Simultaneous gaschromatographic assay of the principal neutral urinary steroids using glass capillary columns

by
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The main points which must be set out currently in a method of gaschromatographic assay of urinary steroids are:

1 - enzymatic hydrolysis

2 - separation and evaluation of each steroid individually, thus obviating many disadvantages of evaluating whole families of steroids ([1]).

Great progress has been made and interest has recently been aroused by the new radio-immunological and protein-binding methods for evaluating protein and non protein hormones.

Because of their sensitivity and speed of execution these methods are already being adopted by many laboratories and replacing, for instance, the methods for evaluating plasma steroid hormones using gaschromatography with electron capture detector.

We believe it to be not improbable that, in the near future, the evaluation of individual hormonal steroids will be done by radio-immunological and protein-binding methods only.

However, gaschromatography has, and will continue to have for a long time, an absolute advantage even over the methods mentioned above, in the simultaneous evaluation of several substances from one test sample.

All reagents employed with the exception of the enzyme are purified by double distillation, washing with ether or rechystallization. In this connection, we regard the reagents we use as sufficiently pure only when, substituting for urine the same amount of distilled water and running through the whole method, we obtain a gaschromatograph recording « completely blank » practically free from interfering peaks ([2, 3]).

HYDROLYSIS

24 hours' urine is collected in a bottle containing one ml of glacial acetic acid.

A twentieth part of the 24 hours' urine is brought to pH 5,2 by glacial acetic acid and then diluted with distilled water to 100 ml.

2 ml are taken, which correspond to 1/1000 of the 24 hours' sample.

0,2 ml of acetate buffer pH 5,2 ([a]) and 20 µl Helix pomatia (H.P.) enzyme, corresponding to 2,000 Fishmann units of beta-glucuronidase and 16,000 Roy units of sulphatase, are added.

They are incubated at 37 °C for 48 hours.

The hydrolized urine is kept at — 20 °C until the next stage: extraction.

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EXTRACTION

To the hydrolized urine 2 μg of cholesterol butyrate and 1 μg of estratetriene as internal standards are added, in addition 0.2 ml of sodium hydroxide 8N are added to bring the pH to a basic value between 12 and 12.5.

The urine is then extracted in two stage, first with 75 ml of double distilled ethylic ether and then with a further 50 ml. The two ethereal fractions (75+50 ml) are taken together and washing is carried out with 2 ml of carbonate buffer pH 10.5 ([b]) followed by a further washing with 0.5 ml of double distilled water.

Any traces of water that may still remain are removed with anhydrous sodium sulphate.

The ether is then evaporated to a volume of about 5 ml in a water bath and then transferred by means of a Pasteur pipette into 10 ml test tubes and dried completely at 42 °C under nitrogen flow.

FORMATION OF DERIVATIVES

To make the steroids stable to chromatography, we adopted in 1966 ([2]) a two stage reaction:

1 - We used Methyloxime hydrochloride in alcohol solution together with anhydrous potassium acetate to produce the Methyloximes.

2 - The dry residue of this reaction was dissolved and caused to react with BSTFA. That is to say MO-TMSi and TMSi were formed. However, for the corticosteroids with the side chain: 17-OH, 21-OH and 20=0, such as tetrahydrocortisone (THE), tetrahydrocortisol (THF) and allo-tetrahydrocortisol (allo-THF), it was seen, with the aid of gaschromatography-mass spectrometry, carried out by Dr. Giovanni Galli, that Methyloxime was not formed in 20 and that the derivative had good characteristics of stability to gaschromatographic analysis.

Figure 1, taken from a 1968 report of ours, shows the packed column gaschromatographic analysis of tetrahydrocortisone (THE) as MO-TMSi and as enol-TMSi.

Speaking later of this derivative, with which we were not acquainted, to Dr. Edmund Chambaz of Grenoble, he suggested and put forward the ideas that enol-TMSi were formed.

In 1971, Chambaz ([4]) et al reported the first studies of the formations of enol-THSi derivatives of some corticosteroids with side chains: 17 - OH, 20=0,21 - OH; and 20=0,21 - OH.

Dr. Zaraga Nicosia et al studied the reaction on a larger number of steroids ([5]).

The reaction conditions which we have used for the past three years are:

Formation of TMSi, enol TMSi: to the dry residue is added a quantity of 5-10 mg anhydrous recrystallized potassium acetate and 100 μl of BSTFA. Tre reaction was carried out in sealed test-tubes at 62 °C for 15 minutes.

It was cooled and then rapidly dried under nitrogen flow at 42 °C.

The residue was dissolved in 100 ml hexamethyldisilazane, which serves the

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([a]) Obtained by mixing 21 cc of a solution of 2M acetic acid and 79 cc 2M sodium acetate solution.

(H.P.) Digestive juice of Helix pomatia. Industrie Biologique Française, 35-49 Quai du Moulin de Cage, Gennevillier (Seine), Paris.

1 cc contains 100,000 Fishmann units of beta-glucuronidase and 800,000 Roy units of sulphatase.

(b) Obtained by mixing 150 cc NaOH 5N with 1,000 cc $NaHCO_3$ at 8%.

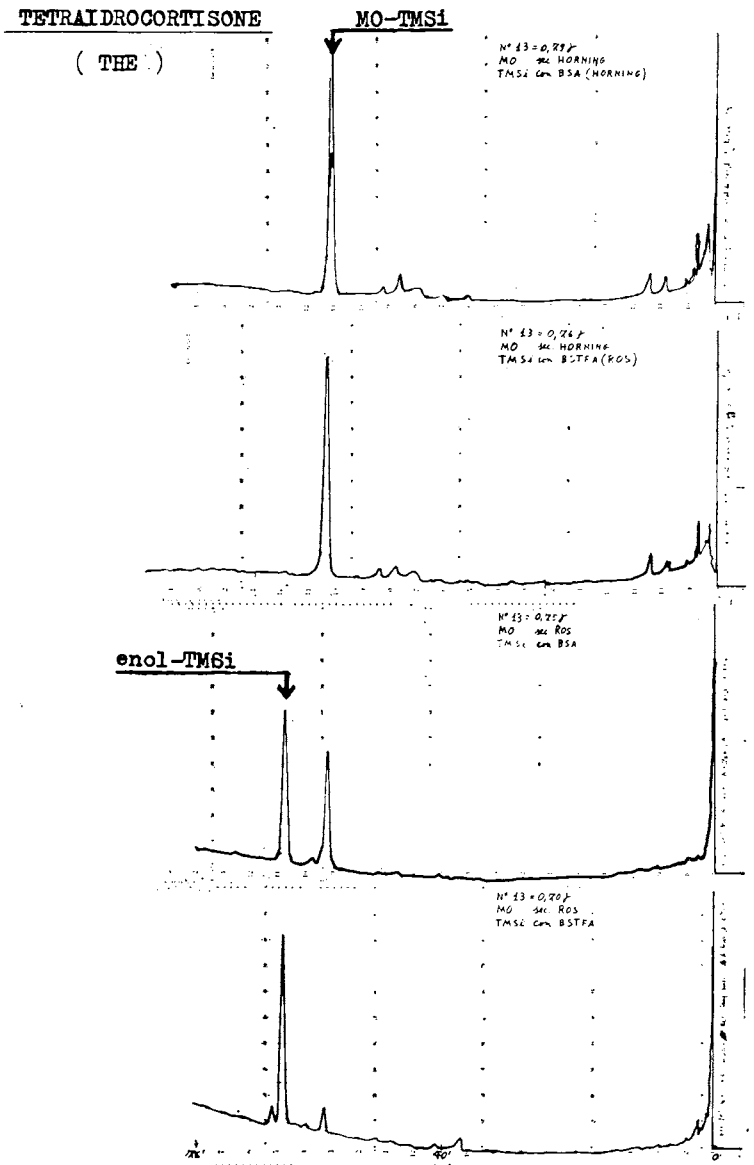

FIG. 1 - See text.

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double purpose of making soluble the steroids which have reacted and keeping them stable and inhibiting, among other things, the further progress of the reaction. We noticed in fact that hexamethyldisilazane added to the BSTFA in sufficient quantity (80% V/V) slows down or prevents the formation of TMSi, enol TMSi.

If the reaction were continued for a longer period it would bring about the formation of TMSi and enol-TMSn in some of the - OH and =O functions of more difficult reactivity. For many of the steroids studied we would thus have two or more peaks on the gaschromatographic analysis.

It was observed, moreover, that if the reaction is carried out at 62 °C for 24 - 48 hours no better gaschromatographic results are obtained. Either because the gaschromatographic separation of the mixture of steroids with all the silanized O, OH, functions is worse, or bacause some steroids containing the groups of difficult reactivity such as 11 - OH, 11=0; 17 - OH react only partially even under the above-mentioned drastic conditions of reaction, which can perhaps favour the breaking of the steroid molecules at the weakest points.

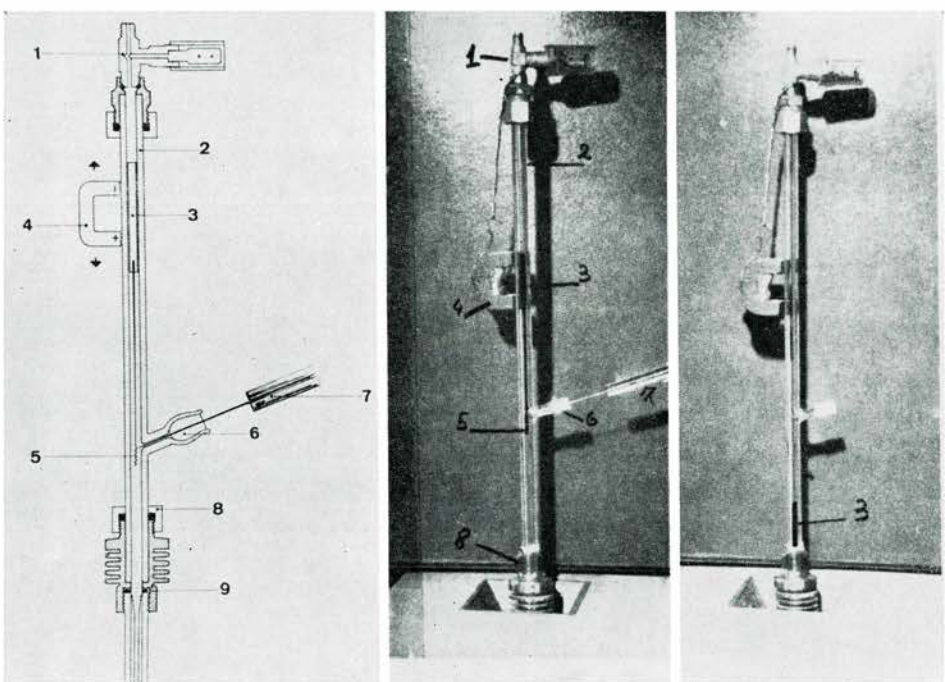

FIG. 2 - See text.

The advantages of enol-TMSi ascompared with MO-TMSi are:

1 - A single reaction without transfer from one test-tube to another.

2 - Total reaction time = 10-15 minutes as compared with 14-20 hours of the MO - TMSi.

3 - Non-formation of isomers.

4 - Formation of a single derivative for each urinary steroid at the selected reaction time. The only exceptions are: 11=0 An. and 11=0 Et, which react to

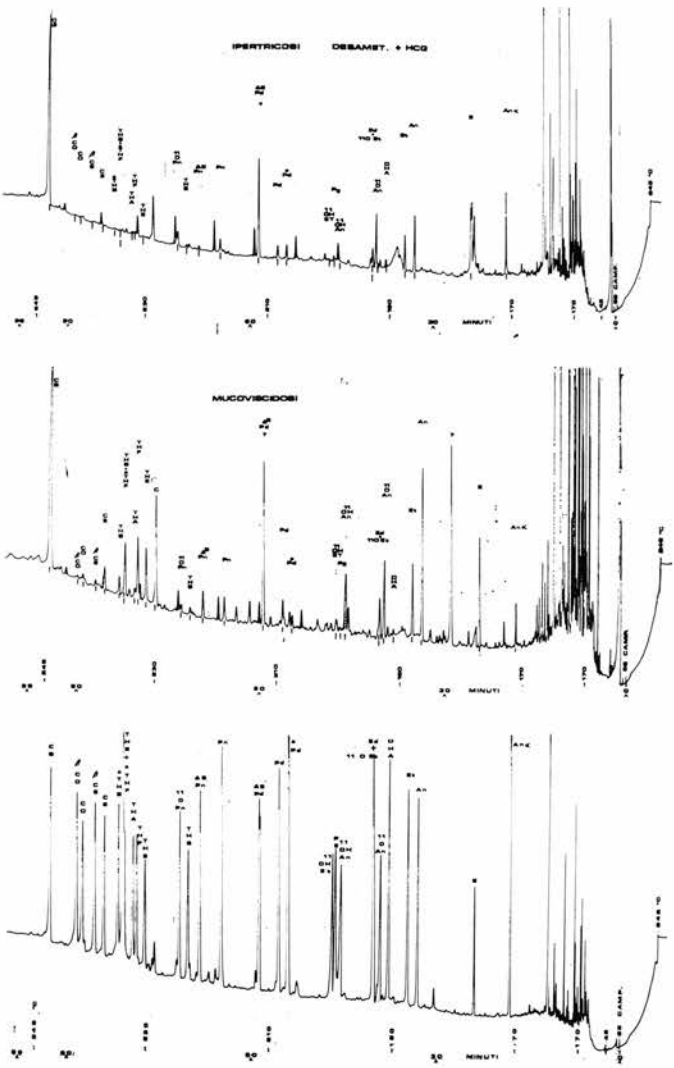

FIG. 3 - Tracings obtained from a mixture of pure steroids studied by us, from the urine of a 20 year old woman affected by mucoviscidosis, and from a case of hypertrichosis after the test with dexamethazone plus HCG.

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Table 1

Abbreviations	Trivial names	Systematic names	Silylated functions	Molecular Weight
An 3α	Androstenol 3α	5α-androst-16-en-3α-ol	3α	346
E	Estratetraenol	Estra-1,3,5,(10),16-tetraen-3-ol	3	326
An	Androsterone	5α-androstan-3α-ol-17-one	3α	362
Et	Aetiocohlanolone	5β-androstan-3α-ol-17-one	3α	362
DHA	Dehydroepian-drosterone	Androst-5en-3β-ol-17-one	3β	360
epi-An	Epiandrosterone	5α-androstan-3β-ol-17-one	3β	362
11-O-An	11-Oxo-Andros-terone	5α-androstan-3α-ol-11, 17-dione	3α,(17)	376,(448)
11-O-Et	11-Oxo-Aetiocho-lanolone	5β-androstan-3α-ol-11, 17-dione	3α,(17)	376,(448)
Ed	Aetiocolandiol	5β-androstan-3α, 17β-diol	3α,17β	
11-OH-An	11-Hydroxy-aetiocholanolone	5α-androstan-3,11β-diol-17-one	3α	378
11-OH-Et	11-Hydroxy-Aetiocholanolone	5β-androstan-3α,11β-diol-17-one	3α	378
Pg	Pregnanolone	5β-pregnan-3α-ol-20-one	3α	390
a Pd	allo-Pregnandiol	5α-pregnan-3α,20α-diol	3α,20α	464
Pd	Pregnanediol	5β-pregnan-3α,20α-diol	3α,20α	464
Δ^5-Pd	Δ^5-Pregnenediol	Pregn-5en-3β,20α-diol	3β,20α	462
Pn	Pregnanetriol	5β-pregnan-3α,17α,20α-triol	3α,20α	480

the extent of 5 - 10% even in position 17, giving TMSi at 3 and Enol-TMSi at 17.

5 - Excellent stability and gaschromatographic separation.

In table 1+1a the steroids we studied are listed.

Gaschromatographic Analysis Apparatus. In our research we used a Fractovap 2400 T (Carlo Erba, Milan) Gaschromatograph provided with temperature programming and automatic dry sampler.

The electronic circuit is arranged so that sampling occurs with a cold column and, after automatic analyses, a timer changes the temperature of the oven in the initial isotherm in order to avoid as far as possible the ageing of the column. *Column.* The 50 m × 0.31 ± 0.01 mm (i.d.) glass capillary columns coated with OV-101 were supplied by Dr. Brechbuller of Zurich (Urdorf im Grüt 30).

The number of satisfactory analyses carried out for each column was on average

200-300. The ageing of the columns is revealed by the progressive shortening of the retention times and enlargement of the peaks, especially those of the more polar steroids (e.g. 11-OH An; 11-O An, etc.).

We are now convinced that 50 metres is too long and indeed in order that the

Table 1 a

Abbreviations	Trivial names	Systematic names	Silylated functions	Molecular Weight
Δ^5-Pn	Δ^5-Pregnentriol	Pregn-5ene-3β-17α, 20α-triol	3β,20α	478
THS		5β-pregnan-3α,17α,21-triol-20-one	3α,17α, 20,21	638
11-O-Pn	11-Oxo-pregnane-triol	5β-pregnan-3α,17α,20α-triol-11-one	3α,20	494
THE	Tetrahydrocort-isone	5β-pregnan-3α,17α,21-triol-11,20-dione	3α,17α, 20,21	652
THF	Tetrahydrocort-isol	5β-pregnan-3α,11β,17α, 21-tetrol-20-one	3α,17α, 20,21	654
THA	Tetrahydrodehy-drocortico-sterone	5β-pregnan-3α,21-diol-11,20-dione	3α,20,21	654
THB	Tetrahydro-corticosterone	5β-pregnan-3α,11β,21-triol-20-one	3α,20,21	566
a-THF	Allotetrahydro-cortisol	5α-pregnan-3α,11β,17α, 21-tetrol-20-one	3α,17α, 20,21	654
a-THB	Allotetrahydro-corticosterone	5α-pregnan-3α,11β,21-triol-20-one	3α,20,21	566
CE	Cortolone	5β-pregnan-3α,17α,20α, 21-tetrol-11-one	3α+	582
β-CE	β-Cortolone	5β-pregnan-3α,17α,20β, 21-tetrol-11-one	3α+	582
CO	Cortol	5β-pregnan-3α,11β,17α, 20α,21-pentol	3α+	584
β-CO	β-Cortol	5β-pregnan-3α,11β,17α, 20β,21-pentol	3α+	584
	Corticosterone	Pregn-4en-11β,21-diol-3,20-dione	20,21	490
	Cortisol	Pregn-4-ene-11β,17α, 21-triol-3,20-dione	17α,20,21	578
	Cortisone	Pregn-4-ene-17α,21-diol-3,11,20-trione	17α,20,21	576

+ Two of the three hydroxy groups of the side chain are silylated.

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analysis must finish at a temperature below 250 °C one is obliged not only to use hydrogen as carrier but also to have a column entrance pressure of 1.8 - 2 kg/cm² which gives a flow and therefore a speed of the carrier greater than the optimum.

(Optimal speed of carrier = 15 - 25 cm/sec for a column of 50 m: 1.6 - 2.1 ml/min of carrier).

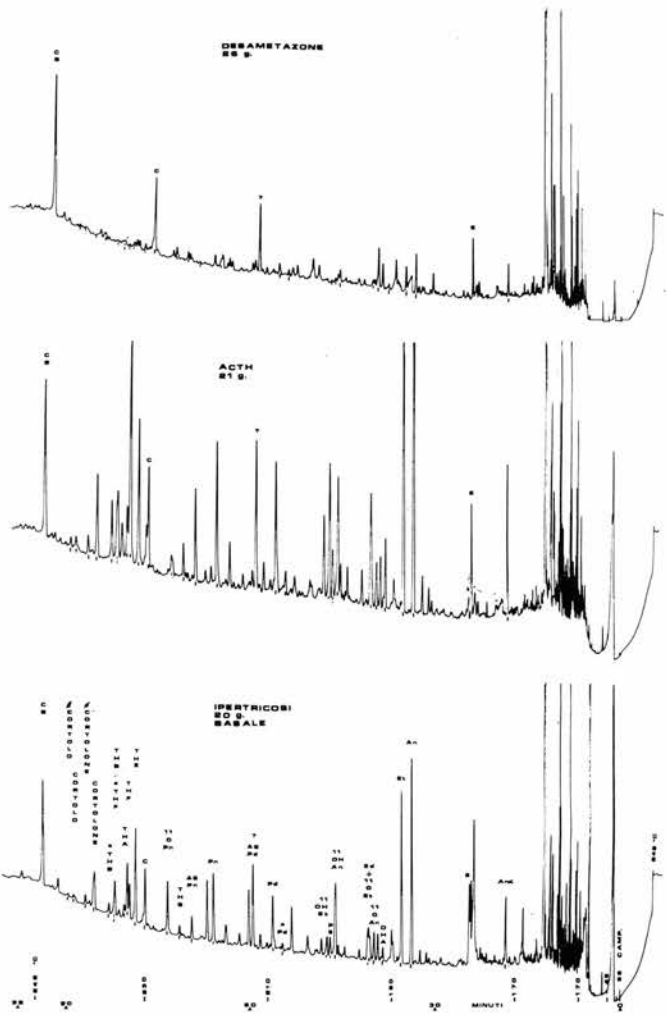

FIG. 4 - Tracings obtained in a case of hypertrichosis: with base-20th day of cycle, after ACTH and after dexamethazone suppression.

ANALYTICAL CONDITIONS

Temperature: flash heater 270 °C; detector 270 °C.

At the moment of sampling the temperature of the column is 55 °C which falls in the next two minutes to 45 °C; then in 5 minutes it rises to 170 °C; it remains

isothermic for 10 minutes and is then increased 1 °C/min up to 248 °C.

Hydrogen for the flame: 0.20 kg/cm^2 = 9 ml/min.

Air 0.80 kg/cm^2 = 190 ml/min.

Carrier gas = hydrogen at an entrance pressure of 1.8 - 2 kg/cm^2 and at a flow rate at 170 °C of 3.8-4.1 ml/min.

Attenuation, with the recorder on:

1 mv - f.s.d. was 1 ×32 = f.s.d. of 1.8 per 10^{-12}A.

SAMPLING

With cold column sampling all steroids are condensed in the first part of the column where they begin to migrate as the temperature is progressively raised.

The result is that the sample volume and the speed at which the sample enters the column no longer need to be kept as low as possible but can reach values which are greater than the optimal ones for normal analysis with isothermic sampling.

Only in this way, we find, is it possible to obtain the best profiles with an automatic sampler which has necessarily a glass flash heater of relatively large capacity as it has to contain 15-20 capillary tubes for the samples.

Since 1969 we have used sampling without splitter in the analysis with capillary columns.

Since 1963 (7.8) we have always used in our research gaschromatography even with packed column dry sampling, with a device which we built ourselves (fig. 2).

It has the great disadvantage that it has to be operated manually and therefore only 4-5 analyses a day can be carried out.

Now with the possibility of using automatic dry sampling, 15-20 analyses can be done in 24 hours and in addition between the first and the last of such analyses there is no variation in separation nor in the shape of the peaks.

For carrying the samples we use small glass tubes graded at 2 µl. Quick filling is achieved by capillarity touching the surface of the liquid to be analysed and they are kept in a nitrogen stream for about 15 minutes, until total drying of the hexamethyldisilazane.

PURE STEROIDS FOR CONTROL

The standards which we took into consideration are listed in Table 1, 1a.

This Table also shows the groups which reacted as TMSi and as Enol-TMSi and their respective molecular weights. For further details reference should be made to the work of Nicosia et al.

Figure 3 shows a gaschromatograph analysis of the mixture of steroids studied. Each peak corresponds to an amount of 40 ng, except: E = 20 ng; aPd = 60 ng.

CALCULATION OF THE RESULTS

1. Into 10 test-tubes each containing 2 µg of C.B. and 1 µg of E, was added the usual quantity of BSTFA and potassium acetate. The reaction proceeded as described. The samples are analysed by gaschromatography, trying to keep the samples as uniform as possible by taking 2 µl per 100 of solvent. Sampling will thus have been done of 40 ng for the C.B. and 20 ng for the estratetraenol.

The average height of the peaks obtained is regarded as the 100% of the internal standards.

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In view of the non participation of the C.B. in the reaction and the quantitative reactivity of the estratetraenol 3 - OH group, and furthermore accepting the total passage in ether of both standards, we can assume that any reduction in the peaks of the standards in successive analyses can be attributed solely to the loss of organic solvent due to the method.

2. 10 mixtures of pure steroids (Ana 2 μg) are extracted from 2 ml of distilled water, after addition of all the reagents including those of hydrolysis.

After completing all the stages of the method 10 profiles of the pure steroids were obtained.

a. Two correction factors are calculated for each profile by taking the ratio between the theoretical value (100%) and that found for the two internal standards.

The average of these two factors is used for correcting the height of all the peaks of the re-extracted steroids.

In this way we obviate the possibility of error due to the loss of organic solvent during the process and of sampling error as such.

b. The average of ten recuperated values of each steroid is regarded as 100% recuperation of same.

The ratio between the nanogrammes and the height in mm corresponding to each steroid constitutes the conversion factor: mm of mass height.

3. *In urinary profiles* the height of each peak of the steroids studied will first be multiplied by the correction factor typical of the profile in question, thus compensating for any possible error of extraction and sampling and giving the relative factor converted into mass expressed as mg/24 hrs.

Table 2. *Repeated analyses (11 findings) of the urine of a normal woman at the 21st day of the cycle.*

	Mean ± SD mg/24 h	Coefficient of Variation (%)
Androstenol	0.34 ± 0.09	7.3
Androsterone	2.44 ± 0.07	2.4
Eticolanolone	3.10 ± 0.05	2.7
11 - OH - An.	0.45 ± 0.07	3.8
allo - Pd.	0.64 ± 0.04	3.4
Pd.	4.12 ± 0.06	2.6
Pregnantriol	1.38 ± 0.05	7.1
Δ^5 Pregnantriol	0.21 ± 0.03	4.3
THE	1.94 ± 0.06	5.3
THF	0.80 ± 0.07	7.1
Cortolone	0.79 ± 0.09	7.4
β Cortolone	0.51 ± 0.08	7.9
Cortol	0.21 ± 0.06	7.3
β Cortol	0.16 ± 0.09	6.5

SPECIFICITY

In Table 2 we give the steroids, the peaks of which, in 11 different urinary extracts of a normal woman in the 21st day of the cycle, and in a urinary extract for each clinical group examined by us, never revealed the presence of impurities by using gaschromatography - mass spectrometry. In all the other peaks of steroids exami-

ned the impurities, when it was possible to record the mass spectrum, were always less than 20%.

For the Δ^5Pd and for the cholesterol = C, the impurities were almost always above 40%, making even a tentative evaluation impossible.

We would also like to stress that when the amount of steroid eliminated in 24 hours is less tha 50 - 100 µg, evaluation must be regarded as for guidance only and not absolute, in the sense that in reality it could be always polluted by other steroids or imputities, which at that level are very numerous in the urine.

The precision of the method has been determined by repeated analyses of the same sample of urine collected on the 21st day of a normal cycle (Table 2).

The results shown in Table 2 seem to us satisfactory. In this connexion we are unable to share the pessemism expressed by Vollmin ([6]), and indeed in an earlier work we also obtained, with the use of capillary columns and TMSi and MO-TMSi derivatives, acceptable standard deviation and coefficient of variation.

Figures 3 and 4 are examples of gaschromatographic analyses.

SUMMARY

The method for simultaneous gaschromatographic assay of the principal neutral urinary steroids using glass capillary columns is described. The advantages of this method are described.

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Therapeutic trials in placental insufficiency

by

P. GRELLA and E. ZARDINI

A study was made of the efficacy of two therapies for placental insufficiency, based on different precepts: the object of the first is to improve the placental blood flow, whereas the purpose of the second is to intervene positively in placental metabolism by the provision of phosphorylated glycide, fructose 1-6-diphosphate.

Evaluation of the functional conditions of the placenta and of its modifications by the two therapies was based on the radio-immunological assay of HCG ([1, 2, 3, 4, 5, 6, 7]) and HPL ([8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19]) by the double antibody method (Kit and CEA-IRESORIN-Sclavo).

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