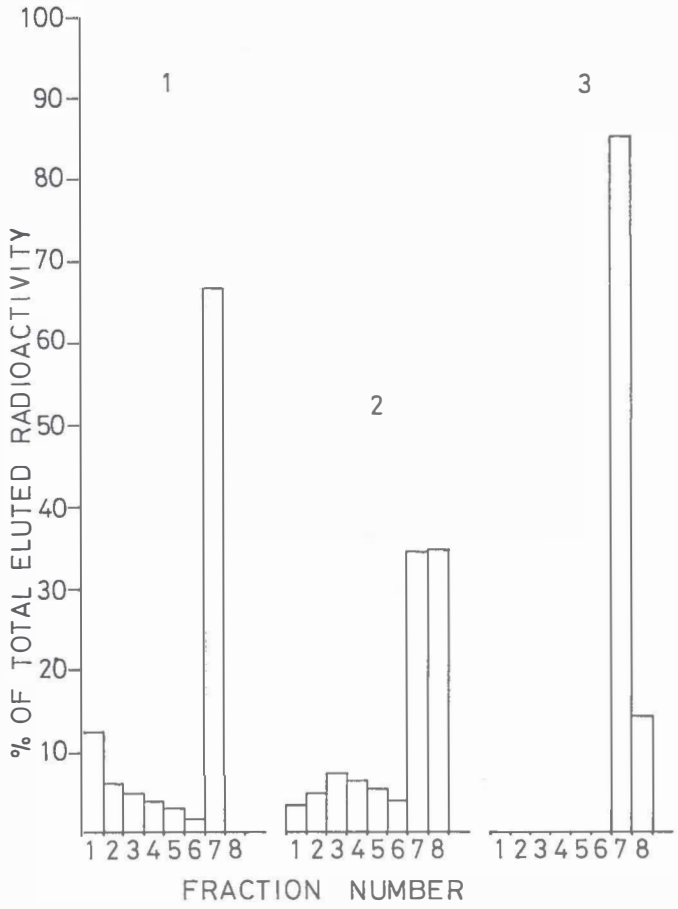


Fig. 2.1 Amberlite XAD-2 elution patterns of radioactive cortisol metabolites.

1. *5 ml of neonatal urine applied to a 1.2 x 13.5 cm Amberlite XAD-2 column (particle size 300-1000 µ), washed with 6 x 25 ml of distilled water (fractions 1-6) and 2 x 25 ml of ethanol (fractions 7 and 8).*
2. *As under 1 with the exception that the particle size of the Amberlite XAD-2 was 100-200 µ.*
3. *As under 1 with the exception that 5 ml of adult urine were applied to the column.*

column, while over all recovery was 97.2%. Although this was a satisfactory recovery, the presence of large amounts of NaCl in the ethanol eluate hampered further

processing, and this procedure was abandoned.

From fig. 2.1 it can be seen that the recovery of the radioactivity in the ethanol eluate could be quite satisfactory when the following three conditions were fulfilled, if Amberlite XAD-2 of particle size 100-200 μ was used, the amount of applied urine was not excessive, and the column was not washed with water. When 40 ml of neonatal urine were processed by Amberlite XAD-2 isolation under the conditions just mentioned, 93.1% of the applied radioactivity was recovered in the ethanol fraction, while the over all recovery was 96.0%. In the course of the experiments a maximum amount of 70 ml of the different aqueous urine fractions, obtained after DEAE-Sephadex chromatography, was applied to 1.4 x 25 cm columns, and recoveries were good (see table 2.1).

DEAE-Sephadex ion exchange chromatography separated the radioactivity present in neonatal urine into four main peaks (see fig. 2.2). The elution pattern of neonatal urine differed considerably from the elution pattern of adult urinary cortisol metabolites. In contrast to the adult urine, there was a relatively large amount of radioactivity eluting with the void volume of the column. This peak will be referred to as the unconjugated (*U*) peak (cf. Hobkirk & Davidson, 1971). In contrast the second peak, which will be referred to as the glucuronide (*G*) peak, contained a relatively small amount of radioactivity, when compared to the adult pattern. The third peak in the neonatal urine was virtually absent in the adult pattern, and this peak is referred to as the "presulphate" (*P*) peak. Peak No. 4 was present in both neonatal and adult urine. As this peak coincided with the peak of (4-^{14}C)cortisol-21-sulphate, which was added to the neonatal urine sample, it was called the sulphate (*S*) peak.

The total amount of neonatal urinary radioactivity eluted from the DEAE-Sephadex column in two experiments

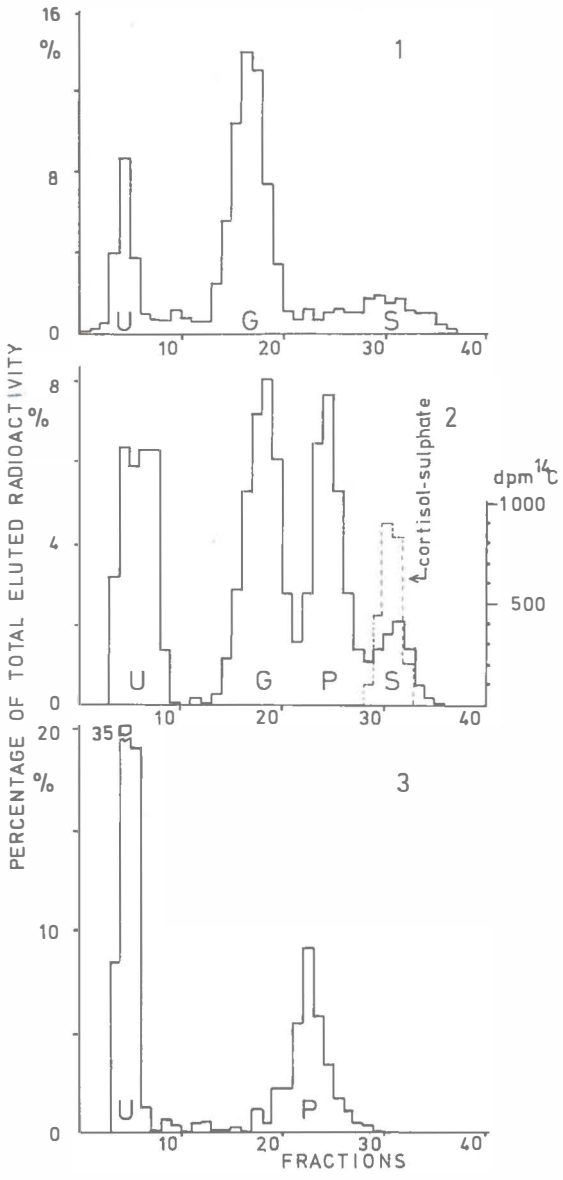


Fig. 2.2 DEAE-Sephadex elution patterns of radioactive cortisol metabolites.

1. *20 ml of adult urine.*
2. *50 ml of neonatal urine + standard (4-^{14}C)cortisol-21-sulphate.*
3. *20 ml of neonatal urine previously subjected to Glusulase hydrolysis.*

U, unconjugated peak; G, glucuronide peak; P, presulphate peak; S, sulphate peak.

was 97.5 and 96.8% of the amount applied to the column. The elution patterns in these duplicate experiments were virtually identical.

The unconjugated, glucuronide and presulphate peaks were collected and processed by Amberlite XAD-2 isolation. The amounts of radioactivity recovered from each fraction are presented in table 2.1.

Table 2.1 Recovery of the radioactivity present in DEAE-Sephadex fractions after Amberlite XAD-2 isolation.

SEPHADEX FRACTION	RADIOACTIVITY IN AMBERLITE XAD-2 ELUATES[a]		
	aqueous	ethanol	over all recovery
unconjugated	3.6	94.7	98.3
glucuronide	n.d.[b]	94.4	94.4
presulphate	n.d.[b]	94.2	94.2

a. Expressed as percentage of the total radioactivity applied to the column.
b. Not detectable.

Hydrolytic experiments.

The neonatal glucuronide fraction (*G*, see fig. 2.3) was subjected to Ketodase hydrolysis, and after incubation was completed the free steroids were separated from the conjugated steroids by DEAE-Sephadex chromatography. 79.3% of the radioactivity present in *G* was eluted in the unconjugated peak (*G-1*) and 20.7% was eluted in the glucuronide peak (*G-2*). The elution pattern indicated that the latter fraction was not homogeneous. The radioactivity present in *G-2* was pooled, isolated by Amberlite XAD-2 and subjected to Glusulase hydrolysis. DEAE-Sephadex separation of the incubation products showed that 23.7% of the applied radioactivity (equivalent to 4.9% of the radioactivity originally present in *G*) was eluted in the unconjugated peak (*G-3*). The material present in the glucuronide peak *G-4* (76.3% of the applied radioactivity) showed

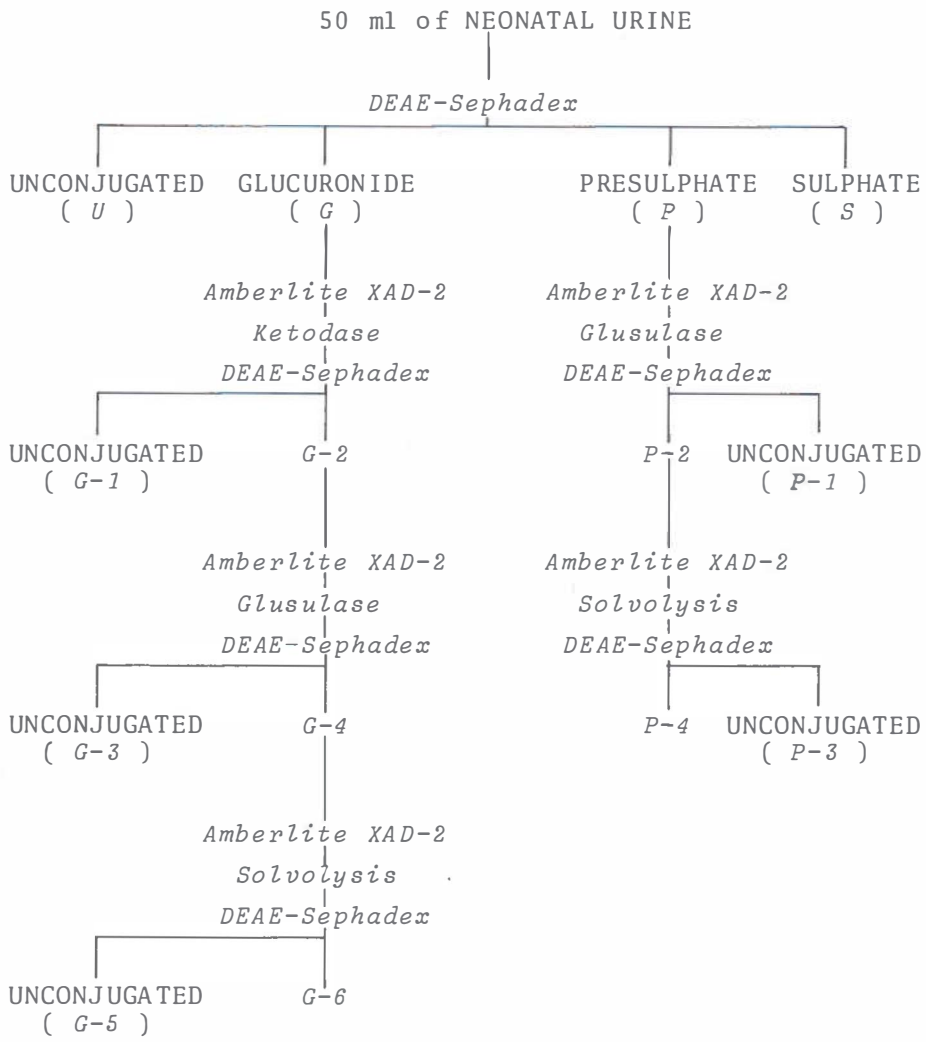


Fig. 2.3 Flow diagram of the hydrolytic experiments.

again a non-homogeneous distribution. *G-4* was pooled, subjected to Amberlite XAD-2 isolation and solvolysed. The solvolysis products were again chromatographed on DEAE-Sephadex and the elution pattern showed that 63.5% of the applied radioactivity (10.0% of *G*) was present in the unconjugated peak (*G-5*). The remainder of the applied radioactivity (*G-6*) eluted earlier than the

original glucuronide peak *G*, suggesting that chemical alterations had taken place.

When 20 ml of the adult glucuronide fraction were subjected to Ketodase hydrolysis, DEAE-Sephadex separation of the incubation products showed that all radioactivity was present in the unconjugated peak.

The presulphate fraction (*P*) was subjected to Glusulase hydrolysis, and after completion of the incubation separated on a DEAE-Sephadex column. Only 9.6% of the applied radioactivity appeared in the unconjugated peak (*P-1*) and the rest was found in the presulphate peak (*P-2*). The latter fraction was pooled, isolated by Amberlite XAD-2 and subjected to solvolysis. DEAE-Sephadex separation of the solvolysis products showed that 83.5% of the applied radioactivity (75.4% of the radioactivity present in *P*) was eluted in the unconjugated peak (*P-3*). Extending the solvolysis time to three days did not increase the amount of radioactivity eluted in the unconjugated peak (*P-3*). In agreement with the observation concerning the solvolysis of *G-4*, the material outside the unconjugated peak (*P-4*) eluted before the elution volume of the original *G*-peak.

The sulphate fraction was not investigated systematically because of the low amount of radioactivity present in this fraction. However, in an experiment in which 20 ml of the original neonatal urine were subjected to Glusulase hydrolysis and were separated on a DEAE-Sephadex column subsequently, no radioactivity could be detected in the sulphate fraction (see fig. 2.2), indicating that the material in this fraction was hydrolysable by Glusulase.

The data presented above are summarised in table 2.2.

Table 2.2 Amounts of radioactivity[a] present in the various fractions after DEAE-Sephadex ion exchange chromatography.

SAMPLE	HYDROLYTIC PROCEDURE	UNCONJUGATED	GLUCURONIDE	PRESULPHATE	SULPHATE	ASPECIFIC[b]
neonatal urine	-	29.6	33.3	27.3	8.7	1.1
adult urine	-	18.6	58.7	6.6	11.8	4.3
adult glucuronide fraction	Ketodase	98.8	-	-	-	-
neonatal glucuronide fraction	Ketodase	79.3	20.7	-	-	-
fraction *G-2*[c]	Glusulase	4.9	15.8	-	-	-
fraction *G-4*[c]	Solvolysis	10.0	-	-	-	5.8
neonatal presulphate fraction	Glusulase	9.6	-	90.4	-	-
fraction *P-2*[c]	Solvolysis	75.4	-	-	-	15.0

a. For the unfractionated original urine expressed as a percentage of the total radioactivity present in the urine; for the urine fractions expressed as a percentage of the radioactivity present in the original fraction after the first separation.
b. Radioactivity not eluted in one of the described fractions.
c. See fig. 2.3.

2.4 Discussion.

Using the methods described above 97% of the radioactivity present in neonatal urine, after the administration of radioactive cortisol to the baby, could be recovered in the free fraction and in the fractions containing the different types of conjugated steroids. The total amount of radioactivity isolated as unconjugated steroids before and after the application of several hydrolytic methods, was more than 90% of the radioactivity present in the urine.

Because of the vast differences between the methods presented here and those published previously (Daniilescu-Goldinberg et al, 1973; Daniilescu-Goldinberg & Giroud, 1974; Migeon, 1959; Aarskog et al, 1964), the results are only comparable to a limited extent. Also, the fact that only one neonatal urine could be studied limits the value of such a comparison. However, some interesting differences can be observed.

The relative amount of radioactivity as it was found by the DEAE-Sephadex method in the unconjugated fraction, is considerably higher (1.5 - 3 times) than the amounts found by Daniilescu-Goldinberg et al and Aarskog et al in their unconjugated fractions after ethyl acetate extraction. The amounts found by Migeon in the unconjugated fraction were much lower. One possible explanation for these differences might be that the conventional extraction methods, on which the observations of these authors were based, could not extract extremely polar cortisol metabolites quantitatively.

The DEAE-Sephadex glucuronide fraction (*G*) contained 26.4% of the total urinary radioactivity as Ketodase hydrolysable material (*G-2*). This is in good agreement with the amounts found by Daniilescu-Goldinberg et al, but somewhat high when compared to the results of

Aarskog et al. This could confirm the presumption mentioned above, that the isolation efficiency of conventional methods is not sufficient for the isolation of extremely polar substances, as according to these authors the glucuronide fraction contains predominantly tetrahydrocortisone (Daniilescu-Goldinberg et al) or "low polar" material (Aarskog et al), which can be extracted easily from neonatal urine by conventional methods.

The most interesting feature of the DEAE-Sephadex separation procedure was the appearance of the presulphate peak in the elution pattern of the neonatal urine. This peak (27.3% of the total urinary radioactivity) contained a large amount of material (20.6% of the total urinary radioactivity) only hydrolysable by solvolysis. This could mean that an important part of the neonatal cortisol metabolites consists of sulphatase resistant sulphates, such as for example 3α- or 6-sulphates (Roy, 1956). It is much more likely that the presulphate fraction does not contain sulphates at all, but some other unknown conjugate which is liable to solvolysis, because this fraction elutes so much earlier than cortisol-21-sulphate. The presence of a small amount (10.0%) of solvolysable material (*G-5*) in the glucuronide fraction, and of a similar small amount (9.6%) of Glusulase hydrolysable material (*P-1*) in the presulphate fraction, could be the result of an incomplete separation of the glucuronides and the presulphates. However, it is not certain that the Glusulase hydrolysable material in the presulphate fraction consisted of glucuronides or a mixture of glucuronides and sulphatase hydrolysable material.

An interesting question is whether there are any C-21 carboxylic acid metabolites of cortisol (Bradlow et al, 1973) present in neonatal urine and, if so, where they would elute in DEAE-Sephadex chromatography.

Unfortunately, reference steroids were not available to answer this question.

The above described methods are a sound basis for further elucidation of the neonatal cortisol metabolism, due to the high isolation efficiency and easy separation of free and several types of conjugated steroids.

CHAPTER 3

A PILOT STUDY; CORTICOSTEROIDS IN THE URINE OF A NEWBORN INFANT RECEIVING CORTISOL THERAPY.

3.1 Introduction.

A major problem in the identification of cortisol metabolites present in the urine of the newborn infant is that these metabolites are present in relatively small amounts compared to other steroids (Mitchell & Shackleton, 1969). Furthermore, as the efficiency of the isolation methods described in chapter 2 is extremely high, the urinary extracts obtained by these methods contain large amounts of non steroidal impurities, which necessitate the involvement of laborious purification methods prior to gas chromatography (Shackleton, Gustaffson & Mitchell, 1973; Schindler, Wuchter, Hoppen & Siekman, 1974).

As a first step towards identification of the neonatal cortisol metabolites it was considered useful to analyse the corticosteroids present in the urine of an infant receiving a relatively high dose of cortisol (25 mg/day) therapeutically. An important advantage of this approach is that the amounts of cortisol metabolites excreted in the urine of an infant during such therapy are about ten times higher than the amounts measured in the urine of a normal untreated infant (Clayton, 1968). A disadvantage is that the administration of such a massive dose of cortisol to an infant could influence the metabolism of cortisol to some extent. However, such influence would presumably be only of a quantitative nature.

In this chapter the analysis of the corticosteroids present in the urine of an infant receiving cortisol therapy is described. Definite or partial identification

was achieved by combined gas chromatography-mass spectrometry. No attempt was made to quantitate the identified steroids.

3.2 Materials and Methods.

Besides the materials mentioned in chapter 2 the following materials were used. Silylating and methyl-oximation reagents were purchased from Pierce Chemical Co., Rockford, Ill., U.S.A. and stationary phases for gas chromatography from Chrompack, Middelburg, The Netherlands.

Collection of urine.

A 12 hours urine collection (110 ml) from a male infant aged 14 days who received 25 mg of cortisol-hemisuccinate/day intravenously was filtered and frozen at -20^{o}C until processed. The baby was suffering from the salt losing form of congenital adrenal hyperplasia and therapy was started 24 hours before the beginning of the urine collection. Besides cortisol-hemisuccinate the baby received 2 mg DOCA and an 0.45% saline infusion. The baby was born after a normal pregnancy and had a birth weight of 3650 g.

Purification of urinary steroids.

50 ml of urine were subjected to DEAE-Sephadex chromatography, and the glucuronide fraction was subsequently hydrolysed by Ketodase. The unconjugated steroids and the steroids liberated by Ketodase from their glucuronides were isolated by Amberlite XAD-2 chromatography, and the ethanol eluates were filtered and taken to dryness in vacuo. The dry residues were dissolved in 0.2 ml of methanol and applied to an 0.5 mm

silica gel thin layer chromatography plate with a Shandon sample streaker. Alongside the extracts a mixture of standard cortisol and tetrahydrocortisol was applied and the plate was developed in the system water-methanol-chloroform 10 : 125 : 865 (v/v/v). After development the marker steroids were stained with blue tetrazolium (Neher, 1964) reagent and the following zones were scraped off: the zone ranging from cortisol to tetrahydrocortisol (zone A), the zone ranging from tetrahydrocortisol to half the R_F value of tetrahydrocortisol (zone B), and the zone ranging from half the R_F value of tetrahydrocortisol to the line of application (zone C). The adsorbent was eluted with methanol-methylene chloride 3 : 7 and the eluates were evaporated under a stream of nitrogen. The dry thin layer chromatographic fractions were dissolved in 1 ml of methanol.

Preparation of O-methyloxime-trimethylsilyl (MO-TMS) derivatives for gas chromatography.

0.5 ml of the different thin layer chromatographic fractions of both the unconjugated and glucuronide fractions was taken to dryness and 50 µl of pyridine containing 2% (w/v) of methylhydroxylamine.HCl were added. These solutions were heated at 60°C for two hours and the solvent was subsequently evaporated under a stream of nitrogen. 20 µl of Trisil-TBT (trimethylsilylimidazole-bis(trimethylsilyl)acetamide-trichlorosilane 3 : 2 : 2) were added and the reaction mixtures were heated at 100°C for four hours.

By this procedure all keto groups, except the keto group at C-11, were converted into O-methyloximes. All hydroxy groups were converted into trimethylsiloxy groups (persilylation; Horning & Eik-Ness, 1968).

Calculation of retention data.

M.U. (methylene units) values were calculated by linear intrapolation of the retention time of a compound between the retention times of the two bracketing even numbered n-alkanes (VandenHeuvel, Gardiner & Horning, 1965). All retention data were determined on a normal gas chromatograph using nitrogen as the carrier gas (see below).

Combined gas chromatography-mass spectrometry.

3 µl of the derivatised samples were injected into a Packard-Becker model 409 gas chromatograph equipped with a flame ionisation detector. A 2 m x 2 mm (i.d.) glass column packed with 3% OV-1 on Chromosorb W-HP 100-120 mesh was used at a flow rate of 18 ml/min (nitrogen). The temperature was programmed from 210-270°C at 1°C/min. After analysis of each sample 3 µl of the same sample were injected again together with 3 µl of a solution of even numbered n-alkanes in hexane.

Also 3 µl of each sample were injected into a Varian Aerograph model 1400 gas chromatograph coupled to a Varian MAT 112 double focusing mass spectrometer. The molecular separator was a Brunnée (1969) type separator with variable-conductance effusion path. A gas chromatographic column of 1.2 mm i.d. was used which was otherwise identical to the column mentioned above. Helium flow rate was 6 ml/min and the temperature was programmed from 200-270°C at 2°C/min. The temperature of both the separator and the ion source was 260°C. Electron energy was 70 eV, and the mass spectra were taken near to the top of the gas chromatographic peaks at a scan speed of 100 a.m.u./sec (linear scan function).

Besides the urinary samples standard mixtures containing the MO-TMS ethers of cortisol, tetrahydro-

cortisone, tetrahydrocortisol, 6β-hydroxy-cortisone, 6β-hydroxy-cortisol, α- and β-cortolone and α- and β-cortol were also chromatographed separately.

3.3 Results.

The M.U. values of the definitely identified steroid MO-TMS ethers are listed in table 3.1. The gas chromatograms as they were recorded from the different fractions are depicted in the figures 3.1, 3.2 and 3.3.

In the unconjugated fraction (thin layer chromatographic zone A) three main compounds were present which were easily identified as the MO-TMS ethers of tetrahydrocortisone (M.U. 29.66), tetrahydrocortisol (M.U. 30.27) and cortisol (M.U. 32.62) on the basis of these retention data and the mass spectra recorded from these compounds. The mass spectrometric data of these compounds are listed in table 3.2 (see also Thompson, Yung, Harten, Springer, Vikho & Sweeley, 1973). A number of relatively small peaks could be observed in the chromatogram (fig. 3.1), but these peaks were not identified. The same compounds as mentioned above were present in the glucuronide fraction (thin layer chromatographic zone A). In this fraction tetrahydrocortisone was the main compound in contrast to the corresponding unconjugated fraction in which cortisol was the predominant compound.

The thin layer chromatographic zone B of the unconjugated fraction showed a great number of gas chromatographic peaks. The compounds at M.U. values 32.88 and 33.14 were identified as the 3-methyloxime syn- and anti isomers of 6β-hydroxy-cortisol MO-TMS ether, and the mass spectra of these peaks (fig. 3.4) were identical to the mass spectra recorded from the authentic compound. 6-hydroxylated 3-keto steroids usually exhibit two gas chromatographic peaks because the syn- and anti isomers

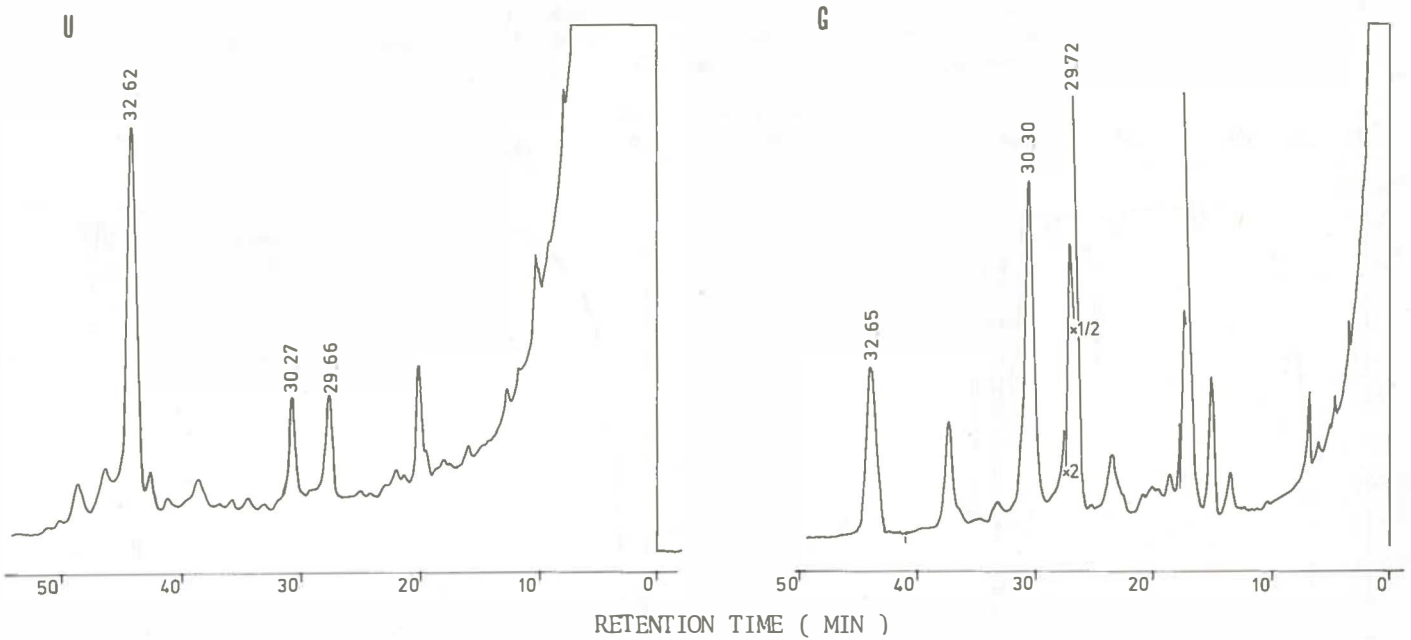


Fig. 3.1 Gas chromatography of the MO-TMS ethers of the steroids present in TLC zone A of both the unconjugated and glucuronide fractions. U = unconjugated; G = glucuronide. The numbers represent the M.U. values of the different peaks.

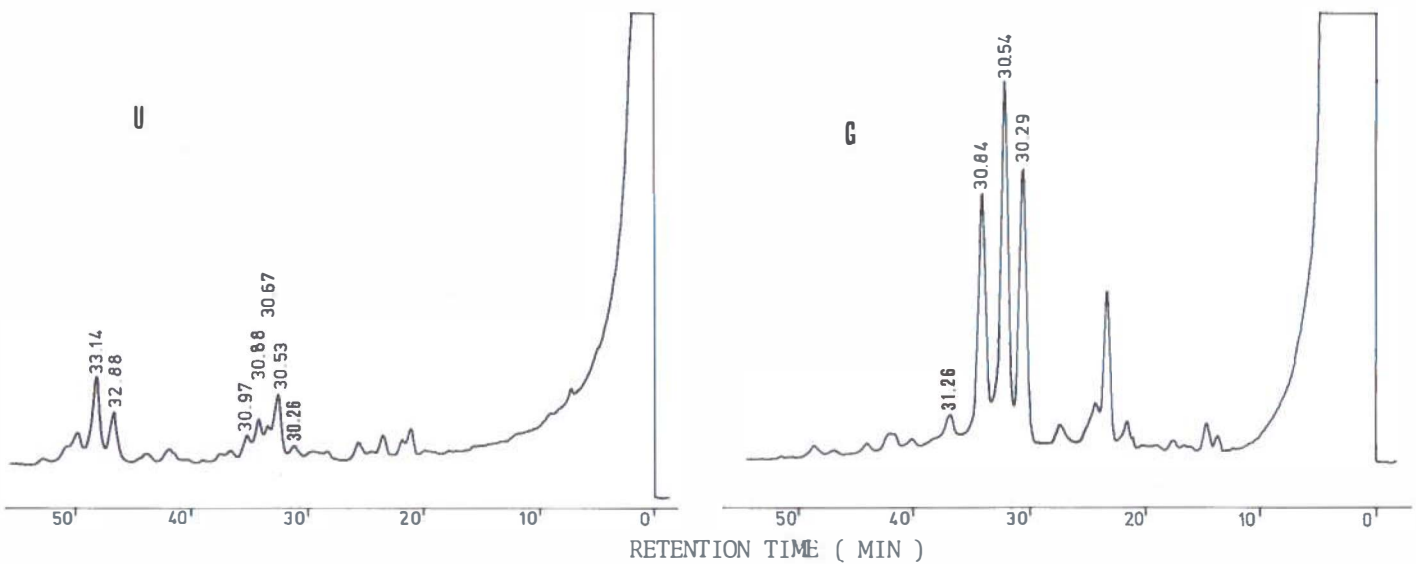


Fig. 3.2 Gas chromatography of the MO-TMS ethers of the steroids present in TLC zone B of both the unconjugated and glucuronide fractions. U = unconjugated: G = glucuronide. The numbers represent the M.U. values of the different peaks.

which are formed when the 3-keto group is methyloximated, are separated on the gas chromatographic column. The peak at M.U. 30.26 was identified as tetrahydrocortisol MO-TMS ether, but the mass spectrum of this compound showed that traces of other compounds were present in the peak. The peak at M.U. 30.53 showed intensive ions at m/e 697 (M^+), 666 (M-31), 594 (M-103), 576 (M-31-90), 486 (M-31-2x90) and 396 (M-31-3x90). Theoretically such a mass spectrum would be expected for the MO-TMS ether of an 11-keto-pregnane-tetrolone (molecular weight 697). The M-31 ion originates from the loss of the methoxy (CH_3-O) group from a methyl-oxime function, and the M-103 ion is frequently seen in the mass spectra of C-21 hydroxy-steroid MO-TMS ethers. This ion originates from cleavage of the C-20 - C-21 bond and the loss of a $(CH_3)_3Si$-O-CH_2 fragment. The consecutive loss of fragments of 90 a.m.u. results from the cleavage of trimethylsiloxyl ($(CH_3)_3Si$-OH) groups from the steroid nucleus and gives rise to the ions at m/e 576, 486 and 396. This fragmentation behaviour is analogous to the behaviour of tetrahydrocortisone MO-TMS ether (Thompson et al, 1973).

The peak at M.U. 30.67 showed also these ions, but an important difference was the relatively high intensity of the M-90 ion (m/e 607) in the mass spectrum of this peak. This fragment ion is not frequently seen as a predominant ion in the mass spectra of the MO-TMS ethers of common corticosteroids (cf. Thompson et al, 1973). In the lower part of this mass spectrum very intensive ions at m/e 196 and 271 were present, and these ions were of far less importance in the mass spectrum of the peak at M.U. 30.53. This indicates that this compound has a trimethylsiloxy-group which is very easily lost in mass spectrometry and has a structure which is considerably different from the structure of the 11-keto-pregnane-tetrolone described above. Shackleton

Table 3.1 M.U. values of the definitely identified urinary steroid MO-TMS ethers compared to authentic compounds.

STEROID	M.U. VALUES	
	urinary	standard
tetrahydrocortisone	29.66	29.69
tetrahydrocortisol	30.27	30.31
cortisol	32.62	32.64
6β-hydroxy-cortisol	32.88 33.14	32.84 33.12
α-cortolone	30.54	30.56
β-cortolone	30.84	30.89
α-cortol	31.26	31.28
β-cortol	30.84	30.89

and Snodgrass (1974) reported the identification of 1β-hydroxy-tetrahydrocortisone in the urine of an infant, and although these authors used a different derivative, the resemblance between the mass spectra of this compound and of the peak at M.U. 30.67 is striking (see fig. 3.4 and ref. Shackleton & Snodgrass, 1974). Therefore, this compound was tentatively identified as 1β-hydroxy-tetrahydrocortisone MO-TMS ether.

The peak at M.U. 30.88 was a rather complex mixture of several compounds, but the peak at M.U. 30.97 showed clearly the ions compatible with the structure pregnane-pentolone MO-TMS ether (molecular weight 771). Again the molecular ion (m/e 771), the M-31 ion (m/e 740) and the ions resulting from the consecutive loss of trimethylsiloxyl fragments (m/e 650, 560, 470 and 380) were observed (see fig. 3.4). This fragmentation behaviour is analogous to that of tetrahydrocortisol MO-TMS ether.

In the thin layer chromatographic zone B of the glucuronide fraction the following compounds were found by gas chromatography-mass spectrometry (see fig. 3.2). The peak at M.U. 30.29 was identical to tetrahydro-

Table 3.2 Mass spectrometric data of the definitely and partly identified steroid MO-TMS ethers.

STEROID	M^+	INTENSIVE FRAGMENT IONS
tetrahydrocortisone	609	578,506,488,398,276,258,246
tetrahydrocortisol	683	652,562,472,382,276,246
cortisol	636	605,515,425,276,246
6β-hydroxy-cortisol	see fig. 3.4	
α-cortolone	654	551,449,359,269,243
β-cortolone	654	551,449,359,269,243
α-cortol	728	638,548,535,523,445,433,343 253,243,217
β-cortol	728	638,625,548,535,523,445,433 343,253,243
1β-hydroxy-tetrahydrocortisone	see fig. 3.4	
11-keto-pregnane-tetrolone (M.U. 30.53)	see fig. 3.4	
pregnane-pentolone (M.U. 30.97, 31.10)	see fig. 3.4	
11-keto-pregnane-pentol (M.U. 31.35)	742	639,537,447,357,267,243,191
11-keto-pregnane-pentol (M.U. 31.62)	742	639,537,447,357,279,267,243 191
1β-hydroxy-20β-cortolone	742	652,537,447,369,357,297,284 271,221,217,209,205,196,181
pregnane-hexol (M.U. 32.00)	see fig. 3.4	

cortisol MO-TMS ether and the peak at M.U. 30.54 showed very intensively the ions of an 11-keto-pregnane-tetrolone MO-TMS ether (fig. 3.4) which was identical to the compound found in the same thin layer chromatographic zone of the unconjugated fraction. In addition, this peak showed also the ions characteristic for α-cortolone TMS ether which elutes at approximately the same retention time (table 3.1). The peak at M.U. 30.84 was identified as β-cortolone TMS ether, but some β-cortol TMS ether was present in this peak. Furthermore, the ions of an 11-keto-pregnane-tetrolone MO-TMS ether

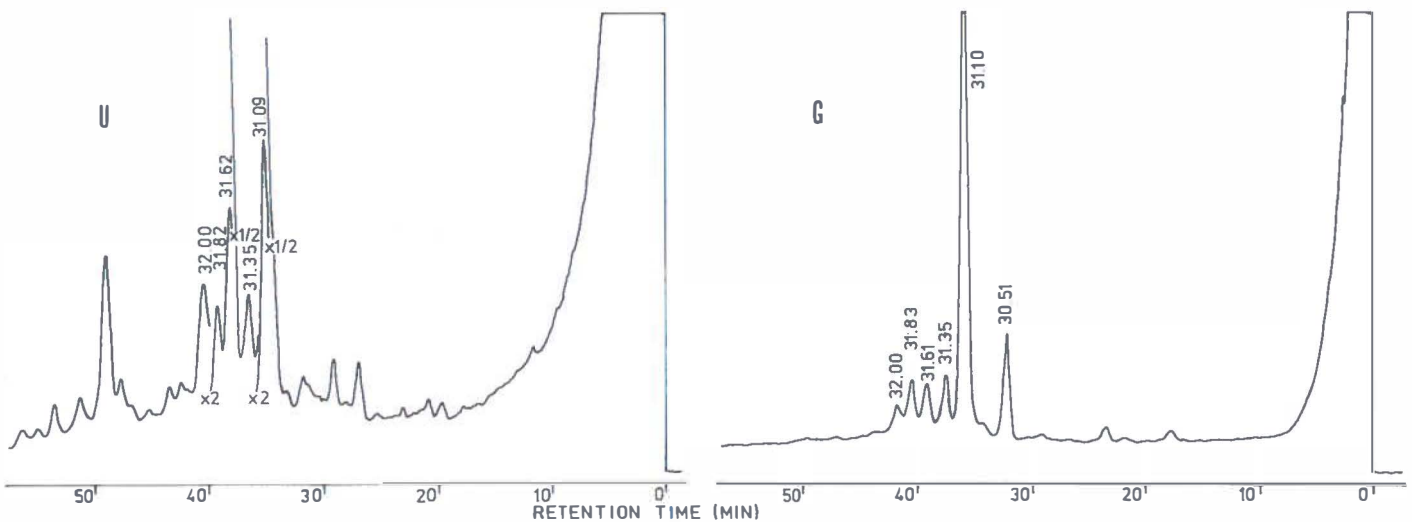


Fig. 3.3 Gas chromatography of the MO-TMS ethers of the steroids present in TLC zone C of both the unconjugated and glucuronide fractions. U = unconjugated; G = glucuronide. The numbers represent the M.U. values of the different peaks.

were, although weakly, present. The small peak at M.U. 31.26 was identified as α-cortol TMS ether.

The unconjugated fraction (thin layer chromatographic zone C) contained several peaks and the two predominant peaks had M.U. values 31.09 and 31.62. The peak at M.U. 31.09 showed very intensively the ions of a pregnane-pentolone MO-TMS ether (fig. 3.4). The major ions of the peak at M.U. 31.62 and also of the peak at M.U. 31.35 were the ions theoretically expected for an 11-keto-pregnane-pentol TMS ether (molecular weight 742). The molecular ion at m/e 742 was weak but visible. The most prominent ions were at m/e 639 (M-103) and 537 (M-205). The loss of a 205 a.m.u. fragment results from cleavage of the C-17 - C-20 bond, and this ion is usually very intensive in the mass spectra of steroid TMS ethers having a glycerol side chain as, for example, the cortolones in which the M-205 ion (m/e 449) is the most abundant ion over m/e 100. The ions at m/e 447, 357 and 267 result from the loss of the other trimethylsiloxy groups present in the molecule. The peak at M.U. 31.82 showed also the ions of an 11-keto -pregnane-pentol TMS ether, but in this mass spectrum the M-90 ion (m/e 652) was relatively intensive. Other important ions were observed at m/e 271, 209, 205 and 196. The differences between the mass spectra of the 11-keto-pregnane-pentol TMS ethers at M.U. 31.62 and 31.82 were of a similar nature as those between the spectra of the 11-keto-pregnane-tetrolone MO-TMS ethers at M.U. 30.53 and 30.67 (unconjugated fraction, thin layer chromatographic zone B). Again a remarkable resemblance is observed between the mass spectra of the 11-keto-pregnane-pentol TMS ether at M.U. 31.82 and of the compound identified by Shackleton & Snodgrass (1974) as 1β-hydroxy-20β-cortolone TMS ether. The mass spectrum of this compound wil be discussed again in chapter 5.

The peak at M.U. 32.00 exhibited all ions expected

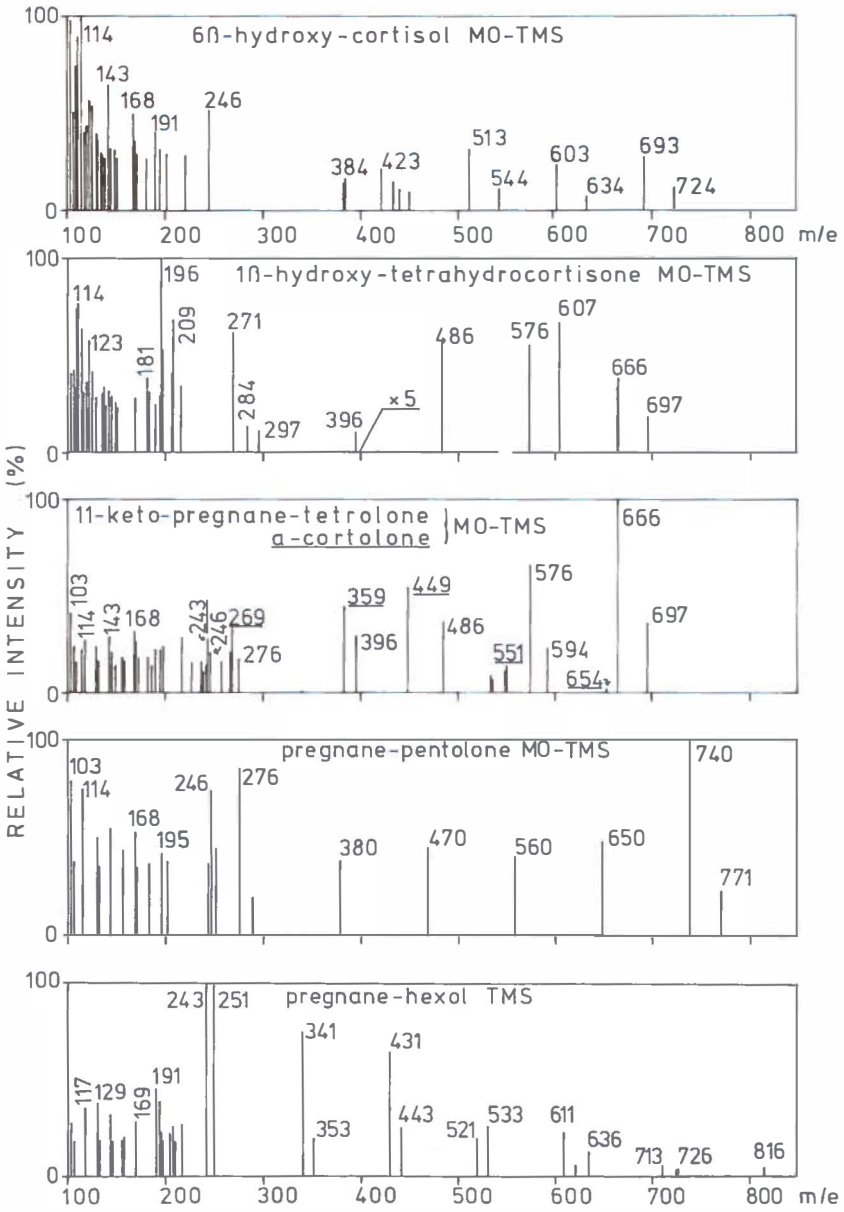


Fig. 3.4 Mass spectra of several corticosteroid MO-TMS ethers found in the urine of a newborn infant receiving cortisol therapy. In the mass spectrum of the mixture of the 11-keto-pregnane-tetrolone and α-cortolone MO-TMS ethers the ions belonging to α-cortolone are underlined.

for a pregnane-hexol TMS ether (molecular weight 816). The molecular ion at m/e 816 was clearly visible, and the M-103 ion (m/e 713) and the M-205 ion (m/e 611) were present. Besides the ions resulting from the consecutive loss of trimethylsiloxyl fragments from the m/e 611 ion (ions at m/e 521, 431, 341 and 251), ions which originated from the loss of similar fragments from the m/e 713 ion (ions at 623, 533, 443 and 353) were also visible. A similar fragmentation behaviour is observed in the spectra of the cortol TMS ethers (cf. Thompson et al 1973). The ions of a pregnane-hexol TMS ether were also weakly present in the peak at M.U. 31.62.

In the glucuronide fraction (thin layer chromatographic zone C) the pregnane-pentolone MO-TMS ether at M.U. 31.10 was the predominant compound. The peak at M.U. 30.51 was an 11-keto-pregnane-tetrolone MO-TMS ether having the same mass spectrometric appearance as the compounds found in other fractions at this M.U. value. The 11-keto-pregnane-pentol TMS ether at M.U. 31.35 was identical to the corresponding compound found in the unconjugated fraction and in the same thin layer chromatographic zone. The peaks at M.U. 31.61 and 31.83 were mixtures, but contained predominantly the compounds found also in the unconjugated fraction at these M.U. values. The mass spectrum of the pregnane-hexol TMS ether at M.U. 32.00 is shown in fig. 3.4.

3.4 Discussion.

The results obtained in this study prove that substantial amounts of ring A reduced corticosteroids having six oxygen functions were present in the urine of an infant receiving a high dose of cortisol. In fact a complete series of compounds having one additional hydroxy group when compared to the well known cortisol metabolites tetrahydrocortisone, tetrahydrocortisol,

cortolone and cortol were found in this urine. Also 6β-hydroxy-cortisol was found in a substantial amount, confirming the results reported previously (Ulstrom et al, 1960; Ducharme, Leboeuf & Sandor, 1970; Daniilescu-Goldinberg & Giroud, 1974). An important part of the steroids described here was found in the unconjugated fraction, which is in agreement with the results described in chapter 2.

It is very likely that the metabolic products of cortisol present in the urine of normal newborn infants are of a similar nature as the steroids described here. The results obtained previously by several authors (Ulstrom et al, 1960; Daniilescu-Goldinberg & Giroud, 1974) support this. The finding of a considerable amount of tetrahydrocortisol, a metabolite scarcely present in the urine of normal newborn infants (Daniilescu-Goldinberg & Giroud, 1974; Shackleton, Honour & Taylor, 1975), indicates that the metabolism of cortisol was altered quantitatively to a great extent by the large dose of cortisol administered to the studied infant.

Two compounds were present the MO-TMS ethers of which showed a striking resemblance to the MO-TMS ethers of 1β-hydroxy-tetrahydrocortisone and 1β-hydroxy-20β-cortolone identified before in the urine of an infant suffering from renal unresponsiveness to aldosterone. 1β-hydroxy-20β-cortolone was also identified in the urine of normal infants (Shackleton et al, 1975). However, these steroids showed a remarkably different fragmentation pattern when compared to the MO-TMS ethers of other 11-keto-pregnane-tetrolones and 11-keto-pregnane-pentols found in the urine of the infant studied here. Furthermore, these 1β-hydroxylated compounds were of minor quantitative importance (figures 3.2 and 3.3), and it can be concluded that hydroxylations at positions other than C-1 play a quantitatively more important role

in the neonatal cortisol metabolism.

6-hydroxylation in Δ-4,3-ketones is a well documented phenomenon in both neonatal (Ulstrom et al, 1960; Reynolds et al, 1962; Ducharme et al, 1970; Daniilescu-Goldinberg & Giroud, 1974; Shackleton et al, 1975) and adult cortisol metabolism (Frantz, Katz & Jailer, 1961; Katz, Lipman, Frantz & Jailer, 1961; Lipman, Katz & Jailer, 1962; Lambert & Pennington, 1965; Burstein, Kimball, Klaiber & Gut, 1967; Dixon & Pennington, 1968 and 1969; Ghosh & Pennington, 1969; Thrasher, Werk, Young Choi, Sholiton, Meyer & Olinger, 1969; Fukushima, Bradlow & Hellman, 1971).

So far no ring A reduced 6-hydroxylated corticosteroids have been definitely identified in human neonatal urine, but several authors (Ulstrom et al, 1960; Daniilescu-Goldinberg & Giroud, 1974) have suggested that such compounds might be present in neonatal urine.

CHAPTER 4

SYNTHESIS AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF RING A REDUCED 6-HYDROXYLATED CORTICOSTEROIDS.

4.1 Introduction.

As pointed out before (section 3.4), considerable evidence was available from the literature that 6β-hydroxylated Δ-4,3-ketones play an important role in the metabolism of cortisol in the human newborn, and to a lesser extent in the adult. This justified an investigation into the presence of saturated 6-hydroxylated corticosteroids in the urine of the newborn infant. As no data on such compounds could be obtained from the literature, it was decided to synthesise the 6-hydroxylated analogs of (allo)tetrahydrocortisol and (allo)tetrahydrocortisone, viz.:

3α,6α,11β,17α,21-pentahydroxy-5α-pregnane-20-one,
3α,6α,11β,17α,21-pentahydroxy-5β-pregnane-20-one,
3α,6β,11β,17α,21-pentahydroxy-5α-pregnane-20-one,
3α,6β,11β,17α,21-pentahydroxy-5β-pregnane-20-one,
3α,6α,17α,21-tetrahydroxy-5α-pregnane-11,20-dione,
3α,6α,17α,21-tetrahydroxy-5β-pregnane-11,20-dione,
3α,6β,17α,21-tetrahydroxy-5α-pregnane-11,20-dione,
3α,6β,17α,21-tetrahydroxy-5β-pregnane-11,20-dione.

The desired compounds were prepared by catalytic hydrogenation of the Δ-4 double bond of the parent 6α- and 6β-hydroxylated Δ-4,3-ketones, and enzymatic conversion of the 3-keto group into a 3α-hydroxy group.

A considerable amount of data was available from the literature about the catalytic reduction of the Δ-4 double bond in structurally related Δ-4,3-ketones having no hydroxy group at C-6 (Loewenthal, 1959; Caspi, 1959; McQuillin, Ord & Simpson, 1963; Harnik, 1963; Combe, Henbest & Jackson, 1967). Large variations in the

relative amounts of 5α- and 5β epimers obtained were observed. The ratio of the 5α- and 5β products depended strongly on such factors as the nature and position of substituents in the investigated compounds, the nature and amount of catalyst used, and the nature of the solvent. However, in solvents of medium to high polarity reasonable amounts of both epimers were usually found.

Enzymatic 3α-hydroxylation was chosen because of its high specificity, thus avoiding the separation of the different isomers obtained in reduction by metal hydrides or Raney nickel.

The stereochemistry at C-5 of the catalytic hydrogenation products was determined by recording the optical rotatory dispersion and circular dichroism spectra of these compounds. Further proof of structure was produced by using microchemical reactions and combined gas chromatography-mass spectrometry. The GC-MS properties of the compounds mentioned above, are also described in this chapter. As the main goal of the work described in this chapter was to obtain qualitative data on these compounds, the quantitative aspects of these syntheses were not considered, except in one case.

4.2 Materials and Methods.

Apart from the materials already mentioned in preceding chapters, the following materials were used. Silica gel columns (Merck Fertigsaüle A and B) were purchased from Merck, Darmstadt, Germany, and 10% palladium om charcoal (w/w) from Koch Light, Colnbrook, Bucks., England. 3α-hydroxy-steroid oxidoreductase was obtained from Nyegaard & Co., Oslo, Norway, and other biochemicals from Boehringer, Mannheim, Germany.

Thin layer chromatography (TLC).

System I: 0.25 mm silica gel plates developed in chloroform-methanol-water 865 : 125 : 10 (v/v/v).
System II: 0.25 mm kieselguhr plates impregnated with 10% ethylene glycol in acetone (v/v) and developed in ethyl acetate saturated with ethylene glycol.

High performance liquid chromatography (HPLC).

Instrumentation: A Waters Associates pump model 6000, a model U6K injector and a model 440 absorbance monitor operated at 280 or 254 nm (254 nm was used only for the detection of Δ-4,3-ketones).
Column: μ-Porasil 30 x 0.4 cm silica gel.
Flow rate: 2 ml/min.
Systems: I: 3.5% methanol and 0.35% water in chloroform.
II: 7.5% methanol and 0.7% water in chloroform.

Preparative column chromatography (PCC).

Column type A: 20 x 1 cm silica gel (Fertigsaüle A) operated at a flow rate of 100 ml/hr. The eluate was collected in fractions of 10 ml.
Column type B: 30 x 2.5 cm silica gel (Fertigsaüle B) operated at a flow rate of 250 ml/hr. Fraction size was 25 ml.
Systems: I: 2% methanol in chloroform.
II: 5% methanol in chloroform.

Combined gas chromatography-mass spectrometry (GC-MS).

Pure gas chromatography and combined gas chromatography-mass spectrometry were carried out as described in section 3.2. The following gas chromatographic columns and operating conditions were used:

Column A: 2 m x 2 mm (i.d.) glass column packed with 3% OV-1 on Chromosorb W-HP, 80-100 mesh; temperature program from 210-270°C at 1°C/min; flow rate 18 ml/min.
Column B: 2 m x 2 mm (i.d.) glass column packed with 3% Silar 10C on Gaschrom Q, 100-120 mesh; isothermal at 220°C; flow rate 10 ml/min.

Circular dichroism and optical rotatory dispersion spectra

These spectra were recorded on a Varian Cary 90 instrument.

Microchemical reactions.

These reactions were used to prove the structures of the synthesised compounds. 0.1-0.5 mg of the steroid to be investigated was subjected to one or more of the reactions described below. Usually one predominant product was obtained in high yield (80-100%). In the chromium trioxide oxidation the nature and the amounts of products formed depended on the stereochemistry of the hydroxy groups to be oxidised, and the reaction time used (cf. Grimmer, 1960).

Acetylation (Bush, 1961). The sample was dissolved in 0.2 ml of a mixture of acetic anhydride and pyridine (1 : 2), and allowed to react for 10 min at room temperature. The reagents were evaporated under a stream of nitrogen. Under these conditions primary hydroxy groups were quantitatively acetylated. Only very small amounts of secondary acetates were formed.

Deacetylation (Modification of the method of Dusza, Joseph & Bernstein, 1962). The sample was dissolved in 0.5 ml of a 0.5% solution (w/v) of sodium methoxide in

methanol, and nitrogen was bubbled through this solution for 1 min. The reaction mixture was allowed to stand for 30 min at room temperature. 20 µl of acetic acid were added, and the solvents were evaporated under nitrogen.

Chromium trioxide oxidation (Bush, 1961). The sample was dissolved in 0.5 ml of glacial acetic acid and 0.35 ml of a 2% solution (w/v) of chromium trioxide in water was added. The mixture was left in the dark at room temperature for 20-60 min. 5 ml of distilled water were added, and the mixture was extracted twice with 20 ml of methylene chloride. The combined extracts were filtered over a water repellent filter and evaporated in vacuo.

Sodium bismuthate oxidation (Bush, 1961). The sample was dissolved in 1 ml of a mixture of acetic acid and water (1 : 1), and 50 mg of sodium bismuthate were added. The suspension was shaken vigorously for 2 hours at room temperature. 5 ml of water were added, and the mixture was extracted twice with 20 ml of methylene chloride. The combined extracts were filtered over a water repellent filter and taken to dryness in vacuo.

Wolff-Kishner reduction (Vandenheuvel, 1974). The sample was dissolved in 0.4 ml of a 15% solution (w/w) of sodium ethoxide in ethanol,and 0.24 ml of a 5% solution (v/v) of hydrazine hydrate (99-100%) in ethanol was added. The vial containing the reaction mixture was placed in a stainless steel pressure vessel as described by Vandenheuvel (1974). 10 ml of ethanol were placed on the bottom of the pressure vessel to maintain a solvent saturated atmosphere inside the reaction vessel. The vessel was sealed, heated at 190^{o}C for 5 hours, and allowed to cool to room temperature. The reaction mixture was diluted with 5 ml of distilled

water and neutralised with acetic acid. The mixture was extracted with 2 x 20 ml of methylene chloride, and the combined extracts were filtered over a water repellent filter and evaporated in vacuo.

4.3 Experimental.

The synthetic pathways and the numbers assigned to the different steroid structures are presented in fig. 4.1.

The preparation of the 21-acetates of 6α-hydroxy-cortisol, 6β-hydroxy-cortisol, 6α-hydroxy-cortisone and 6β-hydroxy-cortisone (Ia, Ib, Ic and Id).

Ia, Ib, Ic and Id were prepared according to the method of Gardi & Lusignani (1967) from cortisol- and cortisone-21-acetates, respectively, via the 3-ethyl-dienol ethers (Serini & Köster, 1938). In this preparation cortisol-21-acetate yielded Ib as the main product, accompanied by about 25% Ia, as was estimated from TLC analysis (system I, detection by UV absorption at 254 nm). The yields from cortisone-21-acetate were 85% for Id and 15% for Ic. Ia and Ib as well as Ic and Id could be separated preparatively by PCC column type B, system I.

To confirm the identity of the products Ia, Ib, Ic and Id, approximately 100 µg of each of these compounds were deacetylated and converted into MO-TMS ethers (see section 3.2). GC-MS analysis (column A) showed that Ib_1 and Id_1 were identical to authentic samples (Steraloids), both compounds exhibiting two gas chromatographic peaks (syn- and anti isomers caused by methyloximation of the 3-keto group), with M.U. values 32.84/33.12 and 32.45/32.72, respectively. The mass spectra of the MO-TMS ethers of Ia_1 and Ic_1 were almost

identical to the spectra of Ib_1 and Id_1 MO-TMS ethers, respectively. The M.U. values were 33.24/33.63 for Ia_1 and 32.58/33.00 for Ic_1.

As further proof for the identity of Ia and Ic, 100 µg of Ia, Ib, Ic and Id were oxidised with chromium trioxide for 60 min. The products obtained were all identical by HPLC, system I (retention time 110 sec, detection at 254 nm, see also fig. 4.2).

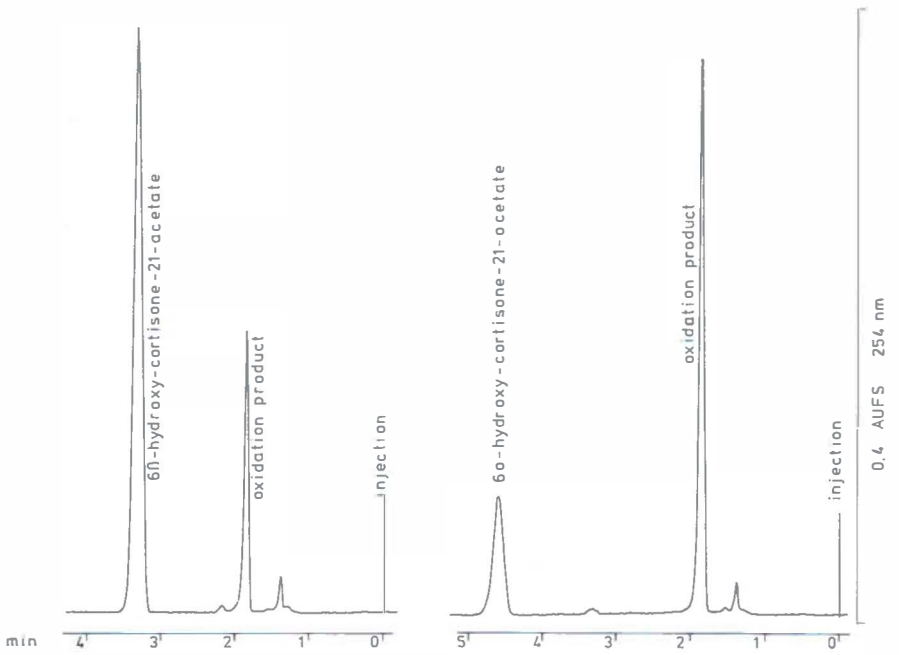
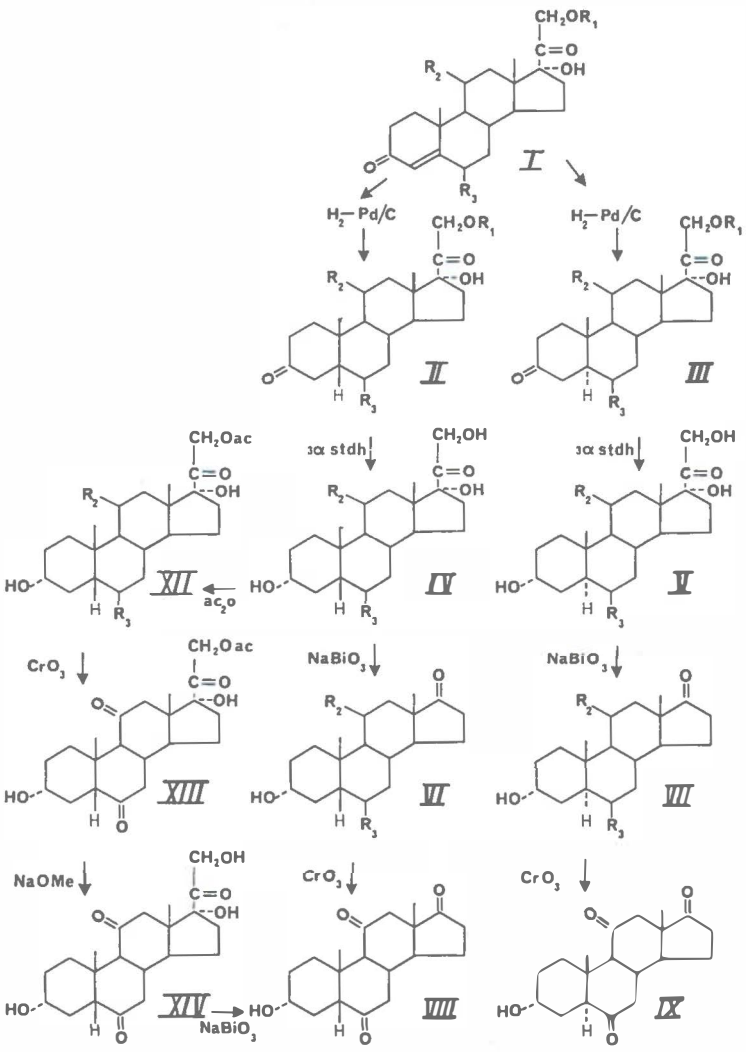


Fig. 4.2 HPLC analysis of the chromium trioxide oxidation products of the 21-acetates of 6α-hydroxy-cortisone and 6β-hydroxy-cortisone.
Reaction time was 60 min. The amount of product formed from the 6β-hydroxy compound (left) was appreciably less than the amount formed from the 6α-epimer. The reason for this is that, in contrast to saturated 6-hydroxylated steroids, the oxidation rate of a 6β-hydroxy group in Δ-4,3-ketones is lower than that of a 6α-hydroxy group (oxidation rates 6β : 6α = 1 : 3.3, Grimmer, 1960).

The synthesis of 6β-hydroxy-tetrahydrocortisol (IVb) and 6β-hydroxy-allotetrahydrocortisol (Vb).

100 mg of 6β-hydroxy-cortisol-21-acetate (Ib) were dissolved in 10 ml of dioxane (freshly distilled from sodium wire) and 200 mg of 10% palladium on charcoal (w/w) were added. Hydrogenation was carried out at room temperature by passing a gentle stream of hydrogen through the solution, until TLC (system I, detection at 254 nm) revealed that Ib was no longer present in the reaction mixture. Analysis of the reaction mixture by TLC system II (detection with blue tetrazolium, Neher, 1964) revealed two spots (R_F 0.36 and R_F 0.40) slightly less polar than Ib (R_F 0.35),and one strong apolar spot (R_F 0.72), presumably a hydrogenolysis product. The reaction mixture was filtered over a Celite pad, evaporated in vacuo and redissolved in 2 ml of methanol-chloroform 2 : 98 (v/v). This solution was applied to a PCC column type A and eluted with the same solvent (PCC system I). The eluate was monitored by analysing aliquots of each fraction of 10 ml by TLC system I (detection with blue tetrazolium). The products slightly less polar than Ib were collected in fractions 19-21 and 27-34, respectively. Fractions 22-26 contained a mixture of both products and these fractions were pooled and chromatographed again to obtain additional amounts of the products.

Approximately 100 µg of both compounds were deacetylated and converted into MO-TMS ethers. GC-MS analysis (column A) showed for each compound two gas chromatographic peaks. The apolar compound had M.U. values 33.11/33.47 and the more polar one 32.10/32.31. Both compounds gave mass spectra consistent with the structure 6β,11β,17α,21-tetrahydroxy-5α/β-pregnane-3,20-dione MO-TMS ether (see table 4.1 compounds IIb_1 and $IIIb_1$).



a R_1=Ac, R_2=β-OH, R_3=α-OH.

b R_1=Ac, R_2=β-OH, R_3=β-OH.

c. R_1=Ac, R_2==O, R_3=α-OH.

d: R_1=Ac, R_2==O, R_3=β-OH.

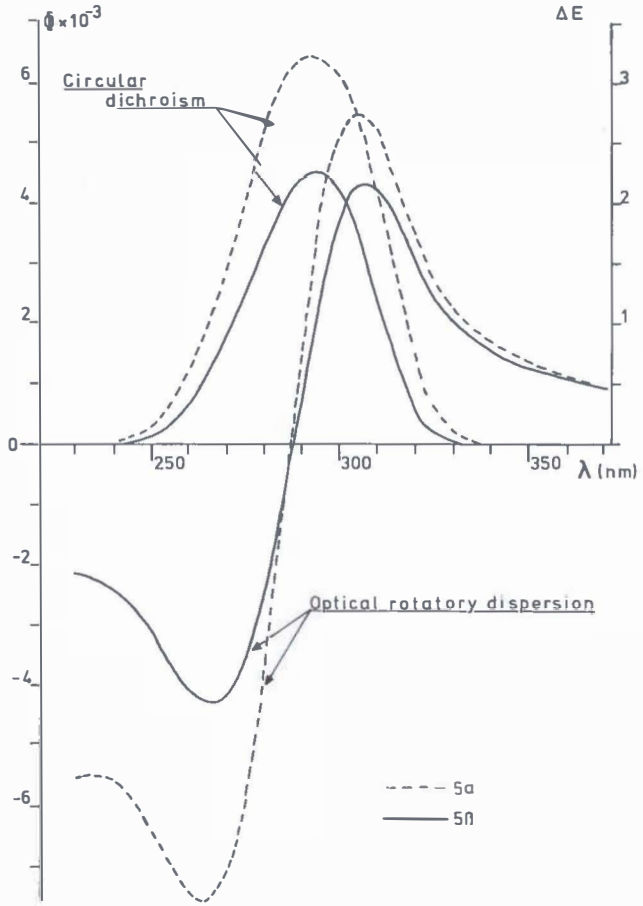
a_1, b_1, c_1, d_1, R_1=H, otherwise as a, b, c, and d respectively

Fig. 4.1 Synthetic pathways.

Recrystallisation of the two products from chloroform -heptane yielded 41 mg of the more polar and 12 mg of the less polar compound.

Circular dichroism and optical rotatory dispersion.

Both hydrogenation products were dissolved in appropriate volumes of methanol and circular dichroism and optical rotatory dispersion spectra were recorded. In fig. 4.3 these spectra and also the corresponding octant diagrams (cf. Velluz, Legrand & Grosjean, 1965) are shown. In this figure the C-21 alcohols are depicted instead of the C-21 acetates, and the octant diagrams refer to the 3-keto group (shown in black). The octant diagrams show that the atoms of the 5α-epimer lie predominantly in the upper positive octant. According to the Octant Rule (Moffit, Woodward, Moscowitz, Klyne & Djerassi, 1961) this results in a positive Cotton effect (see also Velluz et al, 1965). For the 5β-epimer the situation is somewhat less pronounced, but the atoms which are close to C-3 in the molecule lie predominantly in the upper negative octant, resulting in a negative Cotton effect. As the contribution of the 20-keto group is positive and identical for both the 5α- and the 5β-epimer, the net result is a larger positive Cotton effect for the 5α-epimer when compared to the 5β-epimer. The circular dichroism and optical rotatory dispersion spectra (fig. 4.3) show that the less polar compound exhibited an appreciably larger Cotton effect than the more polar epimer. On this basis the less polar epimer was identified as 21-acetoxy-6β,11β,17α-trihydroxy-5α-pregnane-3,20-dione (IIIb) and the more polar epimer as 21-acetoxy-6β,11β,17α-trihydroxy-5β-pregnane-3,20-dione (IIb). This is also in agreement with the experimental results obtained by Velluz et al (1965) for closely



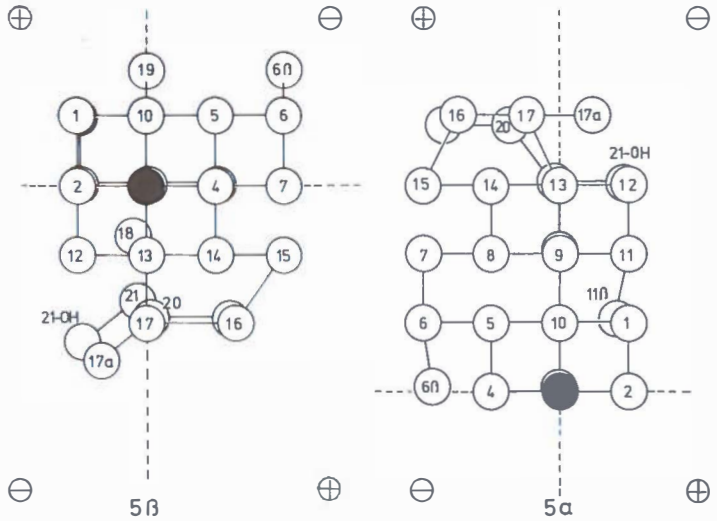


Fig. 4.3 Circular dichroism and optical rotatory dispersion of the catalytic hydrogenation products of 6β-hydroxy-cortisol-21-acetate.
Left: The CD/ORD spectra of these compounds.
solid lines: the more polar compound.
interrupted lines: the less polar compound.
Right: The corresponding octant diagrams.
These diagrams refer to the 3-keto group; the C-21 alcohols are depicted instead of the C-21 acetates.
The UV spectrum of these compounds showed a maximum at 290 nm. For further explanation see text.

related steroids.

Enzymatic 3α-hydroxylation.

10 mg of IIb as well as of IIIb were deacetylated and converted into the 3α-hydroxy compounds by incubation with 1.75 U of 3α-hydroxy-steroid oxidoreductase (3α-STDH, E.C. 1.1.1.50) and 50 mg of NADH in 20 ml of 0.1 M acetate buffer of pH 6.0 according to the equation:

3-keto-steroid + NADH + H^+ ⇆ 3α-hydroxy-steroid + NAD^+

Only if NAD^+ was removed from the reaction mixture could acceptable amounts of the 3α-hydroxy-steroid be obtained (50-70% conversion). This was achieved by adding a solution of 85 mg sodium-L-malate in 5 ml of water adjusted at pH 6.0 and 1100 U of malate dehydrogenase (MDH, E.C. 1.1.1.37) to the reaction mixture. NAD^+ was effectively removed from the reaction mixture according to the equation:

L-malate + NAD^+ ⇆ oxaloacetate + NADH + H^+

in which the equilibrium lies far to the right.

After 24 hours of incubation at 37°C the reaction mixtures were extracted three times with 5 volumes of ethyl acetate, the extracts were combined, dried over anhydrous sodium sulphate and taken to dryness. TLC, system I (detection with blue tetrazolium), showed that the starting compounds were for 50-70% converted into the products IVb and Vb. The crude products were purified by PCC column type A, system II. The yield was 3.6 mg of IVb and 2.9 mg of Vb. Both compounds were pure as was shown by TLC system I. 100 μg of IVb and Vb were converted into MO-TMS ethers and analysed by GC-MS (column A). The mass spectra (fig. 4.4 and 4.5) were consistent with the structure 3α,6β,11β,17α,21-pentahydroxy-5α/β-pregnane-20-one MO-TMS ether, showing the molecular ion (m/e 771), the M-31 ion (m/e 740) and the ions resulting from the loss of trimethylsiloxyl

Table 4.1 Gas chromatographic data of the different steroids (analysed as MO-TMS ethers).

STEROID NUMBER[a]	COMPOUNDS[b] HYDROXY GROUPS	KETONES	TRIVIAL NAME	RETENTION DATA OV-1	SILAR 10C
IIb_1	6β,11β,17α,21	-5β-P-3,20		32.10 32.31	
$IIIb_1$	6β,11β,17α,21	-5α-P-3,20		33.11 33.47	
IId_1	6β,17α,21	-5β-P-3,11,20		31.67 31.81	
$IIId_1$	6β,17α,21	-5α-P-3,11,20		32.89 33.33	
IVb	3α,6β,11β,17α,21	-5β-P-20	6β-OH-THF	30.96	
Vb	3α,6β,11β,17α,21	-5α-P-20	6β-OH-ATHF	31.04	
IVa	3α,6α,11β,17α,21	-5β-P-20	6α-OH-THF	31.12	
Va	3α,6α,11β,17α,21	-5α-P-20	6α-OH-ATHF	31.39	
IVd	3α,6β,17α,21	-5β-P-11,20	6β-OH-THE	30.80	
Vd	3α,6β,17α,21	-5α-P-11,20	6β-OH-ATHE	31.42	
IVc	3α,6α,17α,21	-5β-P-11,20	6α-OH-THE	30.55	
Vc	3α,6α,17α,21	-5α-P-11,20	6α-OH-ATHE	31.30	
XIV	3α,17α,21	-5β-P-6,11,20		30.99	
VIII	3α	-5β-A-6,11,17		1.36[c]	2.14[c] 2.21[c]
IX	3α	-5α-A-6,11,17		1.40[c]	1.72[c]
X	3α,11β	-5β-A		24.91	
XI	3α,11β	-5α-A		24.68	

a: See fig. 4.1.

b: Steroids of the pregnane series are abbreviated to P, and those of the androstane series to A.

c: Retention data are given as M.U. values, except the numbers marked with "c" which are relative retention times (relative to 3α-hydroxy-5α-androstane-11,17-dione MO-TMS ether).

Table 4.2 Mass spectrometric data of intermediate compounds and steroids used in the confirmation of structures (analysed as MO-TMS ethers).

STEROID NUMBER[a]	CHARACTERISTIC IONS AND THEIR RELATIVE INTENSITIES[b]													
	M^+	FRAGMENT IONS												
	726	695	605	515	425	276	246	244	202	172	168	143	114	103
IIb_1	*5*	*54*	*31*	*28*	*22*	*37*	*63*	*40*	*39*	*45*	*52*	*55*	*82*	*100*
	3	*35*	*23*	*28*	*25*	*31*	*60*	*40*	*39*	*44*	*52*	*57*	*75*	*100*
$IIIb_1$	*6*	*83*	*41*	*30*	*23*	*38*	*79*	*44*	*34*	*48*	*48*	*34*	*98*	*100*
	3	*46*	*26*	*20*	*16*	*27*	*70*	*36*	*31*	*42*	*47*	*30*	*79*	*100*
	652	621	531	441	288	280	276	258	246	198	168	143	114	103
IId_1	*14*	*75*	*57*	*41*	*13*	*15*	*14*	*27*	*27*	*51*	*84*	*51*	*51*	*100*
	7	*41*	*38*	*32*	*12*	*12*	*14*	*24*	*25*	*46*	*74*	*49*	*72*	*100*
$IIId_1$	*12*	*69*	*65*	*34*	*13*	*19*	*11*	*22*	*27*	*50*	*68*	*50*	*51*	*100*
	15	*85*	*73*	*33*	*14*	*12*	*12*	*25*	*24*	*49*	*76*	*48*	*46*	*100*
	652	621	531	441	288	276	258	246	198	168	143	129	114	103
XIV	*15*	*93*	*45*	*25*	*18*	*20*	*23*	*25*	*60*	*80*	*40*	*48*	*58*	*100*
	448	433	417	358	343	327	297							
VIII	*94*	*14*	*39*	*47*	*100*	*79*	*45*							
IX	*39*	*5*	*5*	*15*	*100*	*27*	*11*							
	436	421	346	339	331	320	256	241	197	184	169	156	107	
X	*4*	*4*	*31*	*0.5*	*9*	*3*	*100*	*60*	*27*	*27*	*20*	*28*	*19*	
XI	*18*	*6*	*54*	*24*	*40*	*20*	*100*	*98*	*88*	*100*	*64*	*100*	*67*	

a: See table 4.1 and fig. 4.1.

b: Only characteristic ions over m/e 100 are shown. The relative intensities (in italics) are given as percentage of the most abundant ion.

fragments (m/e 650, 560, 470 and 380). The ions m/e 244, 246 and 276 are also observed in the mass spectrum of tetrahydrocortisol MO-TMS ether (cf. Thompson et al, 1973).

The configuration of the 3α-hydroxy group was confirmed by the conversion of 200 μg of IVb and Vb into their 17-keto analogs by sodium bismuthate oxidation. These compounds (VIb and VIIb) were subjected to chromium trioxide oxidation for 20 min to yield VIII and IX, respectively, and the 6- and 17-keto groups were subsequently removed by Wolff-Kishner reduction. Both VIII and IX yielded a 50/50 mixture of X and XI as was shown by GC-MS. The latter compounds were also obtained by Wolff-Kishner reduction of authentic 3α-hydroxy-5β-androstane-11,17-dione and 3α-hydroxy-5α-androstane-11,17-dione, respectively. GC-MS data of compounds VIII-XI are given in tables 4.1 and 4.2.

In the reactions described above the 6β-hydroxy group is virtually as easily oxidised as the 11β-hydroxy group (Schreiber & Eschenmoser, 1955; Grimmer, 1960). The isomerisation of the A-B ring junction at C-5 is a known "risk" of the Wolff-Kishner reduction (House, 1972) as well as the side reaction leading to an 11β-hydroxy group (Vandenheuvel, 1974).

Compounds VIII and IX were analysed on both an OV-1 column and on a Silar 10C column. An interesting phenomenon was that VIII, in contrast to IX, gave rise to two gas chromatographic peaks on Silar 10C.

The synthesis of 6β-hydroxy-tetrahydrocortisone (IVd) and 6β-hydroxy-allotetrahydrocortisone (Vd).

IVd and Vd were prepared from 6β-hydroxy-cortisone-21-acetate (Id) in exactly the same way as IVb and Vb were prepared from Ib. Catalytic hydrogenation of Id yielded approximately 75% IId and 25% IIId, easily

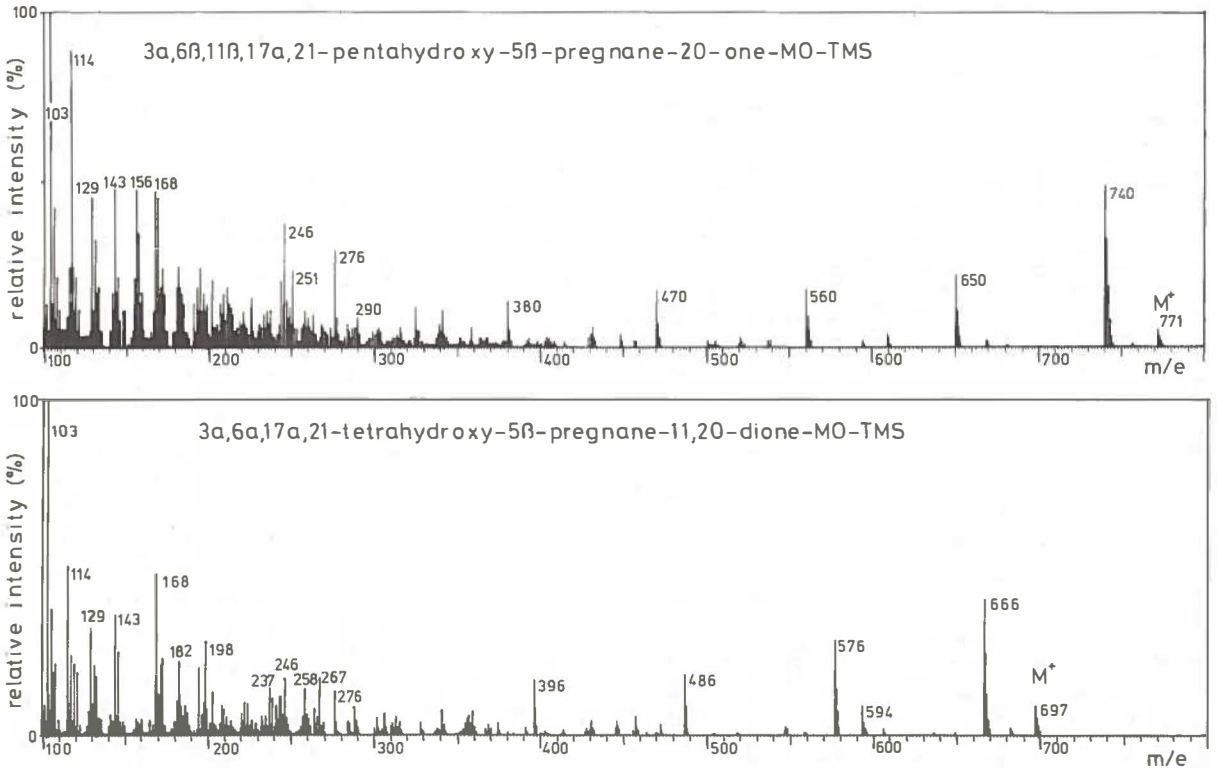


Fig. 4.4 Mass spectra of the MO-TMS ethers of 6β-hydroxy-tetrahydrocortisol and 6α-hydroxy-tetrahydrocortisone.

separable by PCC column A, system I.

The conversion factor for the enzymatic 3α-hydroxylation was again 50-70%, being somewhat better for IVd when compared to Vd. The GC-MS data of the MO-TMS ethers of IVd and Vd are presented in table 4.1 and fig. 4.5. Again these mass spectra show the molecular ion (m/e 697), the M-31 ion (m/e 666) and the ions resulting from the loss of trimethylsiloxyl fragments (m/e 576, 486 and 396). Also the ion resulting from the loss of the primary trimethylsiloxy group at C-21 (m/e 594) can be observed. In the lower part of the spectra many peaks also observed in the spectra of the MO-TMS ethers of IVb and Vb are present.

Proof of structure was accomplished by converting IVd and Vd into their 17-keto analogs (VId and VIId, respectively) and subjecting these compounds to chromium trioxide oxidation for 20 min to give VIII and IX, respectively, identical by GC-MS to the compounds obtained from IVb and Vb previously.

The synthesis of 6α-hydroxy-tetrahydrocortisol (IVa) and 6α-hydroxy-allotetrahydrocortisol (Va).

The same synthetic procedure as described above was applied to 6α-hydroxy-cortisol-21-acetate (Ia). It was, however, not possible to separate the intermediates IIa and IIIa preparatively. Therefore, the mixture of these compounds was converted into the 3α-hydroxy-steroids IVa and Va. The latter compounds were easily separated by HPLC system II. The retention times were 818 and 721 sec, respectively. The concentration of IVa in the mixture was about three times as high as the concentration of Va. GC-MS data of the MO-TMS ethers of IVa and Va are presented in table 4.1 and fig. 4.5.

Va could not be purified completely by HPLC from an impurity having a slightly higher retention time on OV-1

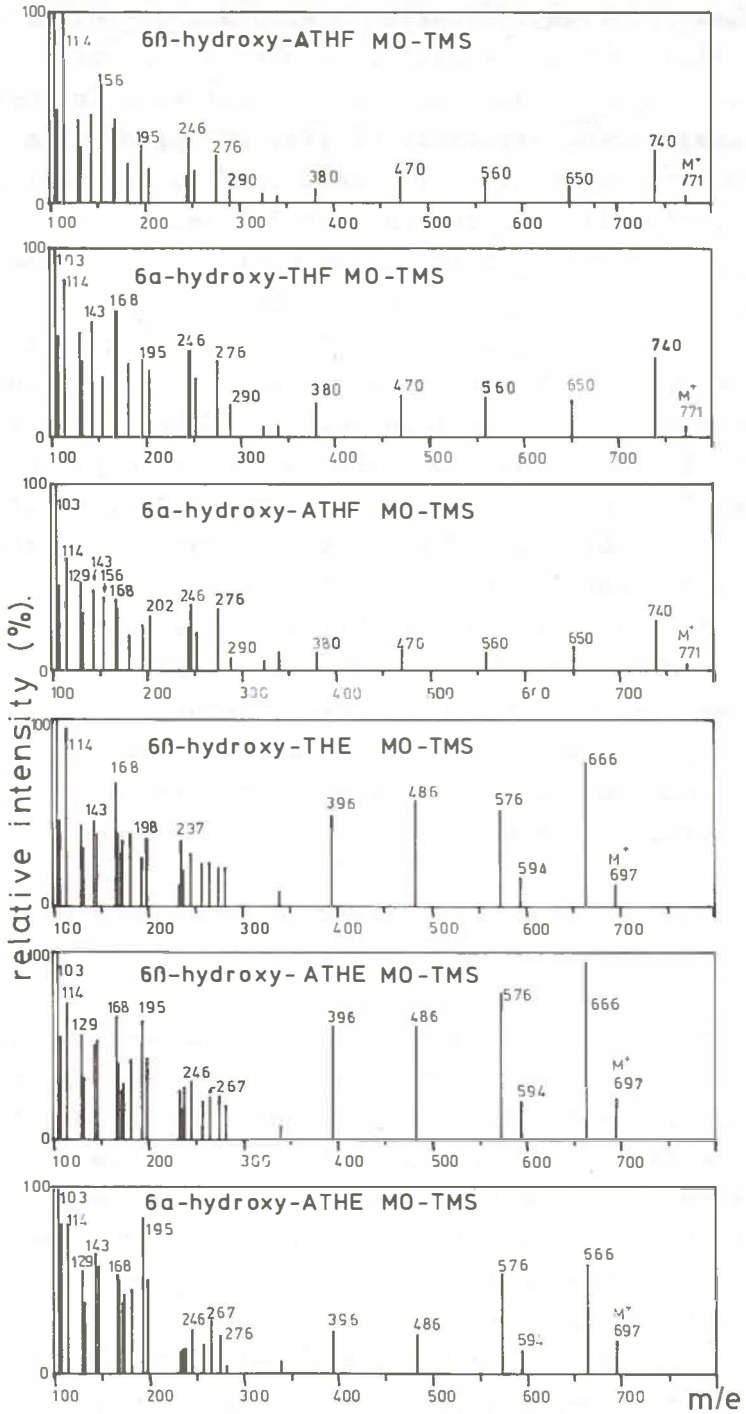


Fig. 4.5 Mass spectra of the synthesised steroids. Only the most abundant ions are shown and isotope ions are not depicted.

and a mass spectrum indicating a pregnane-pentolone MO-TMS ether. As only a minimal amount of Va was available, this compound was not investigated further.

To confirm the structure of IVa, 200 µg of this compound were acetylated. The acetate XIIa was purified by HPLC system II (retention time 341 sec) and subjected to chromium trioxide oxidation for 30 min. The product (XIII) was purified by HPLC system II (retention time 78 sec), deacetylated, and a part of it was converted into its MO-TMS ether. This compound (XIV MO-TMS ether) was identical by GC-MS (tables 4.1 and 4.2) to the product obtained by the same procedure from IVb. The remainder of XIV was converted into its 17-keto analog by sodium bismuthate oxidation and the product was identical to VIII (tables 4.1 and 4.2).

The HPLC purification of XIII showed also that a considerable amount of the 21-acetate of IVc (see below) was present in the reaction mixture. This undoubtedly is due to the lower oxidation rate of a 6α-hydroxy group when compared to 6β- and 11β-hydroxy groups (Grimmer, 1960).

The synthesis of 6α-hydroxy-tetrahydrocortisone (IVc) and 6α-hydroxy-allotetrahydrocortisone (Vc).

IVc and Vc were prepared from 6α-hydroxy-cortisone-21-acetate (Ic) as outlined for IVa and Va from Ia. The mixture of IIc and IIIc was converted into IVc and Vc which were formed in a ratio of 3 : 1, as estimated by HPLC system II (retention times 579 and 492 sec, respectively). 100 µg of IVc and Vc were converted into their MO-TMS ethers and subjected to GC-MS analysis (table 4.1 and figures 4.4 and 4.5).

The structure of IVc was confirmed by converting 200 µg of this compound into XIV according to the procedure described for the conversion of IVa into XIV,

and the final product was found to be identical to the product obtained from IVa by GC-MS. Also the 17-keto analog of this product was identical to VIII.

4.4 Discussion.

Figures 4.4 and 4.5 show that the mass spectra of the different 6-hydroxylated pregnane-pentolone MO-TMS ethers are very similar. Only minor variations in relative ion intensities are observed. The same is true for the mass spectra of the MO-TMS ethers of the different 6-hydroxylated 11-keto-pregnane-tetrolones. It is questionable whether such minor differences could provide a reliable criterion to discriminate between the different isomers of each group of compounds.

On the basis of retention times a discrimination between the different 6-hydroxylated 11-keto-pregnane-tetrolone MO-TMS ethers is very well possible. However, the small differences between the retention times of the MO-TMS ethers of the different 6-hydroxylated pregnane-pentolones (except 6α-hydroxy-allotetrahydrocortisol MO-TMS ether) do not allow an adequate identification on the basis of retention times. In this case high resolution gas chromatography on open-tubular capillary columns might provide the solution.

Another possibility is to combine the separation power of different chromatographic techniques, such as gas chromatography and thin layer chromatography. Unfortunately even TLC system II, which usually gave very good separations, could not separate 6α-hydroxy-tetrahydrocortisol from 6β-hydroxy-tetrahydrocortisol completely. As these compounds also could not be separated by gas chromatography, the combination of thin layer chromatography and gas chromatography was abandoned.

Eventually, high performance liquid chromatography

in combination with gas chromatography provided sufficient separation power to discriminate between the different 6-hydroxylated corticosteroids. This technique will be discussed in detail in chapter 5.

Recently Setchell, Gontscharow, Axelson & Sjövall (1976) reported the identification of 6β-hydroxy-tetrahydrocortisol in the urine of the baboon. Also several other saturated corticosteroids having 6 oxygen functions were found. However, definite evidence for the configuration of the 6-hydroxy group could not be provided because reference steroids were not available. A comparison between the mass spectra in figures 4.4 and 4.5 and the mass spectra given by Setchell et al shows that, except for variations in relative ion intensities, there is good agreement between these spectra.

The same is true for the mass spectra shown in chapter 3 and these spectra will be discussed again in chapter 5.

CHAPTER 5

THE IDENTIFICATION OF SOME POLAR 6-HYDROXYLATED CORTICOSTEROIDS IN THE URINE OF A HEALTHY FULLTERM INFANT.

5.1 Introduction.

The mass spectra of the urinary 11-keto-pregnane-tetrolone and pregnane-pentolone MO-TMS ethers (chapter 3) when compared to the spectra of the corresponding synthetic compounds mentioned in chapter 4, strongly indicate that the urinary compounds are 6-hydroxylated corticosteroids. All major ions present in the mass spectra of the synthetic steroid MO-TMS ethers are also present in the spectra of the urinary compounds. The differences in relative ion intensities which can be observed, are mainly due to varying intensities of the ions between m/e 100 and 200, and such variations can be attributed to minor variations in the operating conditions of the gas chromatograph-mass spectrometer.

As mentioned in section 4.4, it is impossible to assign a definite structure to the urinary pregnane-pentolone (section 3.3) on the basis of the gas chromatographic-mass spectrometric data of the MO-TMS ether of this compound, when compared to those of the synthetic 6-hydroxylated pregnane-pentolone MO-TMS ethers. However, on the basis of similar mass spectra (see sections 3.3 and 4.3) combined with similar retention data the urinary 11-keto-pregnane-tetrolone MO-TMS ether at M.U. 30.54 (section 3.3) can be identified as 6α-hydroxy-tetrahydrocortisone MO-TMS ether.

In this chapter the identification of 6α-hydroxy-tetrahydrocortisone in the urine of a healthy fullterm infant is described. 6α-hydroxy-20α-cortolone and 6α-hydroxy-20β-cortolone were also identified.

Additional evidence for the identity of these three compounds was obtained by analysing the sodium bismuthate degradation products of these steroids by combined gas chromatography-mass spectrometry and mass fragmentography. The urine was systematically investigated for the other 6-hydroxylated cortico-steroids described in section 4.3. By using a combination of high performance liquid chromatography and gas chromatography it was possible to discriminate between the different 6-hydroxylated pregnane-pentolones.

5.2 Materials and Methods.

Besides the materials already mentioned in the preceding chapters 20β-hydroxy-steroid oxidoreductase (E.C. 1.1.1.53), purchased from Boehringer, Mannheim, Germany, was used.

Urine collection.

A 24 hours urine sample was collected during the second day of life from a healthy fullterm infant (birth weight 3550 g). When the baby voided the urine was immediately placed in the 24 hours urine collection bottle at -20°C.

Urine processing.

After mixing the 24 hours collection well, a 20 ml aliquot was subjected to DEAE-Sephadex chromatography as described in section 2.2, and the unconjugated and glucuronide fractions were collected. 9.5 µg of 6β-hydroxy-allotetrahydrocortisone were added to both fractions as a recovery standard. In a pilot study this steroid could not be detected in this urine sample. The unconjugated and glucuronide conjugated steroids were

isolated by Amberlite XAD-2 and the glucuronides were hydrolysed by incubation with Ketodase (section 2.2). The free steroids were again isolated by Amberlite XAD-2.

The ethanol eluates of both fractions containing the unconjugated steroids and the hydrolysed glucuronides were filtered and evaporated in vacuo. The dry residues were transferred to small tubes with methanol, taken to dryness under nitrogen and dissolved in 1.5 ml of methanol-methylene chloride 3 : 7 (v/v). The cloudy solutions were filtered over 0.4 x 4 cm silica gel columns prepared in Pasteurs pipettes. The columns were rinsed with 5 ml of the same solvent and the eluates were evaporated under nitrogen. The dry residues were redissolved in a small volume of methanol and applied on a 0.25 mm silica gel thin layer chromatography plate by means of a Shandon sample streaker. Tetrahydrocortisone was applied alongside the extracts and the plate was developed in the system water-methanol-chloroform 10 : 125 : 865. After the solvent had travelled over a distance of 10 cm, the development was stopped and the lanes in which the tetrahydrocortisone was run were sprayed with blue tetrazolium reagent. The lanes containing the extracts were scraped off from the starting line to just beyond the position of tetrahydrocortisone. The adsorbent was eluted with 6 ml of methanol-methylene chloride 3 : 7 and the eluates were evaporated. The dry residues were transferred to Applied Science MiniAktor vials with methanol and taken to dryness. 100 µl of methanol-chloroform 7 : 93 (v/v) were added and the residues dissolved.

Even after this extensive prepurification of the two extracts the solutions were contaminated with solid material which was precipitated by centrifuging at 3000 r/min for 15 min. Disturbing the solid material as little as possible the solutions were removed from the MiniAktor vials and subjected to HPLC separation.

High performance liquid chromatography (HPLC).

The instrument described in section 4.2 was used. The column was a μ-Porasil 0.4 x 30 cm silica gel column operated at a flow rate of 2 ml/min in the system water-methanol-chloroform 7 : 70 : 923 (v/v/v). Before each of the two extracts were injected into the liquid chromatograph, a standard mixture containing the eight synthetic 6-hydroxylated corticosteroids (see fig. 5.1) was run to determine the retention times of the various fractions to be collected. The prepurified urinary extracts were then injected and after 5 fractions had been collected according to the time schedule determined in the standard run, the column effluent was switched to the composition water-methanol-chloroform 3 : 30 : 67 (v/v/v). The eluate was collected for another 15 min and this fraction is referred to as fraction No. 6.

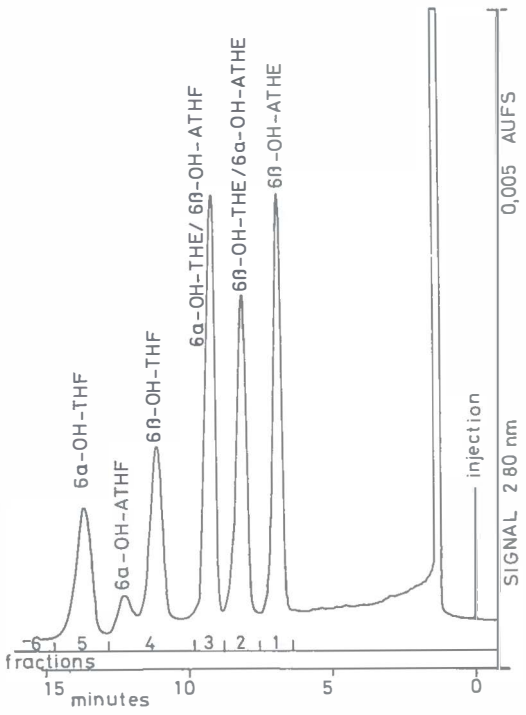


Fig. 5.1 High performance liquid chromatography of eight synthetic 6-hydroxy-corticosteroids. For operating conditions see text.

All fractions of both the unconjugated (denoted by *U-1, U-2,...,U-6*) and the glucuronide (denoted by *G-1, G-2,...,G-6*) extracts were taken to dryness and dissolved in 1 ml of methanol. 2.5 µg of methyl deoxycholate were added as an internal standard to all fractions,except *U-6* and *G-6*. For *U-6* and *G-6* 5.4 µg of 6β-hydroxy-tetrahydrocortisone were used as internal standard, because methyl deoxycholate interfered with the steroids present in these fractions.

Combined gas chromatography-mass spectrometry (GC-MS).

0.5 ml of each of the HPLC fractions were transferred to MiniAktor vials, taken to dryness under nitrogen and converted into MO-TMS ethers as described in section 3.2. From each fraction 3 µl were subjected to gas chromatography and another 3 µl to combined gas chromatography-mass spectrometry. Pure gas chromatography and GC-MS were carried out as described before (section 3.2). The MO-TMS ethers of the intact steroids were chromatographed isothermally at 245°C on an OV-1 column (for details see section 4.2). The trimethylsilyl ethers of the sodium bismuthate degradation products of the corticosteroids (see below) were chromatographed at 220°C on a Silar 10C column.

Mass fragmentography was carried out using the magnetic field switching mode. Usually an integration time of 0.3 sec per channel was used, but when the gas chromatographic peaks were too narrow to obtain sufficient data points per peak shorter integration times were used. The signals of each channel were recorded independently on a multichannel potentiometric recorder.

Retention data.

Relative retention times were calculated using

methyl deoxycholate or 6β-hydroxy-tetrahydrocortisone (for fractions *U-6* and *G-6*) as reference compounds. The relative retention times of standard steroid MO-TMS ethers are presented in table 5.1. From the relative retention times (relative to methyl deoxycholate) the corresponding M.U. values (see chapters 3 and 4) can be calculated using the expression:

$$M.U. = 4.785 \times t_{R,MDC} + 27.057$$

in which $t_{R,MDC}$ denotes the retention time relative to methyl deoxycholate.

This expression was calculated by linear regression of the M.U. values of twelve steroid MO-TMS ethers (the eight 6-hydroxylated corticosteroids, the cortolones and cortols) to the corresponding relative retention times, and is only valid for M.U. values between 30.00 and 32.00.

Table 5.1 Relative retention times of the MO-TMS ethers of 6-hydroxylated standard steroids (OV-1, 245°C).

STEROID	RELATIVE RETENTION TIME	
	MDC (1)	6β-OH-THE (2)
6α-hydroxy-allotetrahydrocortisol	0.912	
6α-hydroxy-tetrahydrocortisol	0.829	
6β-hydroxy-allotetrahydrocortisol	0.827	
6β-hydroxy-tetrahydrocortisol	0.810	
6α-hydroxy-allotetrahydrocortisone	0.891	
6α-hydroxy-tetrahydrocortisone	0.722	
6β-hydroxy-allotetrahydrocortisone	0.912	
6β-hydroxy-tetrahydrocortisone	0.778	
6α-hydroxy-20α-cortolone	0.902	1.160
6α-hydroxy-20β-cortolone	0.967	1.243

(1) Relative to methyl deoxycholate TMS ether.
(2) Relative to 6β-hydroxy-tetrahydrocortisone MO-TMS ether.

Sodium bismuthate oxidation.

From some HPLC fractions 0.25 ml aliquots were taken and subjected to sodium bismuthate oxidation as described in section 4.2. The reaction products were transferred to MiniAktor vials and silylated with a mixture of pyridine, bis(trimethylsilyl)acetamide and trichloroethane 5 : 4 : 1 (v/v/v) at 40°C for 60 min. By this procedure only the relatively unhindered hydroxy groups (including 6β-hydroxy) were silylated and these derivatives will be referred to as uTMS ethers.

5.3 The preparation of reference compounds 6α-hydroxy-20α-cortolone and 6α-hydroxy-20β-cortolone.

300 µg of 6α-hydroxy-tetrahydrocortisone were dissolved in 0.5 ml of methanol and cooled in an ice bath. 20 mg of sodium borohydride were added and the suspension was stirred at 0°C for 30 min (Appleby & Norymberski, 1955). The excess of sodium borohydride was destroyed by the addition of 5 ml of 0.25 M acetic acid in water, and the solution was extracted with 25 ml of ethyl acetate three times. The combined extracts were dried, filtered and evaporated in vacuo (product *A*).

Also, 300 µg of 6α-hydroxy-tetrahydrocortisone were dissolved in 1 ml of 0.1 M phosphate buffer pH 7.4 and 5 mg of NADH and 25 µl of 20β-hydroxy-steroid oxidoreductase (E.C. 1.1.1.53, 18 U/ml) were added (Dixon & Pennington, 1968). The solution was incubated overnight at 37°C and extracted three times with 5 ml of ethyl acetate. The extracts were combined, dried and evaporated in vacuo (product *B*).

Product *A* was purified by HPLC, system water-methanol-chloroform 1 : 10 : 89, and the predominant peak at retention time 634 sec was collected. The eluate was taken

to dryness and the residue was dissolved in 10 ml of methanol (solution *A*). The same procedure was applied to product *B* and the same fraction was collected. After evaporation of the solvent the residue was dissolved in 10 ml of methanol (solution *B*).

From both solutions *A* and *B* 100 µl were taken, evaporated and converted into MO-TMS ethers. Gas chromatographic analysis showed for *A* two peaks with area ratio 1 : 4. The smaller peak (*A-1*) had a relative retention time (relative to 6β-hydroxy-tetrahydrocortisone MO-TMS ether) of 1.160 and the relative retention time of the bigger peak (*A-2*) was 1.242. Product *B* gave only one peak at relative retention time 1.243 (*B-1*). GC-MS analysis showed that *B-1* and *A-2* had identical mass spectra consistent with the structure of a 6α-hydroxy-cortolone-TMS ether (see fig. 5.4). The mass spectrum of *A-1* (fig. 5.4) differed only from those of *A-2* and *B-1* in the relative intensities of the ions.

500 µl of both solutions *A* and *B* and also 25 µg of 6α-hydroxy-tetrahydrocortisone were subjected to sodium bismuthate oxidation, and after silylation the three samples when subjected to GC-MS on Silar 10C gave only one peak each, all having identical retention times and identical mass spectra (fig. 5.2, top).

The data presented above are convincing evidence for the identification of *A-2* and *B-1* as 6α-hydroxy-20β-cortolone and *A-1* as 6α-hydroxy-20α-cortolone (cf. Vandenheuvel, 1975).

5.4 Results.

Recovery of standard 6β-hydroxy-allotetrahydrocortisone.

The peak areas of the MO-TMS ether of 6β-hydroxy-allotetrahydrocortisone which was added to both the unconjugated and glucuronide fraction as a recovery

standard and was collected in fractions *U-1* and *G-1* (see fig. 5.1), were used to determine the amount of this compound recovered from the urine extracts *U* and *G*. These peak areas were compared to the peak area of a known amount of 6β-hydroxy-allotetrahydrocortisone MO-TMS ether which was chromatographed separately. Corrections for injection errors were made on the basis of the internal standard (methyl deoxycholate).

The recoveries were calculated as 65.3% for the unconjugated- and 58.4% for the glucuronide fraction, expressed as a percentage of the amount of 6β-hydroxy-allotetrahydrocortisone added to these fractions.

Identification of 6α-hydroxy-tetrahydrocortisone.

GC-MS on an OV-1 column of the MO-TMS products of fractions *U-3* and *G-3* showed that both fractions contained one predominant peak at relative retention times 0.723 and 0.726 (relative to methyl deoxycholate), respectively. The relative retention time of standard 6α-hydroxy-tetrahydrocortisone was 0.722. The mass spectra of the peaks in fractions *U-3* and *G-3* were identical to the mass spectrum of standard 6α-hydroxy-tetrahydrocortisone MO-TMS ether (fig. 5.3).

0.25 ml portions of both *U-3* and *G-3* were subjected to sodium bismuthate oxidation. An aliquot of a mixture of standard 6α-hydroxy-tetrahydrocortisone, 6β-hydroxy-tetrahydrocortisone and 6β-hydroxy-allotetrahydrocortisone was treated similarly. The products of *U-3*, *G-3* and the standard mixture were analysed as their uTMS ethers by GC-MS, on a Silar 10C column. Identical mass spectra for the oxidation products of *U-3* and *G-3*, and for 3α,6α-dihydroxy-5β-androstane-11,17-dione uTMS were obtained (fig. 5.2). In fig. 5.7 the mass fragmentograms of ions m/e 359, 374 and 464 recorded from the sodium bismuthate oxidation products of fraction *U-3* and the standard

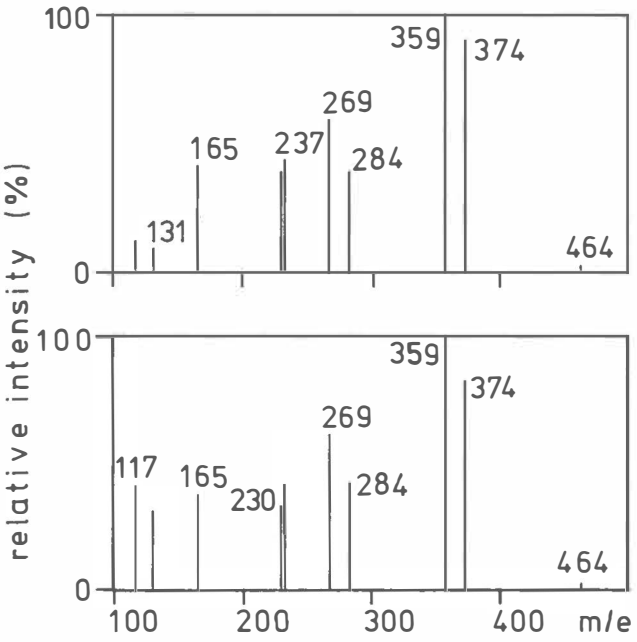


Fig. 5.2 Mass spectra of standard (top) and urinary (fraction U-3) 3α,6α-dihydroxy-5β-androstane-11,17-dione uTMS ether.

mixture, are shown. These fragmentograms show that the intensity ratios of the three ions differ vastly for the three constituents of the standard mixture, in spite of their close structural relationship. However, the ion intensity ratios for standard 3α,6α-dihydroxy-5β-androstane-11,17-dione uTMS ether and the compound in *U-3* are identical. In addition the retention times correspond very well with each other. Mass fragmentography of fraction *G-3* gave identical results.

Identification of 6α-hydroxy-20α-cortolone and 6α-hydroxy-20β-cortolone.

Gas chromatography of the MO-TMS ethers prepared from fraction *U-6* on OV-1 showed three interesting peaks (see

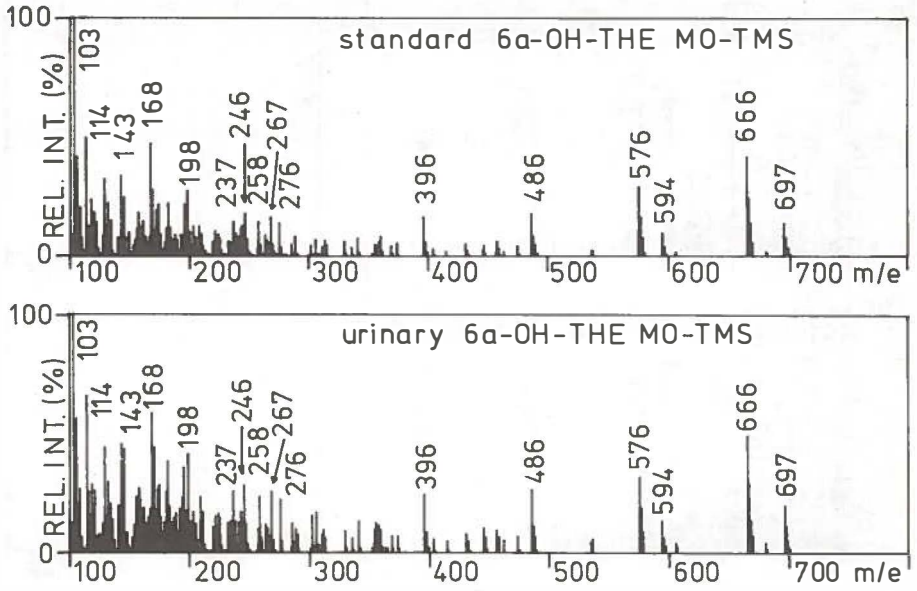


Fig. 5.3 Mass spectra of standard and urinary 6α-hydroxy-tetrahydrocortisone MO-TMS ether.

fig. 5.6A). Peaks 1 and 2 had relative retention times (relative to 6β-hydroxy-tetrahydrocortisone MO-TMS ether) 1.163 and 1.249, respectively, corresponding to the relative retention times of the TMS ethers of 6α-hydroxy-20α-cortolone and 6α-hydroxy-20β-cortolone (1.160 and 1.243, respectively). GC-MS showed that the mass spectra of peaks 1 and 2 were very similar to the mass spectra of 6α-hydroxy-20α-cortolone and 6α-hydroxy-20β-cortolone TMS ethers, respectively (see fig. 5.4). The mass spectrum of peak No. 3 (relative retention time 1.316) was quite different from the mass spectra of peaks 1 and 2, and this mass spectrum was identical to the spectrum of the compound at M.U. 31.82 found in the urine of an infant receiving cortisol therapy (section 3.3). As mentioned in this section this spectrum showed a striking resemblance to the mass spectrum of 1β-hydroxy-20β-cortolone TMS ether described by Shackleton

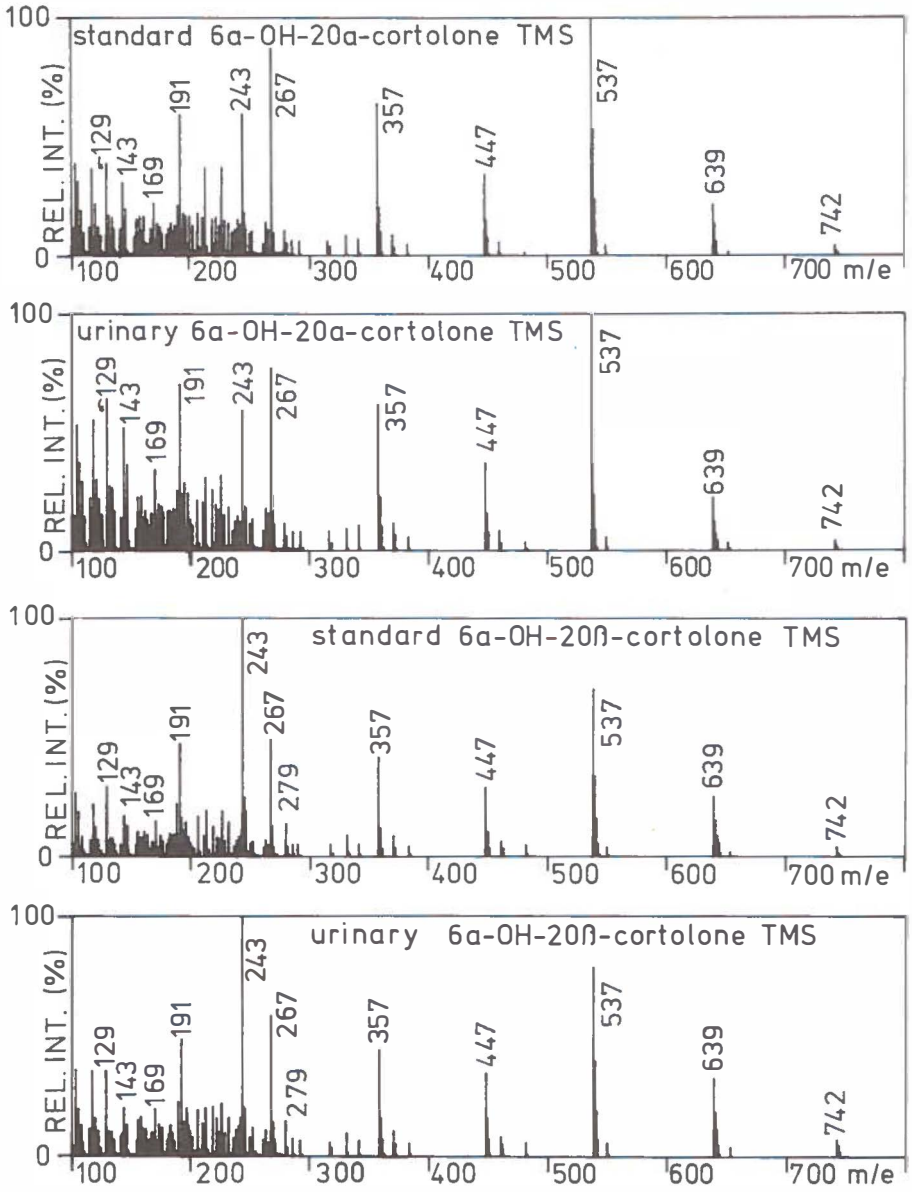


Fig. 5.4 Mass spectra of standard and urinary 6α-hydroxy-20α-cortolone and 6α-hydroxy-20β-cortolone TMS ethers.

and Snodgrass (1974). The mass spectrum of this compound is shown in fig. 5.5. The high mass part of this spectrum can not be compared to the same part of the mass spectrum given by Shackleton & Snodgrass, as these authors used a different silylating procedure leaving the 17α-hydroxy group unsilylated.

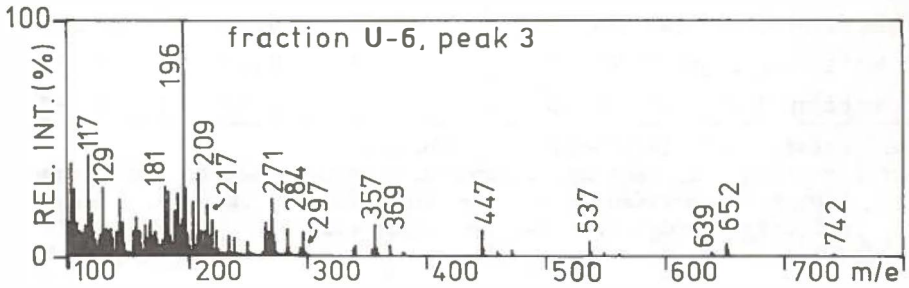


Fig. 5.5 Mass spectrum of fraction U-6, peak No. 3.

Fraction *G-6* showed the same peaks 1, 2 and 3, but in much lower concentrations (relative retention times 1.163, 1.240 and 1.312). Their mass spectra were identical to the mass spectra of the corresponding peaks of fraction *U-6*.

By mass fragmentography the ions m/e 742 (M^+), 639 (M-103) and 537 (M-205) were monitored for both *U-6* and *G-6* and also for standard 6α-hydroxy-20α-cortolone and 6α-hydroxy-20β-cortolone TMS ethers. The ion intensity ratios for the ions m/e 742, 639 and 537 were calculated and are presented in table 5.2. One mass fragmentogram of fraction *U-6* was recorded at very high sensitivity (fig. 5.6B) and this mass fragmentogram shows (arrows) that small amounts of compounds exhibiting these ions, apart from the compounds mentioned above, are present. This could suggest that other 11-keto-pregnane-pentols are present in this fraction, although in low concentrations.

0.25 ml portions of both *U-6* and *G-6* were subjected

Table 5.2 Ion intensity ratios[a] calculated from the mass fragmentograms of standard and urinary 11-keto-pregnane-pentol TMS ethers.

COMPOUNDS	ION (m/e)		
	537	639	742
6α-hydroxy-20α-cortolone	1	0.20	0.04
6α-hydroxy-20β-cortolone	1	0.44	0.08
fraction *U-6* peak No. 1	1	0.18	0.03
fraction *U-6* peak No. 2	1	0.43	0.07
fraction *G-6* peak No. 1	1	0.19	0.03
fraction *G-6* peak No. 2	1	0.40	0.08
fraction *G-6* peak No. 3[b]	1	0.16	0.37

a: relative to the intensity of ion m/e 537

b: fraction U-6, peak No. 3 could not be measured due to the much higher intensities of the monitored ions of peak No. 2 which was only partly separated from peak No. 3.

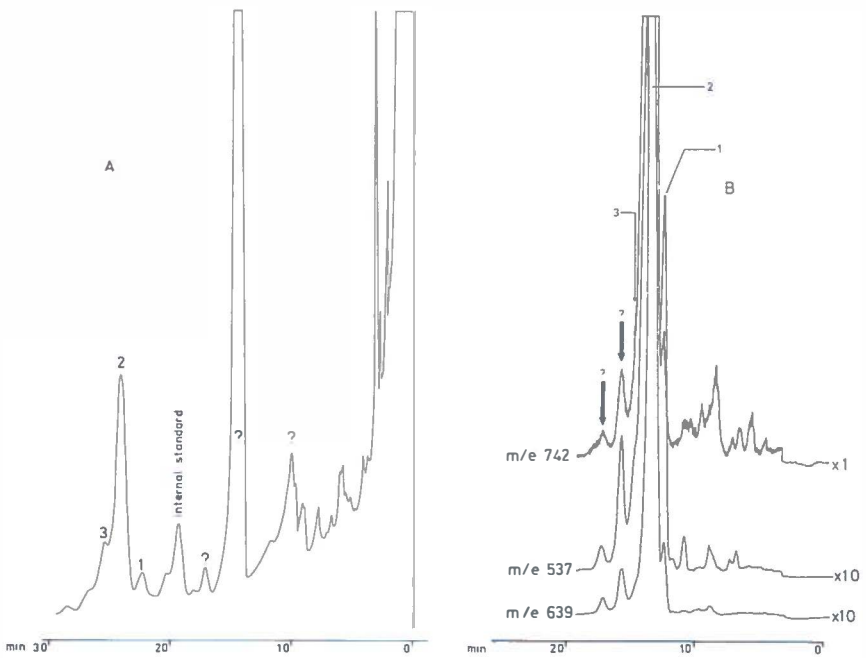


Fig. 5.6 A. Gas chromatogram of the TMS ethers of fraction U-6.
B. Mass fragmentogram of ions m/e 742, 639 and 537 of fraction U-6 TMS ethers; sensitivity was set at such a high level that minor amounts of 11-keto-pregnane-pentol TMS ethers were visible; A and B were recorded under different conditions resulting in different retention times.

to sodium bismuthate oxidation and analysed as their uTMS ethers by mass fragmentography on a Silar 10C column. Ions m/e 464 (M^+), 374 (M-90) and 359 (M-15-90) were monitored. The mass fragmentograms (fig. 5.7) showed that 3α,6α-dihydroxy-5β-androstane-11,17-dione uTMS ether was the main constituent of the sodium bismuthate treated fraction *U-6*. In *G-6* this peak was expectedly much smaller compared to 3α,6β-dihydroxy-5β-androstane-11,17-dione uTMS ether (the oxidation product of the internal standard used in both *U-6* and *G-6*). The peak No. 3 must be the oxidation product of peak No. 3 in the gas chromatogram 5.6A (see also section 5.5).

Other 6-hydroxylated corticosteroids.

Fractions *U-2*, *U-4*, *U-5*, *G-2*, *G-4* and *G-5* did not contain 6-hydroxylated corticosteroids or any other steroids detectable by GC-MS of the MO-TMS ethers. Therefore, these fractions were investigated by mass fragmentography at the ions m/e 740 and 771 (for pregnane-pentolone MO-TMS ethers) and the ions m/e 666 and 697 (for 11-keto-pregnane-tetrolone MO-TMS ethers). 6α-hydroxy-allotetrahydrocortisol was added to these fractions as an internal standard, after it was shown in a pilot study not to interfere with steroids present in these fractions. All relative retention times mentioned below are relative to the retention time of this internal standard.

Fraction *U-2* showed two peaks in the m/e 666 and 697 fragmentograms with relative retention times 0.759 and 0.847, the latter peak being approximately eight times higher than the first one. Also, two peaks in the m/e 740 and 771 fragmentograms were observed with relative retention times 0.870 and 0.900. These peaks were of equal peakheight. The same peaks as mentioned above were barely visible in the fragmentograms of fraction

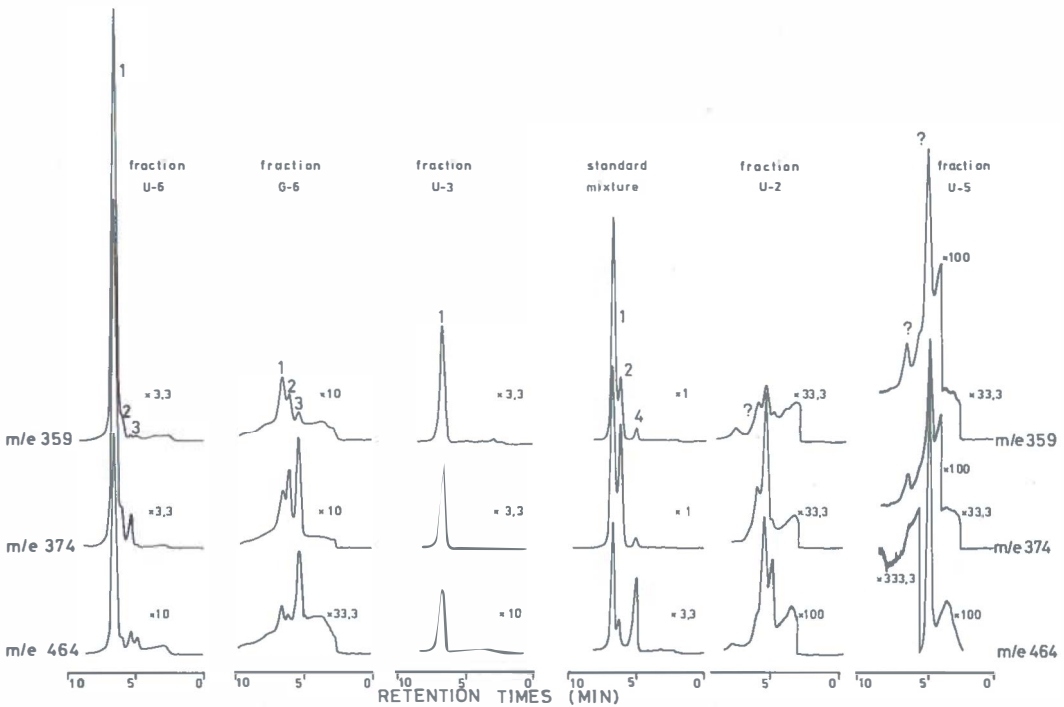


Fig. 5.7 Mass fragmentograms at ions m/e 359, 374 and 464 of the sodium bismuthate oxidation products of a standard mixture and several urinary fractions. 1 = 3α,6α-dihydroxy-5β-androstane-11,17-dione uTMS; 2 = internal standard = 3α,6β-dihydroxy-5β-androstane-11,17-dione uTMS; 3 = probably 1β,3α-dihydroxy-5β-androstane-11,17-dione uTMS; 4 = 3α,6β-dihydroxy-5α-androstane-11,17-dione uTMS. The numbers preceded by "x" refer to the sensitivity levels used.

G-2. The mass fragmentograms of fractions *U-4* and *G-4* were devoid of peaks, but fractions *U-5* and *G-5* showed one peak in the m/e 740 and 771 fragmentograms with relative retention time 0.923.

The m/e 666/697 peak at relative retention time 0.847 and the m/e 740/771 peak at relative retention time 0.923 could be due to 6β-hydroxy-tetrahydrocortisone and 6α-hydroxy-tetrahydrocortisol MO-TMS ethers, respectively. The relative retention times of the corresponding standard steroids were 0.845 and 0.916, respectively.

To obtain additional evidence for this, 0.25 ml of fractions *U-2* and *U-5* were oxidised by sodium bismuthate. The latter reagent was taken from a batch known to oxidise 11β-hydroxy groups (cf. Bush, 1961; Beale, Croft & Taylor, 1967). Mass fragmentography of the uTMS ethers of the oxidation products obtained from these fractions at ions m/e 464, 374 and 359 showed that compounds exhibiting these ions were present in both *U-2* and *U-5* (fig. 5.7). However, none of these compounds could be identified positively as the oxidation product of one of the 6-hydroxylated steroids mentioned before.

From these data the conclusion was drawn that minor amounts (10-100 times less than the amount of 6α-hydroxy-tetrahydrocortisone) of 11-keto-pregnane-tetrolones and pregnane-pentolones were present in this neonatal urine, and that none of these steroids was identical to a 6-hydroxylated corticosteroid.

5.5 Discussion.

This is the first time that 6α-hydroxy-tetrahydrocortisone and the 6α-hydroxy-cortolones have been identified in human neonatal urine. Lewbart & Schneider (1966) produced evidence for the presence of 6α-hydroxy-20β-cortolone and 6β-hydroxy-20β-cortolone in the urine of a human adult, after ingestion of a massive dose of 20β-

cortolone. 6α-hydroxy-20β-cortolone was identified by subjecting the compound to Wolff-Kishner-Barton reduction and comparing the product with authentic 3α,6α,17α,20β, 21-pentahydroxy-5β-pregnane by paper chromatography and melting point determination.

The three 6α-hydroxylated corticosteroids identified in this neonatal urine were identical to the compounds found earlier in the urine of an infant receiving cortisol therapy (section 3.3) at M.U. values 30.54, 31.35 and 31.62 (for 6α-hydroxy-tetrahydrocortisone, 6α-hydroxy-20α-cortolone and 6α-hydroxy-20β-cortolone, respectively). In the latter urine the 6α-hydroxylated steroids were also of major quantitative importance (see figures 3.1-3.3).

The urine of the healthy newborn infant was systematically investigated for other ring A reduced 6-hydroxylated analogs of cortisol and cortisone, but except for 6α-hydroxy-tetrahydrocortisone no such steroids could be detected. There has been some speculation in earlier reports (Ulstrom et al, 1960; Daniilescu-Goldinberg & Giroud, 1974) about the presence of such corticosteroids, following the discovery of important quantities of 6β-hydroxy-cortisol in neonatal urine (Ulstrom et al, 1960).

Traces of other pregnane-pentolones and 11-keto-pregnane-tetrolones and also a compound having a mass spectrum very similar to that of 1β-hydroxy-20β-cortolone (Shackleton & Snodgrass, 1974) were found. 1β-hydroxy-tetrahydrocortisone was not found in amounts detectable by GC-MS, but the compound at relative retention time 0.847 (relative to 6α-hydroxy-allotetrahydrocortisol MO-TMS ether) which was detected by mass fragmentography at ions m/e 666/697 in fraction *U-2*, might be identical to 1β-hydroxy-tetrahydrocortisone MO-TMS ether. The corresponding M.U. value calculated for this compound

(see section 5.2) is 30.75 which corresponds rather well to the M.U. value of the compound tentatively identified as 1β-hydroxy-tetrahydrocortisone MO-TMS ether (M.U. 30.67, see section 3.3).

Additional evidence for this is provided by fig. 5.7. As mentioned before, peak No. 3 in the mass fragmentogram recorded from the sodium bismuthate oxidation products of fraction *U-6* must be the 17-keto analog of 1β-hydroxy-20β-cortolone (section 5.4). In the mass fragmentogram recorded from the oxidation products of fraction *U-2* a peak is observed which has the same retention time and similar relative ion intensities as the peak No. 3 in the fragmentogram of fraction *U-6*. As sodium bismuthate degradation of both 1β-hydroxy-20β-cortolone and 1β-hydroxy-tetrahydrocortisone yields the same product (1β,3α-dihydroxy-5β-androstane-11,17-dione), it is likely that fraction *U-2* contained 1β-hydroxy-tetrahydrocortisone, although in a very low concentration.

In contrast to the urine of the infant receiving cortisol therapy (chapter 3) 6-hydroxylated pregnane-pentolones were not found in the urine of the healthy newborn infant. The presence of a large amount of such a compound in the urine of the infant receiving large amounts of cortisol must be due to changes in the metabolism of cortisol induced by the administration of such a large dose of this steroid. Unfortunately, no more urine of this infant was available for the definite identification of the pregnane-pentolones present in this urine. On the basis of the results described in this chapter, it seems likely that the prominent pregnane-pentolone MO-TMS ether at M.U. 31.10 is identical to 6α-hydroxy-tetrahydrocortisol MO-TMS ether.

CHAPTER 6

THE QUANTITATION OF SOME POLAR CORTICOSTEROIDS IN HUMAN NEONATAL URINE.

6.1 Introduction.

The aim of the study described in this chapter was to investigate the quantitative importance of the newly identified 6α-hydroxylated corticosteroids in relation to a number of corticosteroids already known to be present in neonatal urine. In addition to the three 6α-hydroxylated corticosteroids the following compounds were determined in the urine of six healthy newborn infants: tetrahydrocortisone, tetrahydrocortisol, 6β-hydroxy-cortisone, 6β-hydroxy-cortisol, α-cortolone, β-cortolone and 1β-hydroxy-20β-cortolone (cf. Shackleton & Snodgrass, 1974). Unconjugated and glucuronide conjugated steroids were quantitated separately. The corticosteroids were determined using high performance liquid chromatography, combined gas chromatography-mass spectrometry and mass fragmentography.

6.2 Materials and Methods.

The materials described in the preceding chapters were used. 6β,17α,20β,21-tetrahydroxy-pregn-4-ene-3,11-dione was prepared by incubation of 6β-hydroxy-cortisone with 20β-hydroxy-steroid oxidoreductase.

Subjects.

Urine was collected for 24 hours from six healthy male newborn infants during their second day of life (informed consent was obtained from the parents). The mothers did not receive any medication at the time of

delivery. When the babies voided the urine was immediately placed in the 24 hours urine collection bottles at -20°C. The birth weights of the babies, the 24 hours urine volumes and the 24 hours creatinine excretions are listed in table 6.1.

Table 6.1 Birth weights, 24 hours urine volumes and 24 hours creatinine excretions of the studied newborn infants.

INFANT No.	BIRTH WEIGHT (g)	24 HR. URINE VOLUME (ml)	24 HR. CREATININE EXCRETION (mg)
1	3330	50	19.5
2	3560	29	14.8
3	3550	42	29.4
4	4130	26	20.0
5	4040	90	25.2
6	4090	37	14.0

Urine processing.

20 ml of urine from each of the babies studied was processed as described in chapter 5. The HPLC fractionation was performed as described except that instead of six, eleven fractions were collected as depicted in fig. 6.1. After ten fractions were collected the solvent composition was changed, as described before (section 5.2), and the eluate was collected for 15 min to give fraction No. 11. The HPLC fractions collected from the unconjugated fraction will be referred to as *U-1, U-2,..., U-11* and those from the glucuronide fraction as *G-1, G-2,..., G-11*.

Combined gas chromatography-mass spectrometry.

0.25 ml of each HPLC fraction was taken to dryness and converted into MO-TMS ethers as described in section 3.2. The MO-TMS ethers were analysed by both pure gas

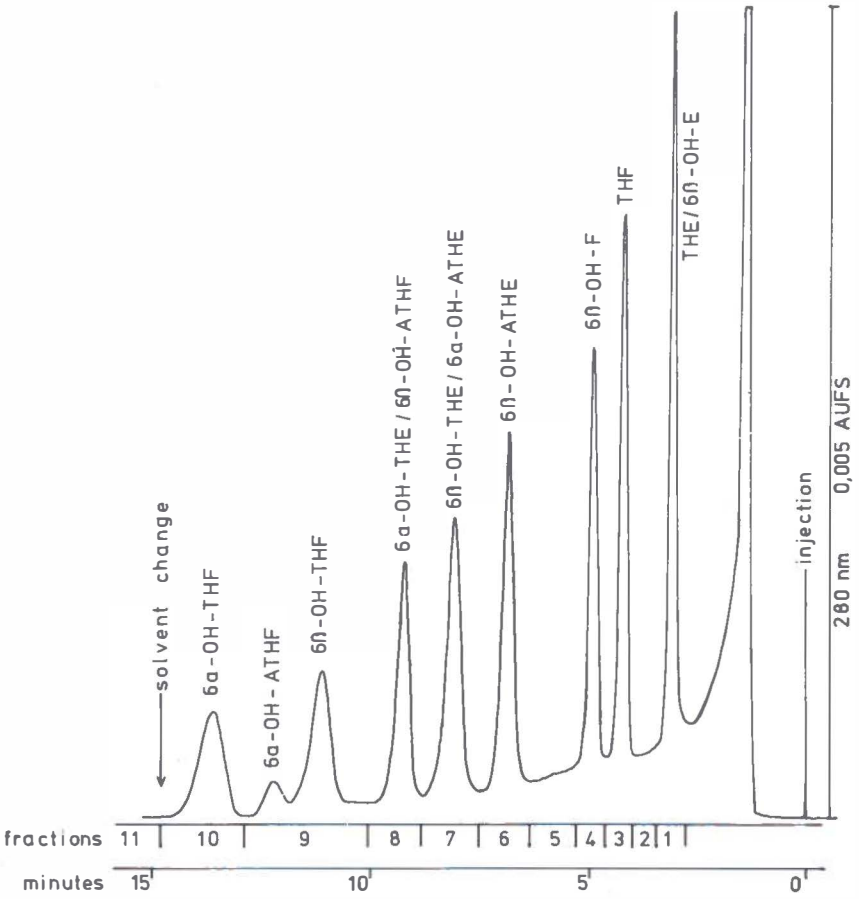


Fig. 6.1 High performance liquid chromatography of twelve standard steroids. α- and β-cortolone were not included in the standard mixture but these steroids elute between 6β-hydroxy-cortisol and 6β-hydroxy-allotetrahydrocortisone.

chromatography (for quantitation) and combined gas chromatography-mass spectrometry (for identification and determination of purity of the gas chromatographic peaks).

In a number of instances it was necessary to quantitate the studied steroids by mass fragmentography, due to interference from extraneous material or insufficient sensitivity in gas chromatography. In these

instances a suitable (see section 6.3) internal standard was added to the HPLC fractions to be analysed, and mass fragmentography was carried out using the magnetic field switching mode. Usually two characteristic ions for each compound to be determined and one ion of the internal standard were monitored. For quantitation the most intensive of the two sample ions was used. The ions monitored in each instance are mentioned in section 6.3. Oven temperatures in mass fragmentography ranged from 240-260^{o}C.

Quantitation.

For the calculation of the 24 hours excretion the recovery of the urinary steroids during the urine work up was determined by assaying the amount of 6β-hydroxy-allotetrahydrocortisone present in HPLC fractions *U-6* and *G-6* (see fig. 6.1),and comparing this value with the amount added to the unconjugated and glucuronide fractions. This steroid was chosen as a recovery standard because it was not present in neonatal urine to any significant extent. As no partitioning steps were present in the urine work up the recovery of this steroid was considered a good measure for the recovery of all steroids studied. No correction was made for losses occurring during DEAE-Sephadex chromatography, but in earlier experiments (see section 2.3) these losses were proven to be virtually negligible.

The over all recovery for the six unconjugated fractions averaged 51.9% ± 10.3 (SD) and for the glucuronide fractions the average recovery was 49.6% ± 5.0 (SD).

To each series of urinary HPLC fractions to be analysed by gas chromatography four standard mixtures (see table 6.2) containing known amounts of the steroids to be assayed were added. Of the 6-hydroxylated

Table 6.2 Relative retention times of the MO-TMS ethers of standard steroids (OV-1, 245°C).

STEROID	IN STANDARD MIXTURE	IN HPLC FRACTION	t_R [a]
tetrahydrocortisone	1	1	0.600
tetrahydrocortisol	1	3	0.687
α-cortolone	1	5	0.744
β-cortolone	1	5	0.792
6β-hydroxy-cortisone	1	1	1.189 1.262
6β-hydroxy-cortisol	1	4	1.343 1.436
6α-hydroxy-tetrahydrocortisone	2	8	0.722
6β-hydroxy-tetrahydrocortisone	2	7	0.778
6β-hydroxy-allotetrahydrocortisol	2	8	0.827
6α-hydroxy-allotetrahydrocortisone	2	7	0.891
6β-hydroxy-tetrahydrocortisol	3	9	0.810
6α-hydroxy-tetrahydrocortisol	3	10	0.829
6α-hydroxy-allotetrahydrocortisol	3	9	0.912
6β-hydroxy-allotetrahydrocortisone	3	6	0.912
6α-hydroxy-20α-cortolone	4	11	0.902
6α-hydroxy-20β-cortolone	4	11	0.967

a. *Relative retention time (relative to methyl deoxycholate TMS ether).*

steroids present in these standard mixtures the 11β-hydroxy-steroids had been standardized by gas chromatography of the MO-TMS ethers against tetrahydrocortisol, and the 11-keto-steroids against tetrahydrocortisone, by comparing their respective peak areas.

The urinary steroid MO-TMS ethers were quantitated by comparing their relative peakheights (relative to the internal standard) to the relative peakheights of the corresponding MO-TMS ethers in the standard mixtures. These standard mixtures contained the same amount of internal standard as had been added to the urinary HPLC fractions. The same procedure was used in mass fragmento-

graphy except that different internal standards were used. These internal standards are mentioned in section 6.3.

6.3 Results.

The amounts of steroids excreted in the urine by each neonate during their second day of life are presented in tables 6.3 and 6.4, and are expressed in µg/24 hr. Steroids were only quantitated by gas chromatography if combined gas chromatography-mass spectrometry showed no extraneous material to be present in the corresponding peaks. The instances in which the use of mass fragmentography was necessary are discussed below.

Tetrahydrocortisone (fractions U-1 and G-1).

The small amounts of tetrahydrocortisone present in the unconjugated fractions were determined by mass fragmentography at ions m/e 609 (M^+) and 578 (M-31), while tetrahydrocortisol MO-TMS ether (m/e 652) was used as internal standard. Although the glucuronide fractions contained large amounts of tetrahydrocortisone, these fractions were also analysed by mass fragmentography because GC-MS showed that in some instances the peak of tetrahydrocortisone MO-TMS ether was contaminated by unknown material.

Tetrahydrocortisol (fractions U-3 and G-3).

The very low amounts of tetrahydrocortisol present in both the unconjugated and glucuronide fractions were determined by mass fragmentography at ions m/e 683 (M^+) and 652 (M-31). Tetrahydrocortisone MO-TMS ether (m/e 609) was the internal standard used.

Table 6.3 Urinary excretion of polar unconjugated corticosteroids by six newborn infants (µg/24 hr).

STEROID	DETERMINED BY[a]:	INFANT No. 1	2	3	4	5	6
tetrahydrocortisone	MF	2.0	3.2	1.0	1.9	2.0	0.6
tetrahydrocortisol	MF	0.3	0.1	n.m.[b]	n.m.[b]	0.1	0.2
6β-hydroxy-cortisone	GC	5.4	9.6	6.6	5.5	6.9	2.7
6β-hydroxy-cortisol	MF	19.5	8.1	32.3	18.7	31.8	7.3
6α-hydroxy-tetrahydrocortisone	GC	27.1	7.3	39.2	13.0	22.6	8.5
	MF	25.5	6.0	39.5	n.m.[c]	20.5	9.4
6α-hydroxy-20α-cortolone	GC	29.4	5.1	22.9	19.7	48.9	15.8
6α-hydroxy-20β-cortolone	GC	102.0	17.0	179.7	53.5	123.9	49.9
1β-hydroxy-20β-cortolone	GC	38.7	7.0	55.2	20.5	42.7	14.0

a. MF denotes mass fraymentography, GC denotes gas chromatography. The steroids were determined by MF when GC gave unreliable results (see text).

b. Not measured.

c. Not measured by MF; mass spectrum indicated that the compound was not contaminated.

Table 6.4 Urinary excretion of polar glucuronide conjugated corticosteroids by six newborn infants (µg/24 hr).

STEROID	DETERMINED BY[a]:	INFANT No. 1	2	3	4	5	6
tetrahydrocortisone	GC	157.4	73.4	56.1	206.6	163.6	89.2
	MF	147.7	41.0	60.3	169.4	178.5	87.5
tetrahydrocortisol	MF	3.0	0.8	n.m.[b]	n.m.[b]	2.5	2.6
α-cortolone	GC	17.8	trace	trace	13.8	14.5	trace
β-cortolone	GC	15.7	trace	trace	31.7	20.4	15.2
6α-hydroxy-tetrahydrocortisone	GC	73.1	40.7	52.7	70.9	124.1	62.0
	MF	69.9	20.3	25.4	n.m.[c]	116.9	35.6
6α-hydroxy-20α-cortolone	GC	7.0	2.5	3.1	16.6	30.1	9.0
6α-hydroxy-20β-cortolone	GC	4.0	1.0	5.7	6.4	9.8	5.5
1β-hydroxy-20β-cortolone	GC	16.5	4.5	20.9	27.3	27.9	16.8

a. MF denotes mass fraymentography, GC denotes gas chromatography. The steroids were determined by MF when GC gave unreliable results (see text).
b. Not measured.
c. Not measured by MF; mass spectrum indicated that the compound was not contaminated.

α-cortol and β-cortol (fractions U-7 and G-7).

These compounds were not detected by normal GC-MS and were not investigated by mass fragmentography.

6β-hydroxy-cortisol (fractions U-4 and G-4).

6β-hydroxy-cortisol was found in all unconjugated fractions, but the gas chromatographic peak of its MO-TMS ether was severely contaminated by a cluster of other peaks (see fig. 6.3). Therefore, this compound was measured by mass fragmentography at ions m/e 724 (M^+) and 693 (M-31), while cortisol (m/e 605) was used as the internal standard. A mass spectrum of the peaks interfering with the peaks of 6β-hydroxy-cortisol MO-TMS ether was very similar to the mass spectrum of 6β,17α,20β,21-tetrahydroxy-pregn-4-ene-3,11-dione MO-TMS ether (fig. 6.2). Mass fragmentography (fig. 6.3) of fractions *U-4* at ions m/e 695 (M^+) and 490 (M-205) revealed several peaks exhibiting these ions, but none of these peaks had a retention time equal to the retention times of 6β,17α,20β,21-tetrahydroxy-pregn-4-ene-3,11-dione MO-TMS ether.

No 6β-hydroxy-cortisol could be detected by mass fragmentography in the glucuronide fractions.

6α-hydroxy-tetrahydrocortisone (fractions U-8 and G-8).

GC-MS of fractions *U-8* and *G-8* showed that the peak of 6α-hydroxy-tetrahydrocortisone MO-TMS ether was pure in the unconjugated fractions, but in some glucuronide fractions there was considerable interference from unknown material. Both the unconjugated and glucuronide fractions were analysed by mass fragmentography at ions m/e 697 (M^+) and 666 (M-31) with 6α-hydroxy-tetrahydrocortisol (m/e 740) serving as internal standard.

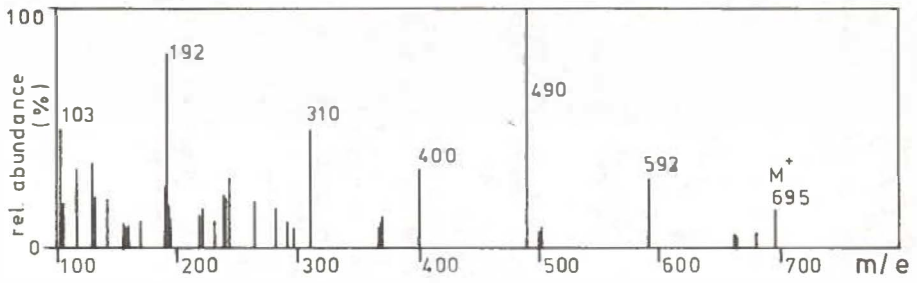


Fig. 6.2 Mass spectrum of standard 6β,17α,20β,21-tetrahydroxy-pregn-4-ene-3,11-dione MO-TMS ether. Only the most abundant ions are shown and isotope clusters are not depicted.

The results of gas chromatography and mass fragmentography agreed very well for the unconjugated fractions, but for the glucuronide fractions of urines No. 2, 3 and 6 mass fragmentography gave appreciably lower values.

1β-hydroxy-20β-cortolone (fractions U-11 and G-11).

For this steroid no reference material was available. Therefore, this compound was quantitated by gas chromatography by comparing its peak area to that of 6α-hydroxy-20β-cortolone TMS ether.

Other 11-keto-pregnane-tetrolones and pregnane-pentolones (fractions U-7, G-7, U-10 and G-10).

As was mentioned in section 5.4 traces of 11-keto-pregnane-tetrolones and pregnane-pentolones were present in fractions *U-7*, *G-7*, *U-10* and *G-10*, and it was shown that none of these compounds was identical to one of the 6-hydroxylated corticosteroids mentioned in chapter 5. The same HPLC fractions of the urines studied here were investigated for these compounds by mass fragmentography at ions m/e 771, 740, 697 and 666 (see section 5.4).

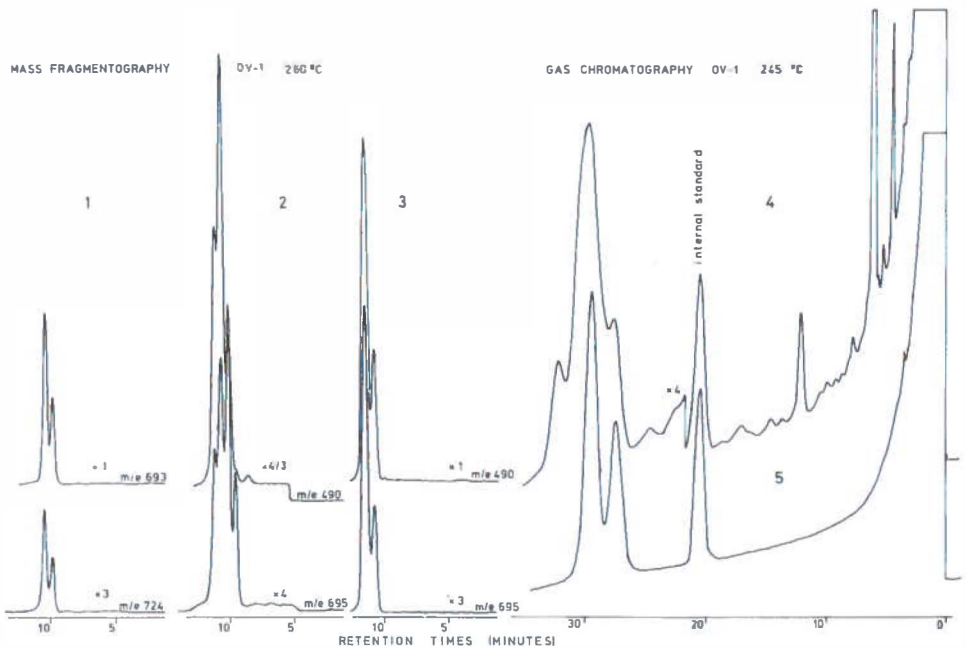


Fig. 6.3 Gas chromatography and mass fragmentography of urine No. 7 fraction U-4.
1. Mass fragmentography at ions m/e 693 and 724 of urinary 6β-hydroxy-cortisol MO-TMS ethers.
2. Mass fragmentography at ions m/e 695 and 490 of urine fraction 4. The first eluting peak in the m/e 695 fragmentogram which is not present in the m/e 490 fragmentogram is due to interference from the M+2 isotope peak of the M-31 ion (m/e 693) of 6β-hydroxy-cortisol MO-TMS ether.
3. Mass fragmentography at ions m/e 695 and 490 of synthetic 6β,17α,20β,21-tetrahydroxy-pregn-4-ene-3,11-dione MO-TMS ether.
4. Gas chromatography of U-4.
5. Gas chromatography of authentic 6β-hydroxy-cortisol MO-TMS ether.

At the retention times mentioned in section 5.4 traces of 11-keto-pregnane-tetrolone and pregnane-pentolone MO-TMS ethers were again detected, but in none of the urines these compounds were quantitatively important; their 24 hours excretions were estimated at a few µg/24 hr, or less.

6.4 Discussion.

The results presented in this chapter clearly indicate that the newly identified 6α-hydroxylated corticosteroids constitute quantitatively a major part of the corticosteroids present in the urine voided by two days old infants. Together with the compound tentatively identified as 1β-hydroxy-20β-cortolone, 6α-hydroxy-tetrahydrocortisone and the 6α-hydroxy-cortolones amount to an average of 60.4% of the steroids found in the unconjugated and glucuronide fractions. Cortisol and cortisone were not investigated in this study, but from the literature it seems unlikely that these steroids are quantitatively important in these fractions (Daniilescu-Goldinberg & Giroud, 1974; Klein et al, 1969).

Evidence for the presence of other unsaturated corticosteroids was provided by the detection of the compounds in fractions *U-4* having mass spectra very similar to 6β,17α,20β,21-tetrahydroxy-pregn-4-ene-3,11-dione MO-TMS ether. The quantitative importance of these compounds was comparable to that of 6β-hydroxy-cortisol.

Apart from 6α-hydroxy-tetrahydrocortisone only traces of other 11-keto-pregnane-tetrolones were found. This is somewhat puzzling, because on the basis of the presence of a compound probably being identical to 1β-hydroxy-20β-cortolone (section 5.4) one would also expect to find the 20-keto analog of this compound in neonatal urine in comparable amounts. As was discussed in section 5.5 traces of 1β-hydroxy-tetrahydrocortisone are probably present in

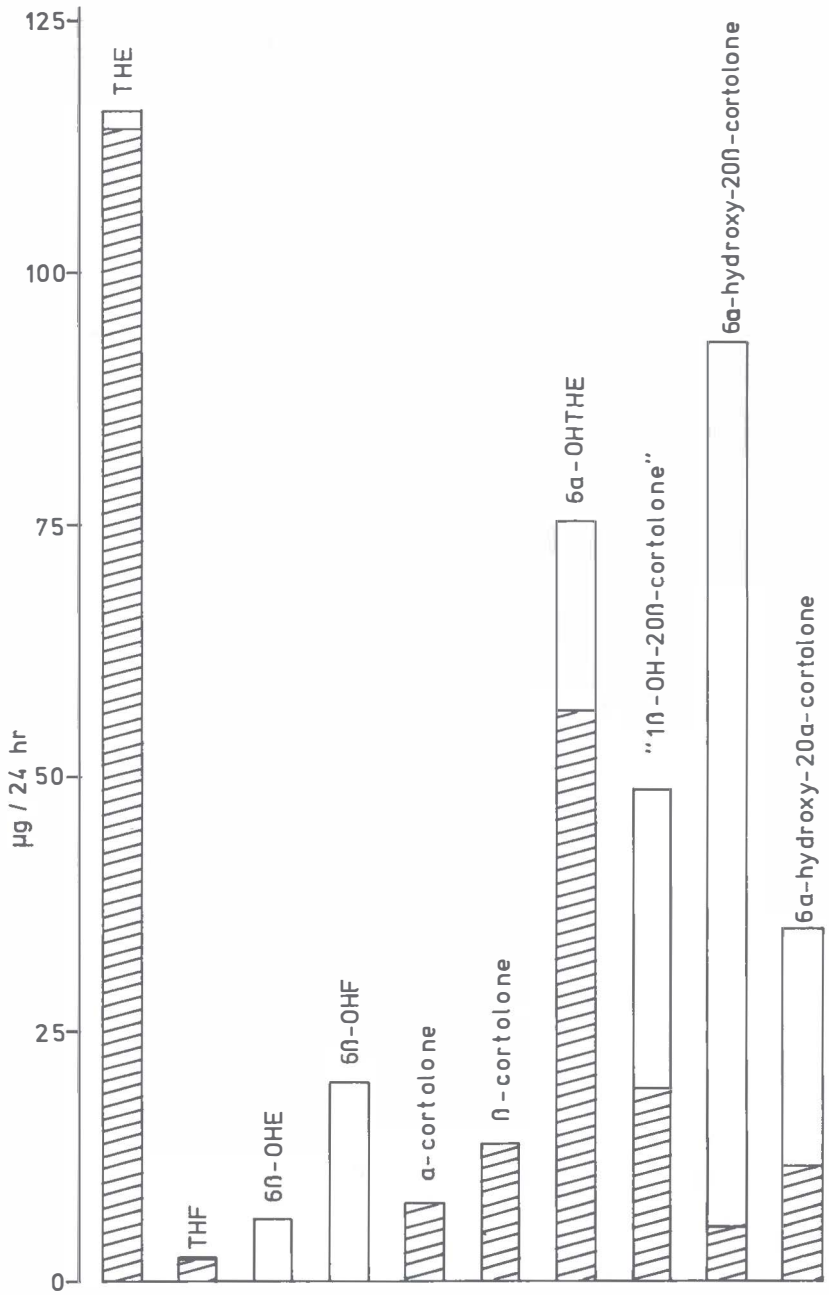


Fig. 6.4 Average total 24 hours excretions of the investigated steroids, as determined in the unconjugated and glucuronide fractions isolated from the urine of six newborn infants. The hatched areas represent the amounts excreted in the glucuronide fraction. The steroids are arranged in order of increasing chromatographic polarity (from left to right).

neonatal urine.

No corticosteroids could be detected in fractions *U-2*, *G-2*, *U-9* and *G-9*.

From the literature only few quantitative data are available which can be compared to the results described in section 6.3. Klein et al (1969) determined the amounts of unconjugated and glucuronide conjugated tetrahydrocortisone and tetrahydrocortisol in the urine of six two days old infants by double isotope dilution. The average 24 hours excretions of the different steroids found by these authors correspond very well to the values mentioned in section 6.3. These authors also determined the amounts of cortisol- and cortisone-sulphate excreted in the urine of these infants, and their results show that the sum of the excretions of these two compounds is of a magnitude comparable to the tetrahydrocortisone excretion. The sulphate and presulphate fractions were not investigated in this study and it is very well possible that the steroid excretion pattern as presented in fig. 6.4 could be altered by the steroids present in these fractions to a considerable extent (cf. Drayer & Giroud, 1965a).

The vast majority of the corticosteroids identified so far in neonatal urine (Daniilescu-Goldinberg & Giroud, 1974; Shackleton, Gustaffson & Mitchell, 1973; Shackleton & Snodgrass, 1974) have the 11-keto configuration in common and this phenomenon probably reflects the relative importance of cortisone compared to cortisol in human neonatal blood (Mitchell & Shackleton, 1969; Buus, Bro-Rasmussen, Trolle & Lundwall, 1966; Klein, Baden & Giroud, 1973).

In fig. 6.4 the 24 hours excretions of the studied steroids are presented. The steroids are arranged in order of increasing polarity from left to right. This figure shows clearly that the fraction of each steroid excreted in the glucuronide fraction decreases with increasing

polarity of the steroid moiety. Exceptions are 6β-hydroxy-cortisone and 6β-hydroxy-cortisol, but this probably is due to the fact that glucuronoconjugation takes place almost exclusively at the 3-hydroxy group which is absent in these steroids (Kornel & Miyabo, 1975). As is shown in fig. 6.4,the major part of 6α-hydroxy-tetrahydro-cortisone is excreted as a glucuronide,and as this steroid is appreciably more polar than 6β-hydroxy-cortisone and 6β-hydroxy-cortisol,the latter steroids would probably have been excreted as glucuronides if they had been good acceptors for the glucuronyl moiety. This makes the theory that 6β-hydroxylation could serve as a compensatory mechanism for the diminished glucuronoconjugation capacity of the neonatal liver (Reynolds et al, 1962) very unlikely. Furthermore, 6β-hydroxylation is quantitatively not important enough to compensate for the diminished glucuronoconjugation,as was also pointed out by Daniilescu-Goldinberg & Giroud (1974).

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS.

As mentioned in chapter 1, it was known from the literature that when radioactive cortisol was given to newborn infants, half of the radioactive urinary cortisol metabolites could not be isolated from neonatal urine. Consequently these metabolites could not be identified. In section 2.1 three possibilities were mentioned which could be responsible for the failure of conventional methods to elucidate cortisol metabolism in the newborn infant. The experimental data presented in chapters 2-6 showed that all three possibilities are at least partly true, and this will be discussed below.

Unknown conjugates.

DEAE-Sephadex chromatography of the neonatal cortisol metabolites (section 2.3) provided evidence that a so far unknown type of conjugate (the presulphate fraction) constitutes a quantitatively important part of the neonatal cortisol metabolites. The experimental data (see also section 2.4) do not exclude the possibility that this conjugate is a type of sulphate which is not liable to enzymatic hydrolysis (cf. Bradlow, 1970). However, it seems not very likely that differences in the site of sulphation would influence the elution behaviour of such a conjugate in ion exchange chromatography to the extent observed for the presulphate fraction (see fig. 2.2). From the hydrolytic experiments it appears also very unlikely that the presulphate fraction contains mixed conjugates or doubly conjugated steroids such as disulphates. Therefore, it must be assumed that the presulphate fraction contains an unknown type of conjugate the nature

of which remains to be elucidated. In their thorough reports on the conjugation of cortisol metabolites Kornel & Saito (1975) and Kornel & Miyabo (1975) mentioned the presence of considerable amounts of "nucleoside complexed polar 6- and 20-hydroxylated cortisol metabolites" in adult urine. Whatever the true nature of this material may be, its electrophoretic behaviour, as described by these authors, is difficult to match with the elution behaviour of the presulphate fraction in ion exchange chromatography. In fact none of the several types of conjugates described by Kornel et al shows corresponding properties when compared to the neonatal presulphate fraction.

Unknown steroids.

The experimental work described in chapters 3-6 was entirely concentrated on the identification of polar corticosteroids present in the free and glucuronide fractions of neonatal urine. Three new corticosteroids (6α-hydroxy-tetrahydrocortisone, 6α-hydroxy-20α-cortolone and 6α-hydroxy-20β-cortolone) were identified. It is very likely that these newly identified corticosteroids, together with the tentatively identified 1β-hydroxy-20β-cortolone, are the unknown polar cortisol metabolites detected by several authors (Ulstrom et al, 1960; Daniilescu-Goldinberg & Giroud, 1974) in neonatal urine. Moreover, these steroids were quantitatively more important than the neonatal cortisol metabolites already known, with the exception of tetrahydrocortisone.

Insufficient isolation efficiency of conventional methods.

In chapter 2 it was mentioned that the amount of free cortisol metabolites isolated by the methods

described in this chapter was relatively high when compared with the amounts isolated by other authors (Migeon, 1959; Aarskog et al, 1964; Daniilescu-Goldinberg et al, 1973; Daniilescu-Goldinberg & Giroud, 1974). The newly identified corticosteroids are extremely polar, and it is virtually impossible to extract such compounds quantitatively with organic solvents generally used in steroid analysis. The relatively low amounts of free cortisol metabolites found by the authors mentioned above can be easily explained by the high polarity of these compounds. The amount of free cortisol metabolites in the neonatal urine described in chapter 2 was of a magnitude comparable to the amount of glucuronide conjugated steroids, and this observation was confirmed by the results presented in chapter 6 (see fig. 6.4).

From the data mentioned above the conclusion can be drawn that apart from the metabolic mechanisms operative in the adult, the neonate has at least three additional quantitatively important pathways of cortisol metabolism at his disposal, i.e.:

- 6α-hydroxylation.
- 1β-hydroxylation.
- Conjugation of the cortisol metabolites to yield an unknown conjugate.

1β- and 6α-hydroxylation result in products which are polar enough to be excreted unconjugated.

Several authors (Migeon, 1959; Ulstrom et al, 1960; Reynolds et al, 1962) suggested that the neonate uses alternative pathways for the disposition of cortisol, because there is a lack or "immaturity" of liver enzymes catalysing the steroid ring A reduction and the glucuronoconjugation in the newborn infant. Reynolds et al (1962) mentioned that the fast disappearance rate of

tetrahydrocortisone from neonatal blood, as described by Bongiovanni et al (1958), is in conflict with a lower glucuronoconjugation rate as described by Migeon (1959). Assuming that 6α-hydroxylation takes place after ring A reduction, for which a considerable amount of evidence is provided by Setchell (1976) and Pepe & Townsley (1976) who studied the metabolism of cortisol in adult and neonatal baboons, then 6α-hydroxylation could excellently account for the high disappearance rate of tetrahydrocortisone from neonatal blood.

An important objection against the theory about diminished A ring reduction in the neonatal liver is the high excretion of saturated corticosteroids as shown in chapter 6. Pepe & Townsley (1976) suggested that in the newborn baboon, in which similar differences from adult cortisol metabolism exist, hydroxylation is a preferential metabolic mechanism, while ring A reduction is not diminished. The prolonged half lives of cortisol in neonatal blood observed by Migeon (1959) and Reynolds et al (1962) conflict with this suggestion, but possibly these prolonged half lives are due to causes other than diminished A ring reduction.

The most interesting question is why cortisol is metabolised by the newborn in a way so different from adult cortisol metabolism. Drayer (1965) reviewed several reports describing the inhibiting action of oestrogens and progestagens on glucuronoconjugation and A ring reduction. As large amounts of oestrogen and progesterone metabolites are excreted by the newborn during the first 3-4 days of life, this could be an explanation for the reduced glucuronoconjugation and, if present, diminished A ring reduction. However, the differences in neonatal cortisol metabolism compared to that in the adult continue beyond the first five days of life (Klein et al, 1969; Shackleton et al, 1973). In the baboon 6β-hydroxylation and diminished

glucuronoconjugation are present even in adult animals (Setchell, Axelson, Simarina & Gontscharow, 1976).

Another reason for the cortisol metabolism of the neonate being so different from that of the adult, could be that the development of enzyme systems involved in the metabolism of cortisol is at that stage specifically suited for the neonatal period. It would be interesting to investigate whether polyhydroxylated corticosteroids pass the placental barrier more easily than steroid glucuronides. If so, the neonatal cortisol metabolism should be regarded as a transition phase between dependence on placental- and renal clearing of cortisol metabolites (cf. Migeon, Bertrand & Gemzell, 1961).

Another interesting possibility which deserves further investigation, is that the determination of 6α-hydroxylated corticosteroids in maternal urine could provide information on foetal adrenocortical function during pregnancy. If the mother herself does not excrete significant amounts of these steroids, the determination of 6α-hydroxylated corticosteroids in maternal urine could be used as a valuable diagnostic tool to discriminate between foetal- and maternal adrenocortical function.

SUMMARY

The aim of this study was to elucidate the metabolism of cortisol in the human newborn which differs considerably from that in the adult.

From the literature (chapter 1) it was clear that the techniques generally used in steroid analysis failed to isolate and identify a major part of the neonatal cortisol metabolites. In chapter 2 an analytical procedure is presented which permitted the isolation of over 90% of the cortisol metabolites present in neonatal urine. Also evidence for the presence of a hitherto unknown steroid conjugate in the urine of the newborn infant is produced.

Chapter 3 describes the discovery of a number of unknown corticosteroids in the urine of an infant receiving large amounts of cortisol therapeutically. The interpretation of the results obtained by combined gas chromatography-mass spectrometry showed that these steroids have six hydroxy- or keto groups. On the basis of the previously reported presence of unsaturated 6β-hydroxylated corticosteroids in neonatal urine, the investigations were concentrated on the identification of saturated 6-hydroxylated corticosteroids.

In chapter 4 the synthesis of eight 6-hydroxylated derivatives of the known cortisol metabolites tetrahydrocortisol and tetrahydrocortisone is described. These new steroids were used as reference compounds to ascertain their presence in the urine of the newborn infant.

One of these synthetic steroids i.e. 6α-hydroxy-tetrahydrocortisone was detected as a major steroid in the urine of a healthy newborn infant (chapter 5). Large quantities of the 20α- and 20β-hydroxy analogs of 6α-hydroxy-tetrahydrocortisone were also detected.

Chapter 6 describes an investigation into the amounts of the newly identified 6α-hydroxylated corticosteroids and of the known cortisol metabolites, such as tetrahydro-

cortisone, excreted in the urine of each of six healthy fullterm 2 days old infants. It was found that the newly identified 6α-hydroxylated corticosteroids constitute about half of the cortisol metabolites excreted by newborn infants as free and glucuronide conjugated steroids. The results prove that these 6α-hydroxylated corticosteroids play an important role in neonatal cortisol metabolism.

It is very likely that these steroids are also the main cortisol metabolites in the foetus. In that case the determination of these steroids in maternal urine could possibly be of great diagnostic value for the assessment of foetal adrenocortical function during pregnancy.

SAMENVATTING

Het doel van het onderzoek beschreven in dit proefschrift was meer inzicht te verkrijgen in het cortisol metabolisme in de pasgeborene, waarvan bekend is dat het sterk afwijkt van dat in de volwassen mens.

Uit de literatuur (hoofdstuk 1) was het duidelijk, dat met behulp van de tot nu toe in de steroid analyse gebruikelijke technieken het niet mogelijk was het merendeel van de cortisol metabolieten, die door de pasgeborene worden uitgescheiden in de urine, te isoleren en te identificeren.

In hoofdstuk 2 wordt een analytische procedure beschreven, waarmee het mogelijk is meer dan 90% van de cortisol metabolieten uit de urine van de pasgeborene te isoleren. Tevens wordt in dit hoofdstuk aangetoond, dat een kwart van deze cortisol metabolieten wordt uitgescheiden in de vorm van een tot nu toe onbekend conjugaat.

In hoofdstuk 3 wordt de isolatie van een aantal tot nu toe onbekende steroiden uit de urine van een baby, die om therapeutische redenen een relatief grote hoeveelheid cortisol ontving, beschreven. Met behulp van gecombineerde gaschromatografie-massaspectrometrie kon worden aangetoond, dat het hier ging om steroiden die gekenmerkt worden door de aanwezigheid van 6 hydroxy- of ketogroepen in het molecule.

Op grond van de in de literatuur beschreven aanwezigheid van 6β-hydroxy-cortisol en 6β-hydroxy-cortison in baby urine, werd besloten om te onderzoeken of de gevonden onbekende steroiden identiek waren met 6-gehydroxyleerde corticosteroiden.

Daartoe was het nodig deze 6-gehydroxyleerde steroïden te synthetiseren en in hoofdstuk 4 wordt de synthese van een achttal 6-gehydroxyleerde derivaten van de bekende cortisol metabolieten tetrahydrocortisol en tetrahydrocortison beschreven.

Een van de gesynthetiseerde steroiden nl. 6α-hydroxytetrahydrocortison bleek in relatief grote hoeveelheden voor te komen in de urine van een gezonde pasgeboren baby (hoofdstuk 5). Tevens werden belangrijke hoeveelheden van de 20α- en 20β-hydroxy derivaten van 6α-hydroxytetrahydrocortison gevonden.

In hoofdstuk 6 is een kwantitatief onderzoek beschreven, waarin de hoeveelheden van deze nieuwe cortisol metabolieten , uitgescheiden in de urine van zes gezonde voldragen babies op hun tweede levensdag, werden vergeleken met de uitscheiding van bekende cortisol metabolieten zoals tetrahydrocortison. Ongeveer de helft van de cortisol metabolieten, uitgescheiden door deze babies als vrije en glucuronide geconjugeerde steroiden, bleek te bestaan uit de boven vermelde 6α-gehydroxyleerde steroïden. Hiermee is aangetoond dat deze nieuwe steroïden een belangrijke rol spelen in het cortisol metabolisme van de pasgeborene.

Het is zeer waarschijnlijk dat deze 6α-gehydroxyleerde steroïden eveneens de belangrijkste cortisol-metabolieten van de foetus zijn. In dat geval is het denkbaar dat de bepaling van deze steroïden in maternale urine van groot diagnostisch belang kan zijn voor de beoordeling van de bijnierschors functie van het ongeboren kind.

TRIVIAL NAMES	
aldosterone	11β,21-dihydroxy-pregn-4-ene-3,20-dione-18-al
allotetrahydrocortisol	3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one
blue tetrazolium	3,3'-dianisole-4,4'-(3,5-diphenyl)tetrazolium chloride
cortisol	11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione
cortisone	17α,21-dihydroxy-pregn-4-ene-3,11,20-trione
α-cortol	3α,11β,17α,20α,21-pentahydroxy-5β-pregnane
β-cortol	3α,11β,17α,20β,21-pentahydroxy-5β-pregnane
α-cortolic acid	3α,11β,17α,20α-tetrahydroxy-5β-pregnane-21-oic acid
β-cortolic acid	3α,11β,17α,20β-tetrahydroxy-5β-pregnane-21-oic acid
α-cortolone	3α,17α,20α,21-tetrahydroxy-5β-pregnane-11-one
β-cortolone	3α,17α,20β,21-tetrahydroxy-5β-pregnane-11-one
α-cortolonic acid	3α,17α,20α-trihydroxy-5β-pregnane-11-one-21-oic acid
β-cortolonic acid	3α,17α,20β-trihydroxy-5β-pregnane-11-one-21-oic acid
dehydroepiandrosterone	3β-hydroxy-androst-5-ene-17-one
Δ-4,3-ketones	steroids having a 3-keto group and a 4-5 double bond
glucuronide	β-D-glucopyranosiduronic acid conjugate
6α-hydroxy-allotetrahydrocortisol	3α,6α,11β,17α,21-pentahydroxy-5α-pregnane-20-one
6β-hydroxy-allotetrahydrocortisol	3α,6β,11β,17α,21-pentahydroxy-5α-pregnane-20-one
6α-hydroxy-allotetrahydrocortisone	3α,6α,17α,21-tetrahydroxy-5α-pregnane-11,20-dione
6β-hydroxy-allotetrahydrocortisone	3α,6β,17α,21-tetrahydroxy-5α-pregnane-11,20-dione
11β-hydroxy-androsterone	3α,11β-dihydroxy-5α-androstane-17-one
6α-hydroxy-cortisol	6α,11β,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione
6β-hydroxy-cortisol	6β,11β,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione
6α-hydroxy-cortisone	6α,17α,21-trihydroxy-pregn-4-ene-3,11,20-trione
6β-hydroxy-cortisone	6β,17α,21-trihydroxy-pregn-4-ene-3,11,20-trione
1β-hydroxy-20β-cortolone	1β,3α,17α,20β,21-pentahydroxy-5β-pregnane-11-one
6α-hydroxy-20α-cortolone	3α,6α,17α,20α,21-pentahydroxy-5β-pregnane-11-one
6α-hydroxy-20β-cortolone	3α,6α,17α,20β,21-pentahydroxy-5β-pregnane-11-one
6β-hydroxy-20β-cortolone	3α,6β,17α,20β,21-pentahydroxy-5β-pregnane-11-one
11β-hydroxy-etiocholanolone	3α,11β-dihydroxy-5β-androstane-17-one
6α-hydroxy-tetrahydrocortisol	3α,6α,11β,17α,21-pentahydroxy-5β-pregnane-20-one
6β-hydroxy-tetrahydrocortisol	3α,6β,11β,17α,21-pentahydroxy-5β-pregnane-20-one
1β-hydroxy-tetrahydrocortisone	1β,3α,17α,21-tetrahydroxy-5β-pregnane-11,20-dione

TRIVIAL NAMES (continued)

6α-hydroxy-tetrahydrocortisone	3α,6α,17α,21-tetrahydroxy-5β-pregnane-11,20-dione
6β-hydroxy-tetrahydrocortisone	3α,6β,17α,21-tetrahydroxy-5β-pregnane-11,20-dione
11-keto-etiocholanolone	3α-hydroxy-5β-androstane-11,17-dione
11-keto-pregnane-pentol	pregnane having an 11-keto- and 5 hydroxy groups
11-keto-pregnane-tetrolone	pregnane with 4 hydroxy- and 2 keto groups (one at 11)
methyl deoxycholate	3α,12α-dihydroxy-5β-cholanic acid methyl ester
pregnane hexol	pregnane having 6 hydroxy groups
pregnane-pentolone	pregnane having 5 hydroxy- and 1 keto group
tetrahydrocortisol	3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one
tetrahydrocortisone	3α,17α,21-trihydroxy-5β-pregnane-11,20-dione

ABBREVIATIONS

a.m.u.	atomic mass unit
CD	circular dichroism
Ci	curie
DEAE	diethylaminoethyl
dpm	desintegrations per minute
E.C.	enzyme cataloque
eV	electron volt
GC	gas chromatography
GC-MS	combined gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
M^+	molecular ion
MDH	malate dehydrogenase
m/e	mass/charge ratio
MO-TMS	O-methyloxime-trimethylsilyl
m.p.	melting point
M.U.	methylene unit(s)
nm	nanometer (10^{-9} m)
ORD	optical rotatory dispersion
PCC	preparative column chromatography
R_F	relative mobility (in TLC)
SD	standard deviation
3α-STDH	3α-hydroxy-steroid oxidoreductase
20β-STDH	20β-hydroxy-steroid oxidoreductase
TLC	thin layer chromatography
TMS	trimethylsilyl
U	unit (of enzyme activity)
uTMS	trimethylsilyl ether (only relatively unhindered hydroxy groups silylated)
UV	ultra violet

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