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GAS CHROMATOGRAPHY OF STEROIDS AND ITS CLINICAL APPLICATIONS, INCLUDING LOADING TESTS WITH DEUTERATED COMPOUNDS[1]

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SUMMARY

Glass capillary columns have been used successfully for the determination of steroids. They are especially suitable for the recognition of the identity of unknown compounds, using a combination of gas chromatography–mass spectrometry (GC–MS).

The practicability of the method is demonstrated by studies of urinary steroids in a patient with 21-hydroxylase deficiency, in a patient with a special 11β-hydroxylase deficiency and in a patient with an obscure enzyme defect in steroid biosynthesis, probably a lipoid adrenal hyperplasia. Performing the metyrapone test, tetrahydro-S (THS) determination with glass capillary columns allows a specific evaluation of hypothalamic-pituitary-adrenal function (normally 100 to 500 fold increase of THS).

The investigation of steroid metabolism using deuterated precursors (oral and intravenous loading tests) has been tried with deuterated pregnenolone and progesterone. Only urinary pregnanediol, 5α-pregnanediol and another unknown steroid were found to be deuterated. An oral loading test with deuterated cholesterol showed no deuteration of urinary steroids except cholesterol, cholest-4-en-3-one and a side chain monohydroxylated cholest-4-en-3-one.

INTRODUCTION

In the last 10 to 15 years, a great deal has been learned about steroid metabolism. Many new methods have been developed, among which gas chromatography, alone or in combination with mass spectrometry, has contributed most to the development in this field. In 1960, Horning reported on the practical applications of separating steroids in biological material on packed columns [1]. Since then, many methods have been worked out and reviews have been published [2, 3]. For a long time, the separation of steroids on capillary columns was an unsolved problem. In 1969 Völlmin first introduced this technique using a "Grob column" [4]. In the last few years, many groups reported the use of different types of glass capillary columns [5–7].

In comparison with packed columns, capillaries give a much better separation and, due to the narrower peak width, also a better sensitivity. Horning *et al.* developed a procedure for the separation of a great number of urinary steroids in one chromatogram (steroid profile) applying simultaneously trimethylsilyl- and methoxime-trimethylsilylether derivatives [8]. Völlmin succeeded in applying this method to capillary columns [9]. Due to the enormous separation capacity of glass capillaries (> 50,000 theoretical plates), the further prepurification steps can be omitted and the time of chromatographic analysis is only 1 h.

Capillary columns are especially suitable for the recognition of the identity of unknown compounds using a combination of GC–MS. Due to the low bleeding of stationary phases, the background in the mass spectra is negligible, thus allowing the registration of small peaks. The combination of glass capillaries with mass spectrometry for the analysis of steroids was also first reported by Völlmin[10].

In the following, the practicability of the method is demonstrated by studies in patients with various types of congenital adrenal hyperplasia, who were investigated by our group.

We have previously described the application of stable isotopes, namely deuterium, in the study of metabolic diseases with GC–MS [11–13]. In these studies, amino acid metabolism was investigated *in vivo* by loading healthy subjects and patients with metabolic disorders with deuterated compounds.

Recently we have started to investigate steroid metabolism with deuterated compounds, namely pregnenolone, progesterone and cholesterol. For the deuteration of progesterone and pregnenolone we performed acid catalysed exchange by the method described by Seibel and Gäumann[14].

The deutero-cholesterol was prepared by exchange with D_2O and deutero acetic acid in the presence of active platinum oxide according to Bloch and Rittenberg[15].

Our investigations were carried out *in vivo* by loading a male control subject intravenously with deuterated progesterone and orally with deuterated pregnenolone and cholesterol.

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Trivial names and abbreviations

Androsterone (AN)	3α-Hydroxy-5α-androstan-17-one
Etiocholanolone (ET)	3α-Hydroxy-5β-androstan-17-one
Dehydroepiandrosterone (DHA)	3β-Hydroxy-androst-5-en-17-one
11-Keto-androsterone (11-Keto-AN)	3α-Hydroxy-5α-androstan-11,17-dione
11-Keto-etiocholanolone (11-Keto-ET)	3α-Hydroxy-5β-androstan-11,17-dione
11-Hydroxy-androsterone (11-OH-AN)	3α,11β,-Dihydroxy-5α-androstan-17-one
11-Hydroxy-etiocholanolone (11-OH-ET)	3α,11β-Dihydroxy-5β-androstan-17-one
Pregnanolone (PN)	3α-Hydroxy-5β-pregnan-20-one
Allo-pregnanediol (a-PD)	5α-Pregnane-3α,20α-diol
Pregnanediol (PD)	5β-Pregnane-3α,20α-diol
Pregnenediol (PD')	Pregn-5-ene-3β,20α-diol
Pregnanetriol (PT)	5β-Pregnane-3α,17α,20α-triol
Pregnenetriol (PT')	Pregn-5-ene-3β,17α,20α-triol
Pregnanetriolone (PTL)	3α,17α,20α-Trihydroxy-5β-pregnan-11-one
Cholesterol (CH)	Cholest-5-ene-3β-ol
Tetrahydro-A (THA)	3α,21-Dihydroxy-5β-pregnan-11,20-dione
Tetrahydro-B (THB)	3α,11β,21-Trihydroxy-5β-pregnan-20-one
Allo-tetrahydro-B (a-THB)	3α,11β,21-Trihydroxy-5α-pregnan-20-one
Tetrahydro-S (THS)	3α,17α,21-Trihydroxy-5β-pregnan-20-one
Tetrahydro-DOC (TH-DOC)	3α,21-Dihydroxy-5β-pregnan-20-one
Tetrahydro-E (THE)	3α,17β,21-Trihydroxy-5β-pregnan-20-dione
Tetrahydro-F (THF)	3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one
Allo-tetrahydro-F (a-THF)	3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one
Cortolone (CORTOLONE)	3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one
β-Cortolone (β-CORTOLONE)	3α,17α,20β,21-Tetrahydroxy-5β-pregnan-11-one
Internal Standard (CHOL-BUT. (IS))	Cholesterylbutyrate
REGISIL™ (BSTFA)	Bis(trimethylsilyl)trifluoro-acetamide
MO-TMS	Methoxime-trimethylsilyl ether derivatives

EXPERIMENTAL

Materials

Reference compounds. Reference steroids were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A., and Ikapharm Ltd., Ramat-Gan, Israel. BSTFA was obtained from Regis Chemical Company, Chicago, Ill., U.S.A., and methoxylaminehydrochloride from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. Glass capillary columns were purchased from H. I. Jaeggi, Chromatographie-Labor, 5642 Mühlau, Switzerland.

Deuterated compounds. Deuterocholesterol was prepared according to Bloch and Rittenberg[15]. Deuteropregnenolone and progesterone were formed by a slightly modified method according to Seibel and Gäumann[14]. Under complete exclusion of moisture, 2 g phosphorus pentachloride is added with vigorous stirring to 8 g deuterium oxide, and the acid thus obtained diluted 1:1 with deuterium oxide. 100 mg of the steroid dissolved in CH_3OD solution and 1 g of the 10% acid solution ($DLI/D_3PO_4/D_2O$) are placed into a small sealed flask and stirred for 12 h at room temperature. The steroid is extracted with ethyl acetate, which is then concentrated, and the exchange repeated with 1 g of fresh acid solution. The product is once more extracted with ethyl acetate and recrystallized from chloroform.

Loading with deuterated compounds

1st Experiment: 100 mg of deuterated pregnenolone was given orally to a male control subject and urine was collected at intervals of 4 h.

2nd Experiment: 4·5 g of cholesterol was given orally to a male control subject and urine was collected at intervals of 4 h.

3rd Experiment: 15 mg of deuterated progesterone was administered into the anticubital vein over a 5-min period in 1 ml of a sterile 1,2-prolyleneglycol–water (3:1) mixture.

Hydrolysis of the urine

Ten ml urine are brought to pH 4·6 (by using 0·1 M HCl or 0·1 M NaOH, respectively) and Helicase is added (10,000 U glucuronidase and 150,000 U sulphatase). Three drops of chloroform and 5 ml of acetate buffer (0·1 mol/l; pH 4·62) are added, and the mixture is incubated for 24 h at 37°C.

Extraction of the urine

After the urine has cooled to room temperature, 200 μl solution containing the internal standard (cholesteryl butyrate in ethanol, 100 mg/l) is added. The urine is extracted 3 times with 20 ml each of the ethyl acetate. The combined ethyl acetate extracts are washed 3 times with 10 ml NaOH (0·1 mol/l) and 3 times with 10 ml double distilled water. The solution is dried with sodium sulphate, filtered and evaporated in a rotary vacuum evaporator. The residue is transferred into a small tube with a ground glass joint, using two portions of 4 ml of ethyl acetate.

Derivatives

The methoxime derivatives are made as follows: the ethyl acetate solution is blown dry with nitrogen, and 200 μl of a solution of methoxylamine hydrochloride in pyridine (10 mg/ml) is added. The formation of the methoximes lasts about 12 h at room temperature. In this process, keto groups in positions 3, 17 or 20 are transformed into methoxime groups. However, the 11 position is not attacked. Finally the mixture is evaporated to dryness on a rotary vacuum evaporator. Silylation is done with 200 μl BSTFA for 1 h at 60°C in closed tubes. In this reaction, hydroxyl groups in positions 11 and 17 are not silylated.

Gas chromatography

A Carlo Erba Mod. GI (flame ionization detector) with glass capillaries (20 m long, 0·3 mm i.d.) was used. The stationary phase was OV-101. The following conditions in the separation column were used: 2 ml/min helium; injection temperature 260°C; column temperature for the first 15 min 160°C, then progressing by 2·5°C/min to 240°C. The technique of splitless injection (the so-called solvent bypassing) according to Grob[16, 17] was used. The injected amount was 1 μl solution. In principle glass capillaries can be used in every gas chromatograph but care must be taken to bring the columns as near as

possible to the detector in order to achieve optimal separations. An inlet splitter or a solid insert is necessary to introduce the samples.

Quantitative determination

One μl of a steroid reference mixture (AN, ET, DHA, 11-O-ET, PD, PT, PT', PTL, THS, THE and IS) containing 50 ng of each steroid is injected daily into the gas chromatograph. The factor area steroid/area IS serves for the correction in the chromatograms of the urinary steroids.

Gas chromatography–Mass spectrometry

The same columns were used in a combination gas chromatograph–mass spectrometer LKB 9000. The gas chromatographic conditions were as follows: carrier gas flow 2 ml/min, the temperatures were: injection port: 260°C; column: 10 min 180°C, progr. 3°/min, 250°C; separator: 280°C; ion source: 290°C. A streamsplitting inlet block from a Carlo Erba Model GI gas chromatograph was mounted instead of the normal inlet part. The first stage of the jet separator was omitted and an additional helium gas stream of 20 ml/min was added at the end of the column to allow proper flow conditions in the separator. The injection was also carried out without stream-splitting [16, 17]. The total ion current was recorded at 20 eV, the mass spectra at 70 eV.

RESULTS AND DISCUSSION

Gas chromatography on glass capillary columns

It will be shown that glass capillary columns can be used successfully for the determination of steroids. Their high resolution saves complicated prepurification steps when biological samples are studied.

With the splitless injection technique, quantitative analyses are also possible. Using a combination GC–MS, capillary columns are especially suitable for recognizing the identity of unknown compounds.

Figure 1 shows a capillary GC-separation of urinary steroids from a normal male. It should be noted that, according to Grob[16, 17], the technique of splitless injection was used.

The separations have been carried out on a glass capillary column coated with OV-101. The respective retention times of steroids are very similar on stationary phases OV-101 and SF-96. Starting with 10 ml of urine the final sample for gas chromatography is obtained by a simple extraction procedure without any prepurification step. This method involves the enzymatic hydrolysis of glucuronide and sulphate conjugated steroids, extraction with ethyl acetate and formation of methoxime-trimethylsilyl ether derivatives.

The reproducibility of the method in 8 independent determinations of the same urine is shown in Table 1. It should be emphasized, that the variation coefficients represent the whole analytical procedure including hydrolysis and extraction.

A second internal standard appearing in the first part of the gas chromatogram helps to improve the variation coefficient markedly.

Clinical applications

The practicability of the method is demonstrated by studies in a patient with 21-hydroxylase deficiency, in a special patient with 11β-hydroxylase deficiency and in a patient with an obscure enzyme defect in steroid biosynthesis.

A profile of urinary steroids from a boy (age 8 years) with congenital adrenal hyperplasia due to a

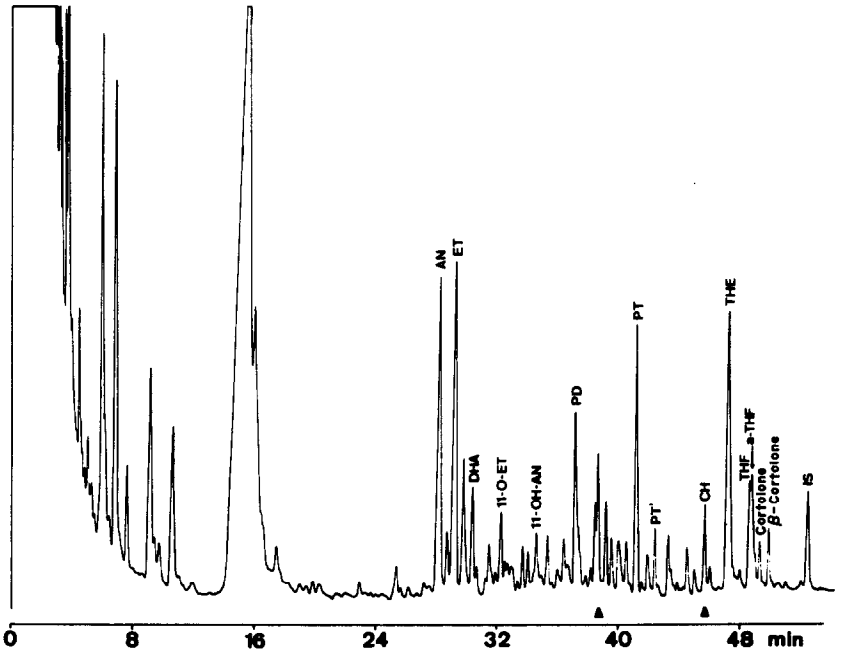

Fig. 1. Urinary steroid profile (MO-TMS) of a normal man (age 34 years).

Table 1. Reproducibility of 8 independent determinations of the same urine (μg/24 h)

	1	2	3	4	5	6	7	8	$\bar{x}$	±	s	VC (%)
AN	1485	1947	2607	1518	1353	1683	1221	1749	1695	±	433	25
ET	2244	2178	2673	1881	1881	2112	1650	2046	2083	±	305	15
DHA	462	495	528	396	396	429	264	363	416	±	82	20
11-Keto-ET	528	576	720	528	480	576	336	528	534	±	107	20
PD	768	928	928	800	672	736	672	896	800	±	106	13
PT	1575	1935	1890	1710	1575	1710	1170	1575	1642	±	236	14
PT'	385	340	540	440	385	385	275	385	412	±	29	7
THE	6420	5885	7169	6206	6099	6420	4387	5885	6058	±	790	13

VC = Variation coefficient.

21-hydroxylase deficiency (insufficiently treated with prednisone) is shown in Fig. 2. Androsterone, etiocholanolone and pregnanetriol are markedly increased. Characteristically for this condition, pregnenetriolone is also found in considerable amount. On the other hand cortisol metabolites are absent or very low.

Figure 3 shows urinary steroids from an infant girl with congenital adrenal hyperplasia due to a 11β-hydroxylase deficiency. In comparison with a normal child of the same age, all steroids oxygenated in position 11 are only detectable in small quantities or are not present. On the other hand THS is markedly increased. The secretion rate of S was extremely high and that of F was low, while the secretion rates of DOC and B and the aldosterone excretion were within normal limits, therefore only the conversion of compound S to compound F is inhibited, but not the conversion of DOC to B. This supports the assumption that there is more than one enzyme system involved in steroid 11β-hydroxylation [18].

Our subsequent kinetic studies on 11β-hydroxylase preparations obtained from human adrenals up to 12 h after death, suggest 11β-hydroxylase of compound S and of DOC as two systems with at least one different component [19].

Figure 4 shows the urinary steroid profile from an infant who died 10 days after birth. The gas chromatogram shows only cholesterol and another component with a different retention time which has approximately the same mass spectrum as cholesterol. The gas chromatographic profile is similar to that expected in lipoid adrenal hyperplasia. Theoretically, in this condition the conversion of cholesterol to pregnenolone is deficient due to the enzyme defect of either 20α-hydroxylase, 22R-hydroxylase or 20–22 desmolase. However, the autopsy findings do not agree with this diagnosis, because no lipid storage could be observed in the adrenals of this patient.

Figure 5 shows current concepts of pregnenolone formation according to Degenhart *et al.* [20].

The advantage of glass capillary columns is clearly shown in this case, since the separation of cholesterol and the unknown substance would be impossible on packed columns.

The structure of the second component showing a different retention time but practically the same

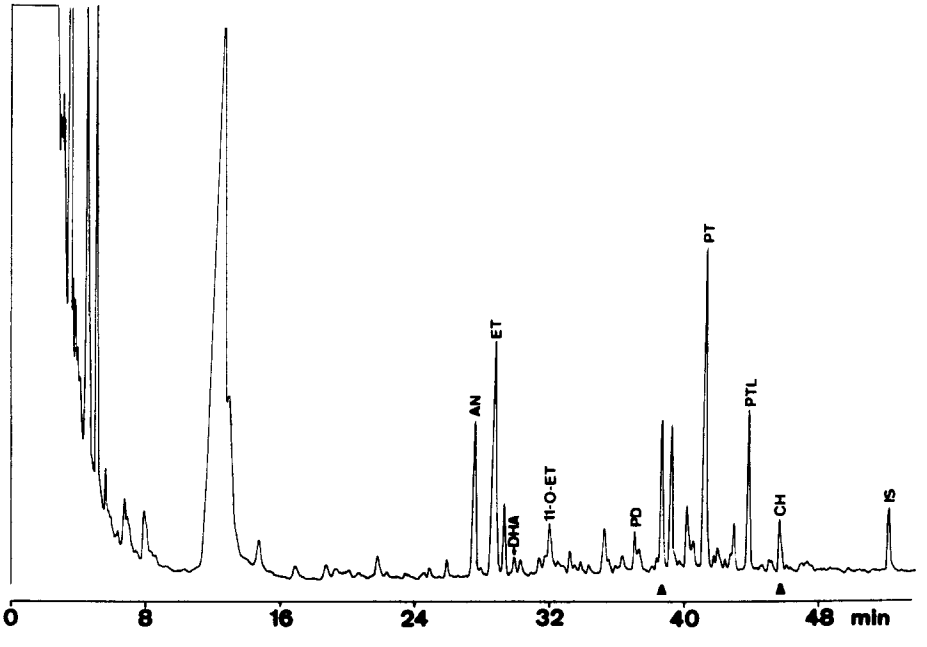

Fig. 2. Urinary steroid profile (MO-TMS) of a boy with congenital adrenal hyperplasia due to a 21-hydroxylase deficiency (age 8 years).

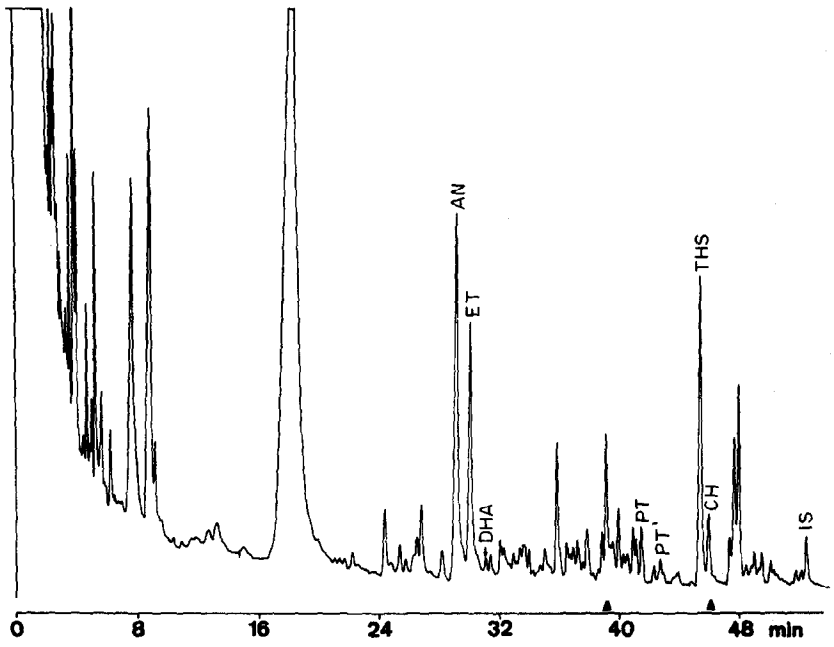

Fig. 3. Urinary steroid profile (MO-TMS) of an untreated girl with congenital adrenal hyperplasia due to an 11β-hydroxylase deficiency (age 3 months).

mass spectrum as cholesterol, is not yet clarified. We expect it to be a cholesterol isomer. At present, an explanation of these findings is difficult. Incubation studies using an enzyme preparation obtained from the patients adrenal tissue are now in progress.

THS determination

The estimation of urinary THS on glass capillary columns is also useful for an abbreviated, sensitive and specific modification of the metyrapone test.

Figure 6 shows urinary steroids of a normal man (age 31 years) before and after metyrapone.

Since the peak of the THS-excretion after a single dose of metyrapone (500 mg/m^2) is reached after 8 h, the collection of a 12-h urine sample after ingestion of the drug is sufficient to evaluate hypothalamic-pituitary-adrenal function. Using this method, the mean THS-response in endocrinologically normal subjects was 1078 $\pm$ 268 μg/m^2/12 h and that in patients with proven ACTH-deficiency was 84 $\pm$ 23 μg/m^2/12 h [21].

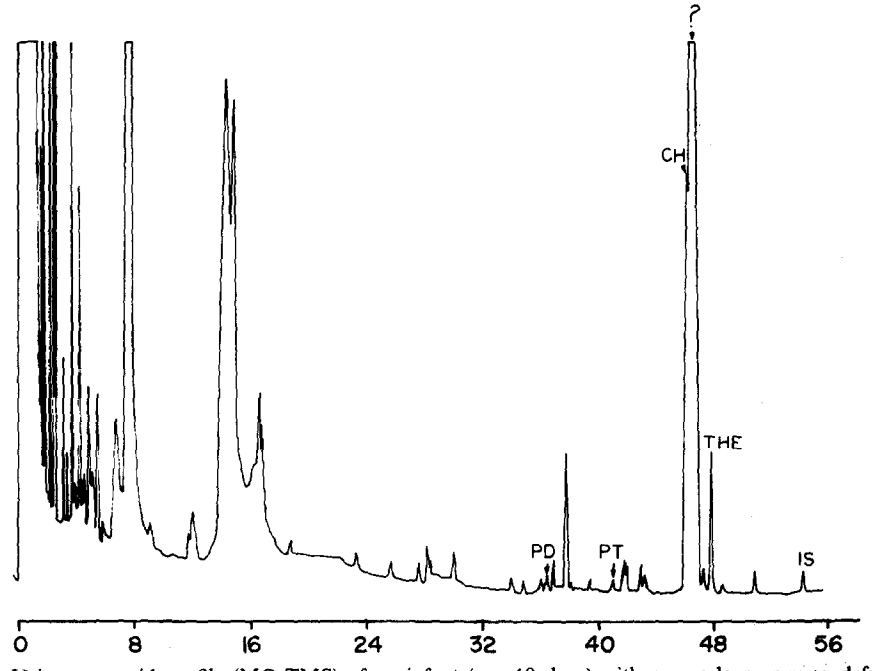

Fig. 4. Urinary steroid profile (MO-TMS) of an infant (age 10 days) with an unclear enzyme defect in steroid biosynthesis.

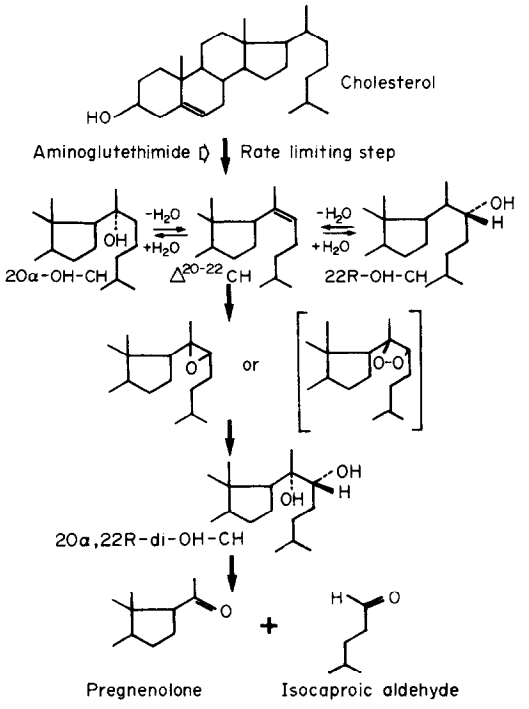

Fig. 5. Mechanism of cholesterol side chain cleavage according to Degenhart *et al.* [20].

Application of stable isotopes

The investigation of steroid metabolism using deuterated precursors has been tried with deuterated pregnenolone, progesterone and cholesterol.

Deuteration of steroids

The deuteration of steroids presents some major problems. Hydrogen atoms in the neighbourhood of a keto-group can be activated and easily labelled by the action of acid or base [22]. Hydrogen atoms at the branched positions 20 and 25 can be exchanged by catalytic deuteration [15].

However, most of these positions may be exchanged during normal metabolism. Stable positions are found, for instance, at C-1, C-7 and C-19, but such deuterated compounds can be synthesized only by more complicated and expensive techniques. Therefore, in preliminary studies, we used the more convenient technique of acid or catalytic exchange.

The mass spectrum of deuterated pregnenolone made by acid catalyst compared with the undeuterated compound is shown in Fig. 7.

It is obvious that 4 deuterium atoms are incorporated. From the mass spectrum of the isolated pregnanediol and from the literature it can be concluded, that 3 hydrogen atoms have been exchanged at position 21 and one at position 17 [23].

Figure 8 shows the mass spectrum of progesterone, deuterated and undeuterated. The mass spectrum indicates that 9 deuterium atoms are incorporated.

From the literature it can be concluded, that in addition to deuteration at positions 21 and 17, two hydrogen atoms have been exchanged at position 2, one hydrogen atom at position 4 and 2 hydrogen atoms at position 6 [23].

The mass spectrum of deuterated cholesterol prepared by catalytic exchange according to Bloch and Rittenberg[15] indicates that 2 deuterium atoms are incorporated. We assume that only hydrogen at positions 20 and 25 could be exchanged.

Unfortunately the deuterium labelling of the 20 and 25 positions is lost during the oxidation of C-20 and the splitting off of the side chain and does not allow us to follow the metabolism further than a side chain monohydroxylated cholest-4-en-3-one.

Loading tests

After oral administration of 100 mg of deuteropregnenolone to a male control subject, investigation of the urinary steroids by GC–MS showed that only pregnanediol, allo-pregnanediol and another unknown steroid, probably pregnadiene-diol, were deuterated. Intravenous loading with 15 mg of deuterated progesterone showed only pregnanediol and allopregnanediol to be deuterated. These results were quite unexpected because we had hoped to follow the cortisol metabolism with the aid of deuterated precursors. One possible explanation of these results is that the label of our precursors was too easily exchanged during metabolism. We are now trying to synthesize steroids deuterated in the more stable positions 1 and 7.

An oral loading test with 4·5 g of deuterated cholesterol showed no deuteration of urinary steroids except cholest-4-en-3-one, cholesterol and a side chain monohydroxylated cholest-4-en-3-one in decreasing quantity, which confirms our comments above.

A practical result of these investigations is that intravenous loading with deuterated progesterone can

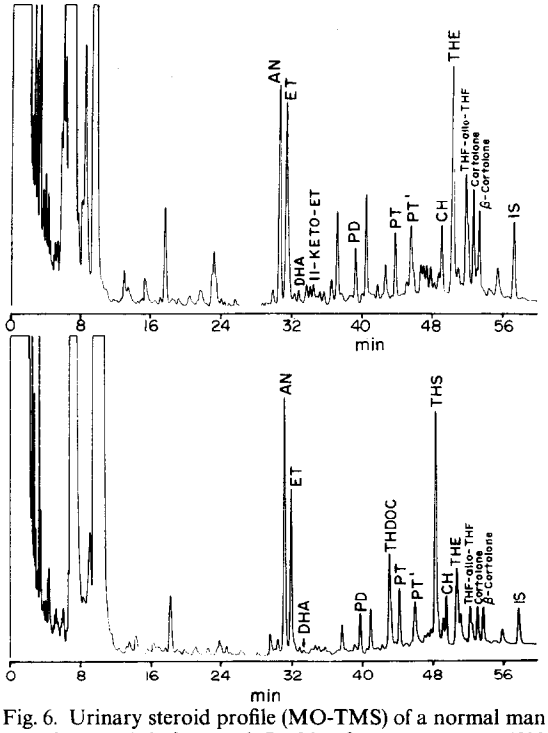

Fig. 6. Urinary steroid profile (MO-TMS) of a normal man (age 31 years) before and 7–12 h after metyrapone (500 mg/m²).

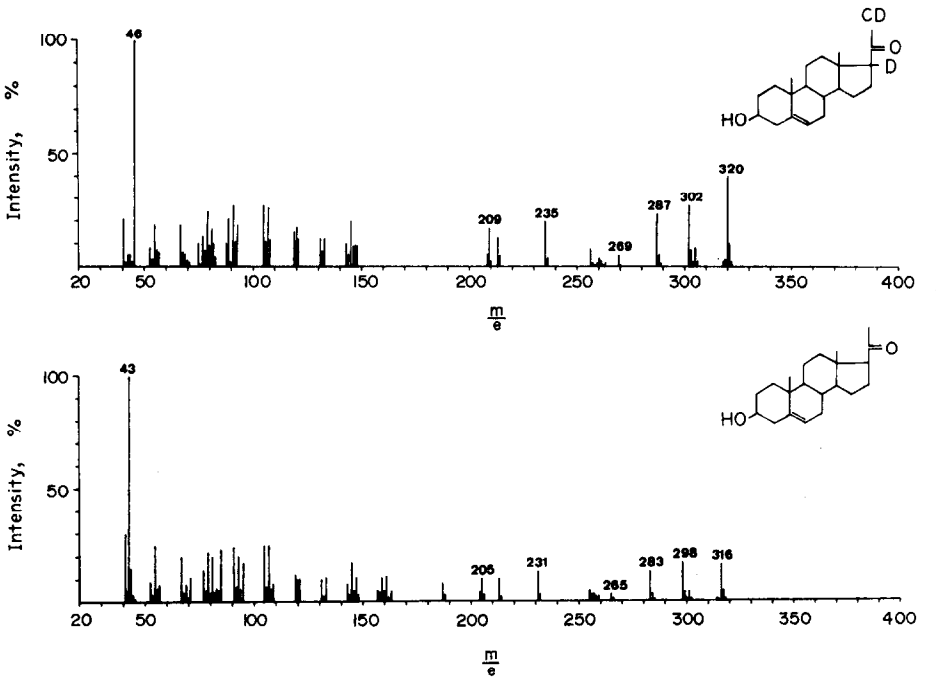

Fig. 7. Mass spectrum of pregnenolone, deuterated (above) and undeuterated (below).

be used for the determination of the secretion rate of progesterone.

Gas chromatography with glass capillary columns is a powerful instrument for the diagnosis of enzyme defects in steroid metabolism. Using a combination of GC–MS, capillary columns are especially suitable for the identification of unknown compounds. By applying the stable isotope technique (oral or intravenous loading of deuterated precursors) secretion rates can be determined. The labelling of steroid precursors in more stable positions should allow the investigations of steroid metabolism *in vivo* without any risk to the patient.

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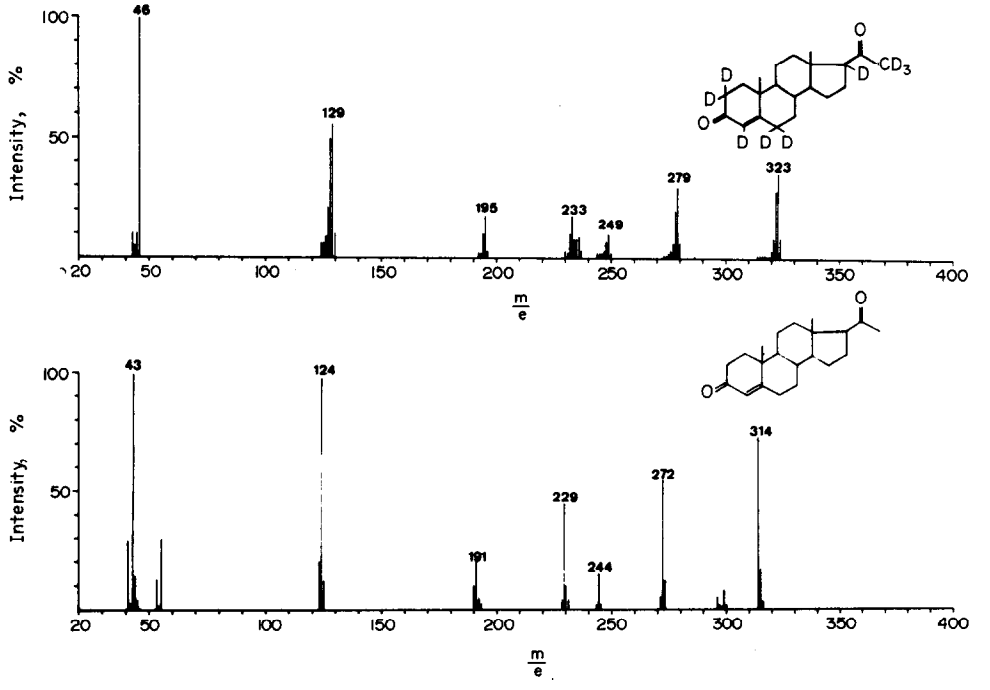

Fig. 8. Mass spectrum of progesterone, deuterated (above) and undeuterated (below).

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