# GAS–LIQUID CHROMATOGRAPHY OF 17-KETOSTEROIDS AND PROGESTERONE METABOLITES OF URINE: COMPARISON OF DIFFERENT METHODS OF HYDROLYSIS

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## INTRODUCTION

Among the many methods used at present for the quantitative and semi-quantitative determination of urinary ketosteroids and progesterone metabolites, gas chromatography has lately become more and more important[1–21]. The main difficulties encountered with all procedures currently in use are the conditions of hydrolysis as has already been shown by several workers[1–9]. Therefore it is not surprising that results based upon different hydrolysis techniques often cannot be compared.

The conventional colorimetric procedures for the determination of urinary steroid compounds are neither sufficiently specific nor sensitive enough[23, 24] to allow a comparison between different methods of hydrolysis. Since the method of double-isotopes is very time-consuming, it was not considered for the present study. Because of its high sensitivity and specificity gas chromatography seemed to be more suitable for studies of different methods of hydrolysis. In addition, it allows detection of by-products and breakdown products.

In the work to be presented here, 10 different hydrolysis procedures have been compared using gas chromatographic analysis of up to 22 different urine specimens. Aspects of the extraction of steroids from the urine and of the purification of the extracts prior to gas chromatography were also studied. On the basis of the results obtained, suitable methods of hydrolysis are described and compared in detail.

## MATERIALS

### *Equipment*

Continuous extraction apparatus (Kutscher-Steudel); fractometer, model F-20 (Perkin-Elmer); gas chromatograph, model 1520 (Aerograph); syringe 10.0 $\mu$l capacity (Hamilton Co. Inc., U.S.A.); TLC equipment (Desaga, W. Germany).

### *Reagents*

All solvents were of reagent grade and were distilled before use.

Aluminum oxide, activity grade 1, neutral (Woelm); aluminum oxide for

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chromatography: 0.36 ml $H_2O$ and 6.0 g aluminum oxide, agitated vigorously and left 20 min; 4 g are taken for a chromatographic column.

Tetrahydrofuran (Merck, W. Germany and Fluka, Switzerland) was twice shaken with 40 % potassium hydroxide, tested for peroxides by potassium iodide, dried over sodium sulfate and distilled over sodium.

$\beta$-Glucuronidase (Sigma), type I, bacterial.

0.5 *M* phosphate buffer, pH 6.2.

Helicase (Industrie Biologique Française SA, Gennevilliers, France).

2 *M* acetate buffer, pH 5.2.

Silica Gel G (Merck, W.Germany).

Silylation mixture: 3 ml of hexamethyldisilazane + 0.1 ml of trimethylchlorosilane.

Dehydroepiandrosterone sulfate was purchased from Sigma, U.S.A.; androsterone glucuronide from Messrs. Ikapharm, Israel; some steroids were given to us by Prof. W. KLYNE, Chemistry Department, Westfield College, Hampstead, London, England. The remaining steroids were purchased from Messrs. Ikapharm, Israel and Merck, W.Germany.

*Standards*

*Internal standards.* (a) 10 mg pregnanolone dissolved in 100 ml of ethanol; (b) 10 mg testosterone dissolved in 100 ml of ethanol.

*Test mixture.* 10 mg each of allopregnanediol, pregnanediol, androsterone, etiocholanolone, dehydroepiandrosterone, pregnanolone, pregnanetriol, 11-ketoandrosterone, 11-ketoetiocholanolone, 11$\beta$-hydroxyandrosterone, 11$\beta$-hydroxyetiocholanolone and pregnanetriolone dissolved in 100 ml of ethanol.

## METHODS

*Hydrolysis and solvolysis techniques*

(1) *Hydrolysis with hydrochloric acid*

50 ml of urine and 40 ml of toluene are brought to boiling. After addition of 5 ml of concentrated hydrochloric acid, the solution is boiled for 15 min under reflux, cooled to room temperature and the water phase is carefully neutralized with 2 *N* sodium hydroxide.

(2) *Hydrolysis with $\beta$-glucuronidase*

50 ml of urine are adjusted to pH 6.2. After addition of 10 ml phosphate buffer and 5,000 U $\beta$-glucuronidase, the mixture is placed in an incubator for 24 h at 37°. Bacterial decomposition is avoided by the addition of three drops of chloroform.

(3) *Hydrolysis with $\beta$-glucuronidase and continuous ether extraction at pH 0.8*[21,25]

The same procedure as in (2) is applied to 50 ml of urine. After extraction with ether, the water phase is adjusted with sulfuric acid to pH 0.8 and filled into a continuous extraction apparatus. Into the round bottom boiling flask of the apparatus, 300 ml of ether are added and the water phase continually extracted at 40° for 72 h. The water–ether phase remaining in the apparatus is rinsed into a separatory funnel and re-extracted three times with 50 ml of ether. The ether extract is added to the

ether from the round bottom boiling flask of the continuous extraction apparatus and washed with 0.1 *N* sodium hydroxide and water as described under "extraction". After drying with sodium sulfate, the ether is evaporated.

((4)) *Hydrolysis with β-glucuronidase and hydrochloric acid hydrolysis of the water phase*

50 ml of urine are treated as in procedure (2). After extraction with ether, the water phase is handled as described in (1).

((5)) *Hydrolysis with helicase* (*β-glucuronidase/sulfatase from Helix Pomatia*[28])

50 ml of urine are adjusted to pH 5.2. 2.5 ml of acetate buffer and 50,000 U of helicase are added and the mixture is incubated for 24 h at 37°. Then, 25,000 U of helicase are added for another 24 h incubation. Bacterial decomposition is avoided by the addition of three drops of chloroform.

((6)) *Hydrolysis with β-glucuronidase/solvolysis with sulfuric acid in ethyl acetate*[31]

50 ml of urine are treated as in (2). After ether extraction, the water phase is adjusted to pH 4.0. After addition of 6 g ammonium sulfate, extraction is performed with three times 50 ml of ethyl acetate. The extract is dried with sodium sulfate, filtered, evaporated to dryness and transferred in acetone into a suitable vessel. To the dried urine extract 50 ml ethyl acetate and 0.4 ml of 2 *M* sulfuric acid are added successively. After a 16 h solvolysis at 39° the ethyl acetate is transferred into a separatory funnel and shaken out with 1 *N* sodium bicarbonate to alkalinity. The mixture is washed to neutrality with distilled water. The ethyl acetate is dried with sodium sulfate and evaporated in a rotary evaporator.

((7)) *Hydrolysis with β-glucuronidase and solvolysis with sulfuric acid in tetrahydrofuran*[26]

The procedur is the same as in (6) but solvolysis is performed with sulfuric acid in tetrahydrofuran ((0.4 ml of 2 *M* sulfuric acid in 50 ml peroxide free, freshly distilled tetrahydrofuran)) instead of ethyl acetate and the mixture is washed to neutrality with saturated sodium chloride solution.

((8)) *Solvolysis with perchloric acid in tetrahydrofuran*

((a)) *Low perchloric acid concentration*[3,27]. 50 ml of urine are saturated with ammonium sulfate ((approx. 30 g)) and extracted three times with 25 ml of tetrahydrofuran. The combined tetrahydrofuran extracts are dried with sodium sulfate and evaporated in a round bottom flask on a rotary evaporator. To the dried extract, a mixture of 15 ml tetrahydrofuran and 0.05 ml of 70 % perchloric acid are added for a 3 h solvolysis at 50°. After addition of 5 ml of 0.1 *N* sodium hydroxide the tetrahydrofuran is blown off with a stream of nitrogen. The alkaline water phase is extracted 3 times with 15 ml of ether. The combined ether extracts are washed to neutrality with distilled water, dried over sodium sulfate and evaporated.

((b)) *High perchloric acid concentration*. The procedure is the same as in (8a) but 0.1 ml of 70 % perchloric acid in 30 ml of tetrahydrofuran are used for solvolysis and 5 ml of 0.5 *N* sodium hydroxide are added for neutralization.

(9) *Solvolysis with perchloric acid in ethyl acetate*[30]

25 g of sodium sulfate are added to 50 ml of urine with the pH adjusted to 4.0. After three extractions with 50 ml of ethyl acetate, the combined ethyl acetate extracts are dried with sodium sulfate. 140 $\mu$l of 70 % perchloric acid (0.01 *M*) are added to the dried extract for a 3 h solvolysis at room temperature. Thereafter, the solution is extracted three times with 15 ml of 10 % potassium hydroxide, washed to neutrality with two 15 ml portions of water, dried over sodium sulfate, filtered and evaporated to dryness. The residue is left standing in 3 ml of 1 % potassium hydroxide in ethanol at room temperature for 1 h. 10 ml of water are added and extraction is performed twice with 15 ml of ether. The ether is washed to neutrality with 5 ml portions of water, dried over sodium sulfate, filtered and evaporated.

*Extraction with ether*

The hydrolysates of (2) and (5) and the aqueous phase of the continuous ether extraction (3) are shaken up three times with 50 ml of ether. The combined ether extracts are further extracted, twice with approx. 20 ml of 0.1 *N* sodium hydroxide and five times with 20 ml of water. The organic phase must be neutral. The ether is dried with sodium sulfate and evaporated *in vacuo*.

*Extraction with toluene*

The water phases of the HCl hydrolysis of (1) and (4) are extracted twice with 50 ml of toluene. The toluene extracts are combined with the 40 ml of toluene added before hydrolysis. The combined toluene extracts are shaken twice with approx. 20 ml of 0.1 *N* sodium hydroxide and five times with 20 ml of water. The organic phase must be neutral. The toluene is dried with sodium sulfate and evaporated *in vacuo*.

*Aluminum oxide chromatography*

The two extracts which have been obtained in methods (3), (4), (6) and (7) are combined; *e.g.* in technique (4) the ether extract after glucuronidase hydrolysis is combined with the toluene extract after HCl hydrolysis.

4 g of neutral aluminum oxide in benzene are filled into a column. The urine extracts (1 to 9) are dissolved in 5 ml of benzene and placed on the column. After washing twice with 4 ml benzene, chromatography was carried out with 25 ml benzene. The solvent is evaporated to dryness (fraction 1). Further chromatography is performed with 70 ml of benzene–ethanol (99.5:0.5) followed by evaporation (fraction 2), and then successively eluted with:

10 ml benzene–ethanol, 99:1
10 ml benzene–ethanol, 97:3
10 ml benzene–ethanol, 95:5
20 ml benzene–ethanol, 90:10
20 ml benzene–ethanol, 80:20

These eluates are combined and evaporated to dryness (fraction 3). To fraction 2, 2.5 ml of acetone are added and 1 ml portions are pipetted into two small test tubes, immediately closed and evaporated. One sample is then silylated and chromatographed. Depending on the result of this analysis, either 50 $\mu$g pregnanolone or 100 $\mu$g testosterone dissolved in 0.5 ml of ethanol are added as the internal standard to the

second test tube. To fraction 3 an internal standard of 50 $\mu$g pregnanolone in 0.5 ml of ethanol is added and evaporated to dryness. Fractions 2 and 3 are normally silylated separately.

*Silylation*

The dried samples are dissolved in 0.5 ml of silylation mixture, pipetted into a test tube with a ground glass stopper and placed in an incubator for 1 h at 60°. The silylation mixture is evaporated at approx. 40° under completely dry nitrogen (pure nitrogen conducted through several washing flasks filled with concentrated sulfuric acid). The residue is taken up in 0.1 ml of absolute hexane and chromatographed.

*Gas chromatography*

Gas chromatography was performed on a Perkin-Elmer (model F-20) fractometer equipped with flame ionization detector. For the preparative collection of samples an Aerograph (model 1520) gas chromatograph with a 90:10 split between column and flame ionization detector was used.

*Column conditions*

XE-60, 3 % on Gas-Chrom P, 80–100 mesh; 2 m glass tube, I.D. 2.7 mm; $T_c$ 225°, $T_i$ 280°; $N_2$, 50 ml/min.

QF-1, 3 % on Chromosorb W, 80–100 mesh; 2 m glass tube, I.D. 2.7 mm; $T_c$ 210°, $T_i$ 275°; $N_2$, 50 ml/min.

*Calculations*

General principles for the quantitation of gas chromatograms are given in ref. 20.

The use of calibration curves for evaluation turned out to be practical. Samples containing different amounts of steroids and a constant concentration of an internal standard are analyzed and the peak areas are calculated by multiplying peak height times peak width at half height. The factor "$F$" is calculated from the area of the unknown ($A_x$) and the area of the standard ($A_{std}$).

$$F = \frac{A_x}{A_{std}}$$

"$F$" values are plotted (ordinate) against the concentration of the sample (abscissa). The calibration curve thus obtained is used for the quantitation of the samples containing unknown amounts of steroids and a known amount of the standard. It has been obtained by subjecting the test substances to all of the analysis steps, thus eliminating errors due to losses.

*Standard method*

(*a*) *Hydrolysis*

50 ml of urine are adjusted to pH 6.2. 10 ml of phosphate buffer and 5000 U of $\beta$-glucuronidase are added and the mixture is placed in an incubator for 24 h at 37°. Bacterial decomposition can be avoided by the addition of three drops of chloroform.

(*b*) *Extraction with ether and hydrolysis of the water phase*

The hydrolysate is extracted three times with 50 ml of ether. The water phase is

further treated as in (3) with the aid of a continuous extraction apparatus. The ether phase is shaken twice with approx. 20 ml of 0.1 *N* sodium hydroxide and five times with 20 ml water. The organic phase must be neutral. The ether is dried using sodium sulfate and evaporated *in vacuo*.

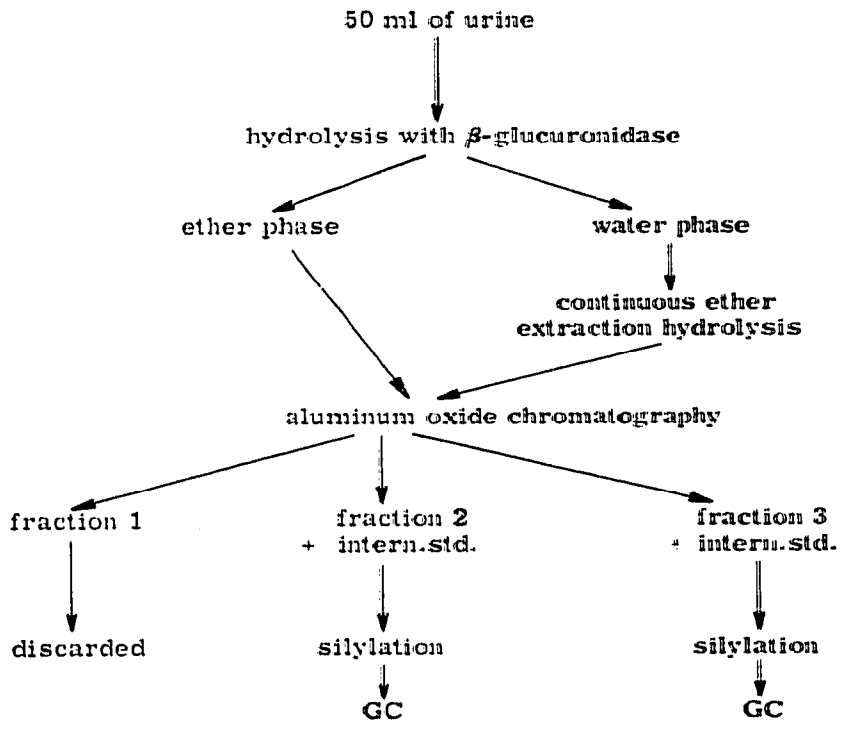
(*c*) *Aluminum oxide chromatography*

The ether extracts after both hydrolysis procedures are combined. The dried extracts are dissolved in 5 ml of benzene and added to an aluminum oxide column (4 g aluminum oxide). After two washes with 4 ml of benzene, chromatography was started with 25 ml of benzene and further handled as described under aluminum oxide chromatography. The three fractions obtained are evaporated. 50 $\mu$g pregnanolone in 0.5 ml of ethanol are added to fraction 3 as the internal standard and evaporated to dryness. To fraction 2 are added 2.5 ml of acetone; 1 ml samples are pipetted into 2 test tubes and evaporated. One of these samples is silylated and gas chromatography was carried out on XE-60. From this chromatogram we can distinguish whether pregnanolone and epiandrosterone in addition to dehydroepiandrosterone are present in the sample. If this is not the case, 50 $\mu$g pregnanolone in 0.5 ml of ethanol are added to the second test tube and evaporated to dryness. If disturbing amounts of pregnanolone or epiandrosterone are present in the sample, 100 $\mu$g testosterone in 0.5 ml of ethanol are added as internal standard and evaporated to dryness. If disturbing quantities of testosterone are found in the chromatogram the height of the original peak must be taken into account.

(*d*) *Silylation*

The dried samples containing an internal standard are dissolved in 0.5 ml of silylation mixture and further handled as described under silylation.

DIAGRAM



*(e) Gas chromatography*

If epiandrosterone and dehydroepiandrosterone are to be determined, gas chromatography is concluded with a QF-1 column; in all other cases a XE-60 column is used.

## RESULTS AND DISCUSSION

### *Extraction, purification, and gas chromatographic procedures*

*Extraction of steroids from urine*

Since estrogens could not be extracted with 2 *N* sodium carbonate, estriol and phenolic substances were removed by washing the ether extract with 0.1 *N* sodium hydroxide. We detected only small amounts of estrone and estradiol in the sodium hydroxide phase, even after using 2 *N* sodium hydroxide.

*Purification of extracts prior to gas chromatography*

The extract was purified by means of aluminum oxide column chromatography as already applied by ZACHMANN[21]. In this step, a great number of impurities were eliminated and only small losses were encountered (see Table I). Moreover, the sepa-

TABLE I

| | *Original amount* | *Recovery* | |
|---|---|---|---|
| | *µg* | *µg* | % |
| Allopregnanediol | 50 | 51.4 | 103 |
| Pregnanediol | 50 | 50.0 | 100 |
| Androsterone | 50 | 49.1 | 98 |
| Etiocholanolone | 50 | 47.6 | 95 |
| Dehydroepiandrosterone | 50 | 49.0 | 98 |
| Pregnanetriol | 50 | 48.6 | 97 |
| Pregnanetriolone | 50 | 49.0 | 98 |

TABLE II

RETENTION TIMES RELATIVE TO PREGNANOLONE (P) AND TESTOSTERONE (T)

| *No.* | *Steroids as TMSi ethers* | *XE-60 rel. to P* | *QF-1* | | *Aluminum column fractions* |
|---|---|---|---|---|---|
| | | | *Rel. to P* | *Rel. to T* | |
| 1 | Allopregnanediol | 0.463 | 0.534 | | 2 + 3 |
| 2 | Pregnanediol | 0.529 | 0.562 | | 3 |
| 3 | Androsterone | 0.612 | 0.685 | | 2 |
| 4 | Etiocholanolone | 0.730 | 0.754 | | 2 |
| 5 | Dehydroepiandrosterone | 0.850 | 0.850 | 0.473 | 2 |
| 13 | Epiandrosterone | | | 0.531 | 2 |
| 6 | Pregnanolone | 1.000 | 1.000 | 0.559 | 2 |
| 7 | Pregnanetriol | 1.165 | 1.220 | | 3 |
| 8 | 11-Ketoandrosterone | 1.420 | 1.435 | | 2 + 3 |
| 9 | 11-Ketoetiocholanolone | 1.655 | 1.451 | | 3 |
| 10 | 11β-Hydroxyandrosterone | 1.790 | 1.343 | | |
| 11 | 11β-Hydroxyetiocholanolone | 2.180 | 1.452 | | 3 |
| 12 | Pregnanetriolone | 2.715 | 2.740 | | 3 |
| 14 | Testosterone | | | 1.000 | 2 |

ration into fractions of different polarities during the adsorption step resulted in a more suitable distribution of compounds in the gas chromatograms.

Table I shows the recovery of seven steroid compounds after aluminum chromatography.

*Gas chromatography procedures*

The choice of suitable internal standards for gas chromatographic analysis is still under discussion. In our experience, pregnanolone could generally be used as an internal standard in routine procedures with satisfactory results; for the chromatography of dehydroepiandrosterone on a QF-1 column, testosterone was used. When testosterone and pregnanolone are analysed, an additional chromatogram without internal standard has to be performed. In Table II, the retention times of fourteen steroids under the described column conditions are listed.

Fig. 1 shows the gas chromatograms of the silyl ethers of twelve steroids on XE-60.

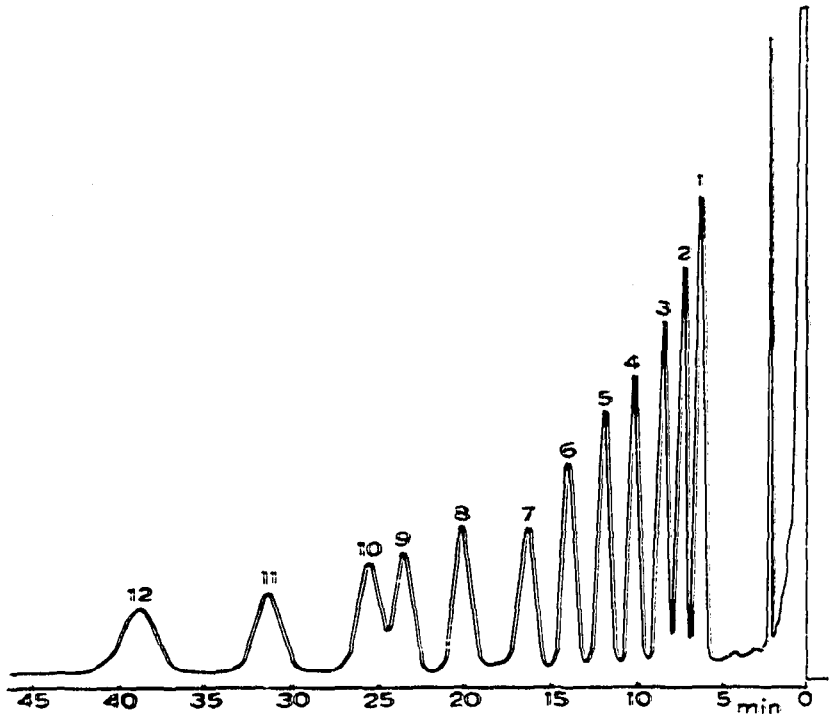



Fig. 1. Chromatogram of a standard mixture of twelve steroids (TMSi derivative). XE-60; Column conditions as described under Materials. The numbering of the fractions corresponds to the one in Table III and is uniform throughout this paper.

Fig. 2 shows the gas chromatograms of fractions 2 and 3 of a urine after aluminum oxide chromatography and silylation according to the standard method.

The 17-ketosteroids were found in fraction 2 (Fig. 2a), *i.e.* androsterone, dehydroepiandrosterone, etiocholanolone, as well as testosterone, pregnanolone and a part of 11-ketoandrosterone and allopregnanediol. In fraction 3 (Fig. 2b), the 11-keto- and 11-hydroxy-17-ketosteroids, the progesterone metabolites pregnanediol, pregnanetriol and pregnanetriolone and the remaining allopregnanediol and 11-ketoandrosterone were observed.

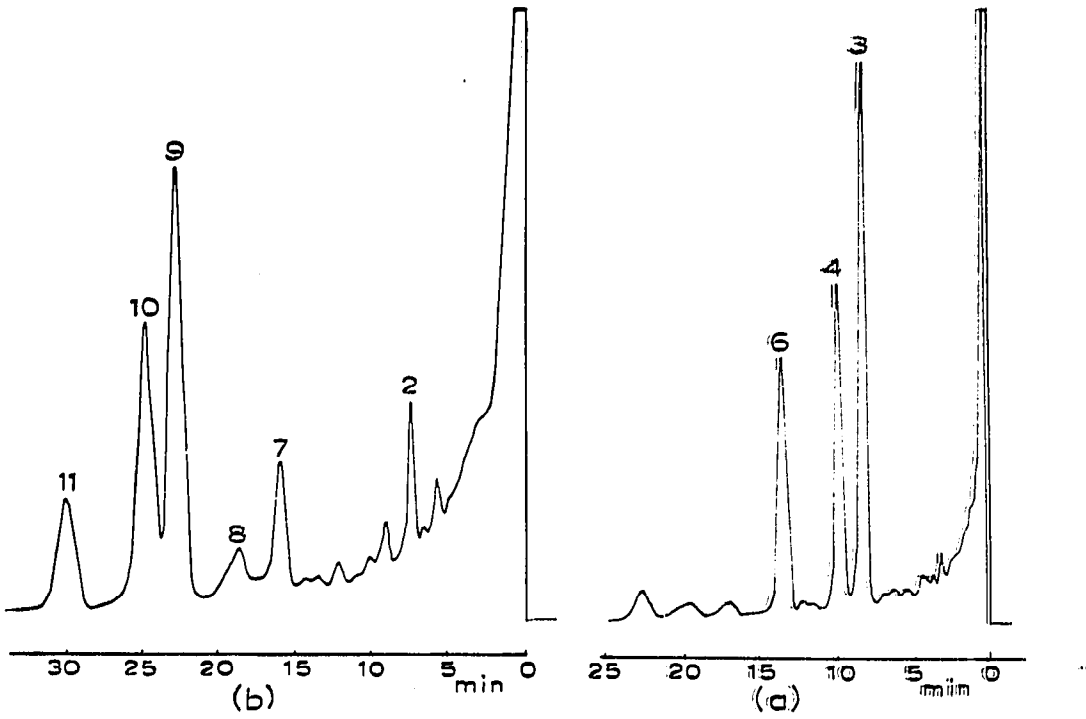



Fig. 2. Chromatograms of urinary steroids (TMSi derivative). XE-60.

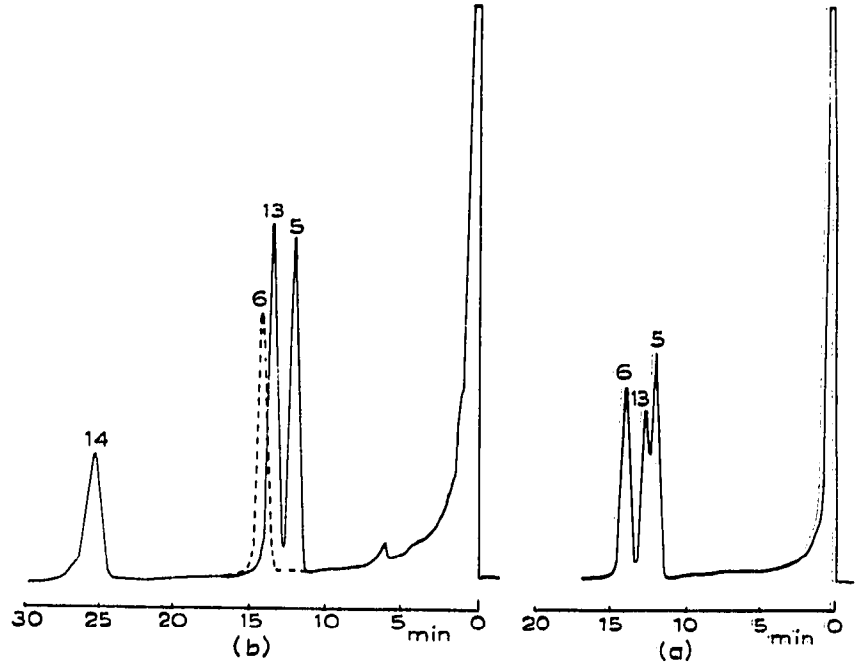



Fig. 3. Chromatograms of standard mixtures of dehydroepiandrosterone and epiandrosterone (TMSi derivative). (a) XE-60; pregnanolone was the internal standard; (b) QF-1; testosterone was the internal standard.

In Fig. 3 the gas chromatograms of epiandrosterone and dehydroepiandrosterone obtained with QF-1 and XE-60 columns are shown. With the use of the QF-1 column, dehydroepiandrosterone could be separated clearly from epiandrosterone. However,

in this system, epiandrosterone interfered with pregnanolone. Therefore, when QF-1 columns were used, testosterone replaced pregnanolone as the internal standard.

Table III gives the results of dehydroepiandrosterone estimations in the urines of ten healthy adults. The urines were treated according to the standard method given in the experimental part.

TABLE III

CONCENTRATION OF DEHYDROEPIANDROSTERONE IN THE URINES OF TEN HEALTHY ADULTS
The numbering of the fractions is uniform throughout this paper.

| *Fraction No.* | *Dehydroepiandrosterone* | |
|---|---|---|
| | *μg/100 ml* | *mg/24 h* |
| 1 | 20 | 0.194 |
| 2 | 105 | 1.09 |
| 3 | 222 | 2.38 |
| 4 | 208 | 2.08 |
| 5 | 4.25 | 0.045 |
| 6 | 1.93 | 0.017 |
| 7 | 20 | 0.326 |
| 8 | 27.1 | 0.257 |
| 9 | 19 | 0.296 |
| 10 | 271 | 2.60 |

*Results of hydrolysis methods*

In order to compare different hydrolysis methods, pure steroids and urine samples were first subjected to special hydrolysis conditions, followed by gas chromatography.

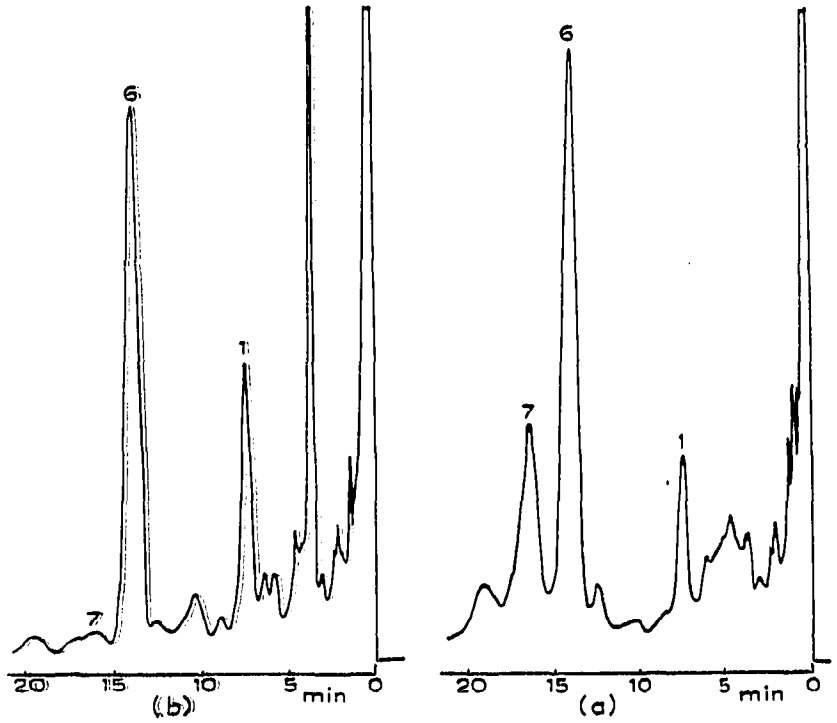



Fig. 4. Chromatograms of urinary steroids (TMSi derivative). XE-60. Decomposition of pregnanetriol occurred after HCl hydrolysis (4b) but not after β-glucuronidase (4a).

*Hydrochloric acid hydrolysis*

In Fig. 4, the chromatograms of pregnanetriol in urine after hydrochloric acid hydrolysis (1) and after β-glucuronidase hydrolysis (2) are shown.

Hydrochloric acid hydrolysis is a well-known method, often used according to the modification of VESTERGAARD AND CLAUSSEN[29]. Further alterations to this method have recently been examined by RUCHELMAN AND COLE[2].

In the present investigation, partial decomposition of pregnanetriol and pregnanetriolone was observed. Peaks caused by decomposition products will often interfere with the proper analysis of steroids. Therefore, this hydrolysis technique cannot be recommended for gas chromatographic analysis of steroids.

*β-Glucuronidase*

This method can be recommended for glucuronides, as no decomposition was observed.

*β-Glucuronidase/continuous ether extraction at pH 0.8*

A urine sample of one individual was examined four times for nine steroids according to the standard method; the results are recorded in the Table IV.

TABLE IV

RESULTS OF REPEATED ANALYSIS OF URINARY STEROIDS AFTER HYDROLYSIS WITH β-GLUCURONIDASE/CONTINUOUS ETHER EXTRACTION

The number of experiments does not allow a statistical comparison.

| | 1 (μg) | 2 (μg) | 3 (μg) | 4 (μg) |
|---|---|---|---|---|
| Pregnanediol | 152 | 172 | 180 | 180 |
| Androsterone | 448 | 540 | 476 | 480 |
| Etiocholanolone | 298 | 344 | 316 | 342 |
| Dehydroepiandrosterone | 168 | 178 | 171 | 195 |
| Pregnanetriol | 300 | 306 | 308 | 312 |
| 11-Ketoandrosterone | 124 | 138 | 136 | 145 |
| 11-Ketoetiocholanolone | 160 | 170 | 154 | 148 |
| 11-Hydroxyandrosterone | 148 | 136 | 140 | 134 |
| 11-Hydroxyetiocholanolone | 72 | 94 | 86 | 102 |

With this method, which is time-consuming, the recovery of most steroids yielded the highest rates. Decomposition products did not arise. For these two reasons it was chosen as the standard method.

Recovery experiments were made using dehydroepiandrosteronesulfate: The recovery amount of three experiments was 85 % ± 5 %. Other experiments to test the recovery of androsterone from androsterone glucuronide* gave about the same values.

*β-Glucuronidase/hydrochloric acid hydrolysis of the water phase*

Fig. 5 shows the gas chromatogram of a urine treated with β-glucuronidase and then subjected to hydrochloric acid hydrolysis of the water phase. The androsterone peak had a "shoulder".

* The androsterone glucuronide used in our experiments was not free of impurities.

The androsterone peak in aluminum oxide fraction 2 was collected with the help of a split in front of the flame ionization detector in a 15 cm long and 3 mm wide capillary tube, the silyl ethers were hydrolyzed and TLC of the steroid in ethyl acetate–toluene (4:1) was performed. The thin layer chromatogram revealed the existance of an unidentified compound in addition to androsterone.

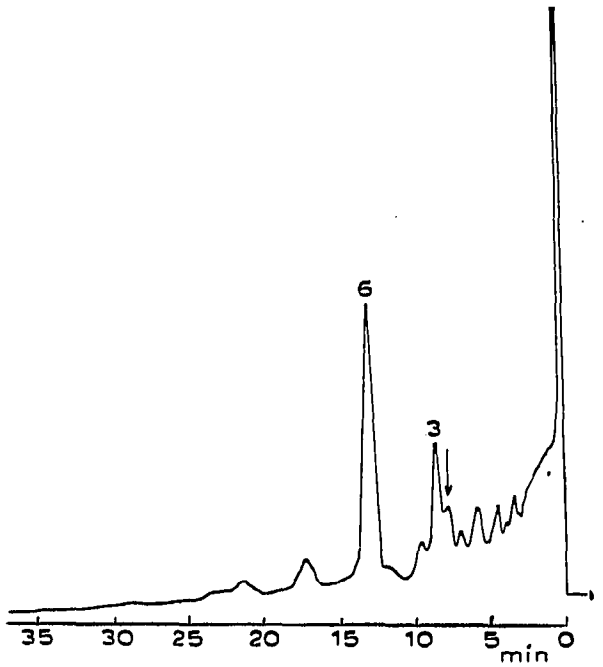



Fig. 5. Chromatogram of urinary steroids (TMSi derivative). XE-60. Arrow points to extra peak.

In all urines examined we have observed this peak which was overlapped by androsterone; it probably represented a decomposition product. Nevertheless, this method seems to be suitable for all other steroids under investigation.

*Hydrolysis with helicase (β-glucuronidase/sulfatase from Helix Pomatia)*

No decomposition products were observed. This method was suitable for all steroids but some losses occurred by emulsification. Other losses may be attributed to the specificity of sulfatase for the 5β-configuration of the steroid sulfates. In a recent paper, VANDENHEUVEL[4] proposed a ten-fold concentration of helicase in order to avoid losses. However, with the helicase used here, very poor separation of the layers was obtained due to emulsification.

*β-Glucuronidase/solvolysis with sulfuric acid in ethyl acetate*

Fig. 6 shows the gas chromatograms of the aluminum oxide fraction 2 of dehydroepiandrosterone after solvolysis of the sulfate with sulfuric acid–ethyl acetate and with sulfuric acid–tetrahydrofuran.

Solvolysis with sulfuric acid in ethyl acetate yielded only 57 %, and an unidentified peak appeared in fraction 3 after pregnanolone.

Since dehydroepiandrosterone sulfates were not completely hydrolyzed by this technique, it cannot be recommended for steroid analysis.

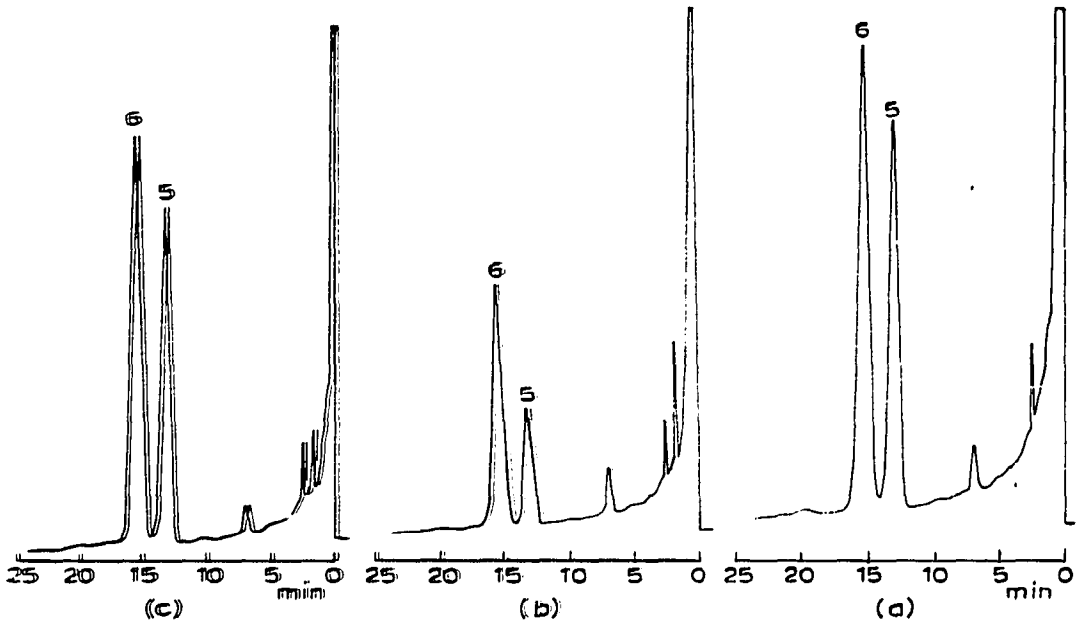



Fig. 6. Chromatograms after solvolysis of dehydroepiandrosterone sulfate (TMSi derivative). XE-60. (a) 62 $\mu$g dehydroepiandrosterone and 100 $\mu$g pregnanolone (100% value); (b) 100 $\mu$g dehydroepiandrosterone sulfate and 100 $\mu$g pregnanolone after solvolysis with sulfuric acid–ethyl acetate (aluminum oxide chromatography (fraction 2) and gas chromatography); (c) 100 $\mu$g dehydroepiandrosterone sulfate and 100 $\mu$g pregnanolone after solvolysis with sulfuric acid–tetrahydrofuran (aluminum oxide chromatography (fraction 2) and gas chromatography).

*β-Glucuronidase/solvolysis with sulfuric acid in tetrahydrofuran*

In contrast to the preceding procedure, the yield after solvolysis with sulfuric acid–tetrahydrofuran was almost 100% and no decomposition peak was found. However, when urine was solvolyzed with sulfuric acid–tetrahydrofuran, decomposition occurred (Fig. 7) yielding an extra peak on XE-60, with the retention time of

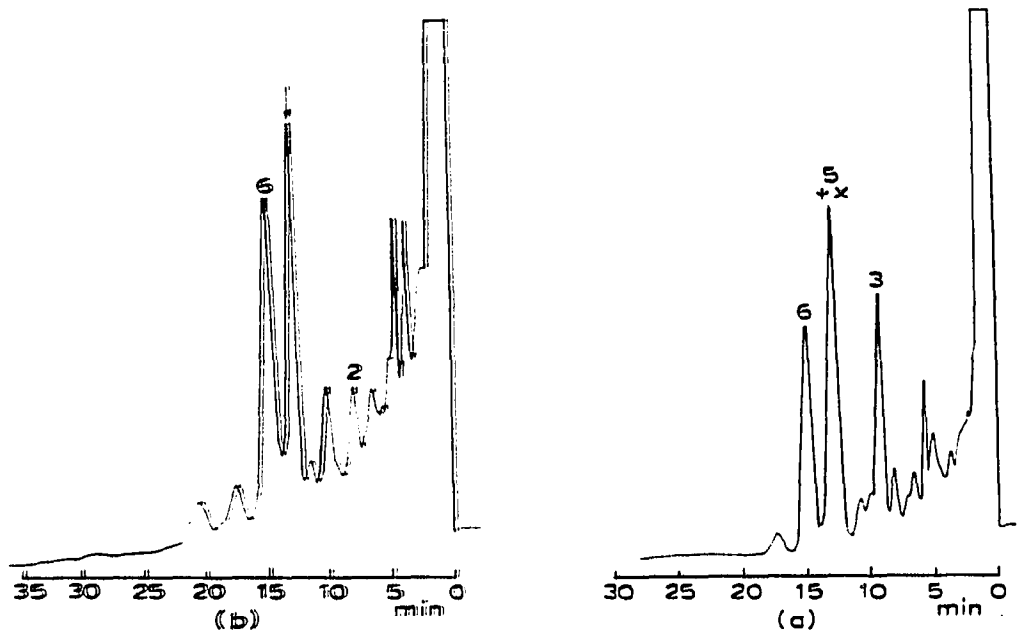



Fig. 7. Chromatograms of urinary steroids (TMSi derivative). XE-60. (a) Aluminum oxide fraction 2; (b) aluminum oxide fraction 3. Arrow points to extra peak.

dehydroepiandrosterone. Most of this unidentified substance was found in the aluminum oxide fraction 3 and a smaller portion in the aluminum oxide fraction 2, overlapping dehydroepiandrosterone.

The dehydroepiandrosterone peak in aluminum oxide fraction 2 was again collected, the silyl ethers were hydrolyzed and TLC of the steroid was performed. The thin layer chromatogram revealed the existance of an unidentified compound in addition to dehydroepiandrosterone.

This method was suitable for the hydrolysis of all other steroids.

*Solvolysis with perchloric acid in tetrahydrofuran*

Fig. 8 shows the gas chromatogram of one urine solvolyzed according to procedures (8a) and (8b). Solvolysis according to procedure (8a) as proposed by different

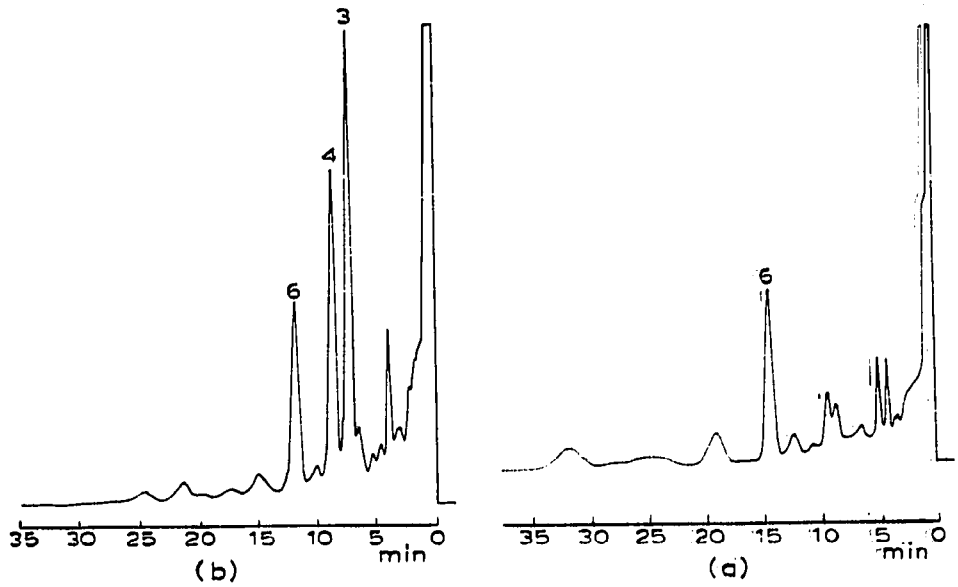



Fig. 8. Chromatogram of urinary steroids (TMSi derivative). XE-60. (a) Solvolysis method 8a; (b) solvolysis method 8b.

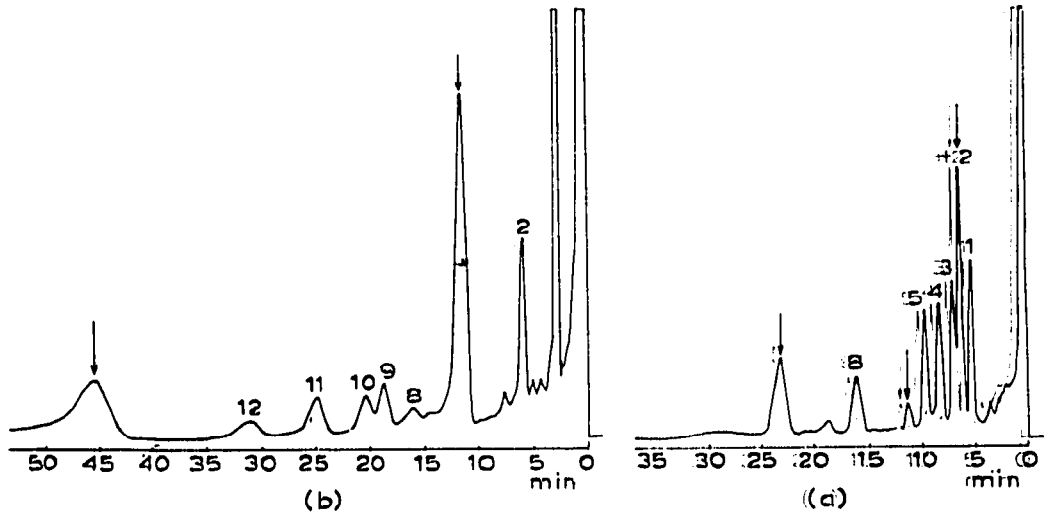



Fig. 9. Solvolysis of a standard mixture of twelve steroids with perchloric acid in tetrahydrofuran (TMSi derivative). (a) Aluminum oxide fraction 2; (b) aluminum oxide fraction 3. Arrows point to extra peaks.

authors[3,27] was not applicable to urine since the perchloric acid concentration was found to be too low and, as a consequence, recovery of steroids was poor.

This method, although quoted in the literature, cannot be recommended for steroid analysis by gas chromatography.

An increase in perchloric acid concentration (procedure 8b) gave a more satisfactory result; however, after this solvolysis additional peaks due to decomposition of tetrahydrofuran appeared (Fig. 9).

In the gas chromatogram of the aluminum oxide fraction 2, an additional peak, appeared between pregnanediol and androsterone; in the chromatogram of the aluminum oxide fraction 3, an additional peak was found between pregnanolone and pregnanetriol, impairing the separation of these two steroids.

It was found that the extra peaks were the result of breakdown products of tetrahydrofuran in the presence of perchloric acid. The steroids themselves were not decomposed under the conditons used here. Because of the occurrence of tetrahydrofuran breakdown products, this method was found to be unsuitable for the analysis of steroids.

*Solvolysis with perchloric acid in ethyl acetate*

Solvolysis with perchloric acid–ethyl acetate has been used by other workers[30]. For our purposes, the method was not suitable.

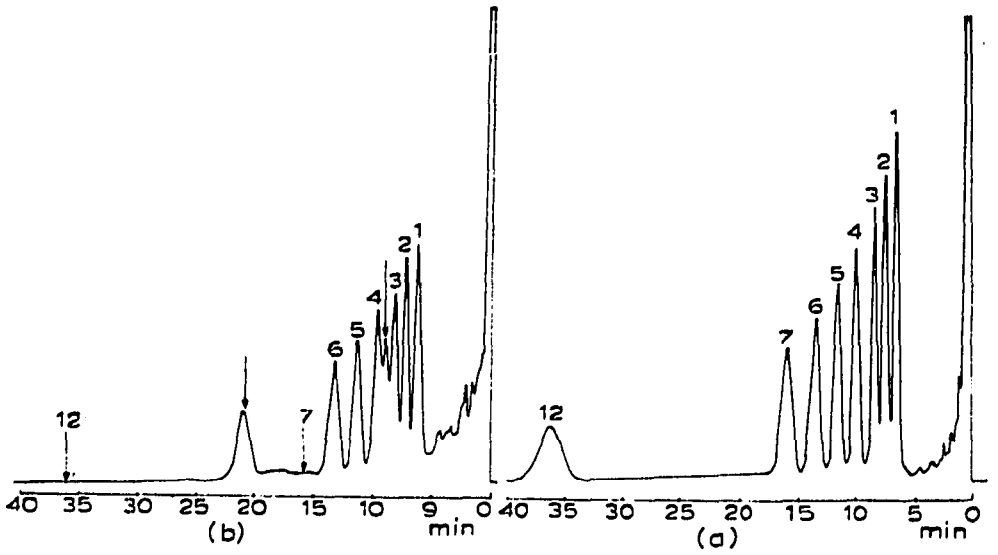



Fig. 10. Chromatograms of a mixture of eight standard steroids (TMSi derivative). (a) Before solvolysis; (b) after solvolysis. Arrows point to extra peaks and lost fraction.

As can be seen (Fig. 10), pregnanetriol and pregnanetriolone are decomposed and peaks caused by decomposition products appear after peak 7.

*Comparison of the different methods of hydrolysis*

Among the five suitable methods of hydrolysis, four were chosen for comparison in two urine specimens. For most steroids the yield was best using the standard method, followed by the helicase method, which gave also satisfactory results.

The sulfatase contained in the helicase preparation, however, is specific for hydrolysis of 5$\beta$-sulfates only. Androsterone sulfate is therefore not hydrolyzed and

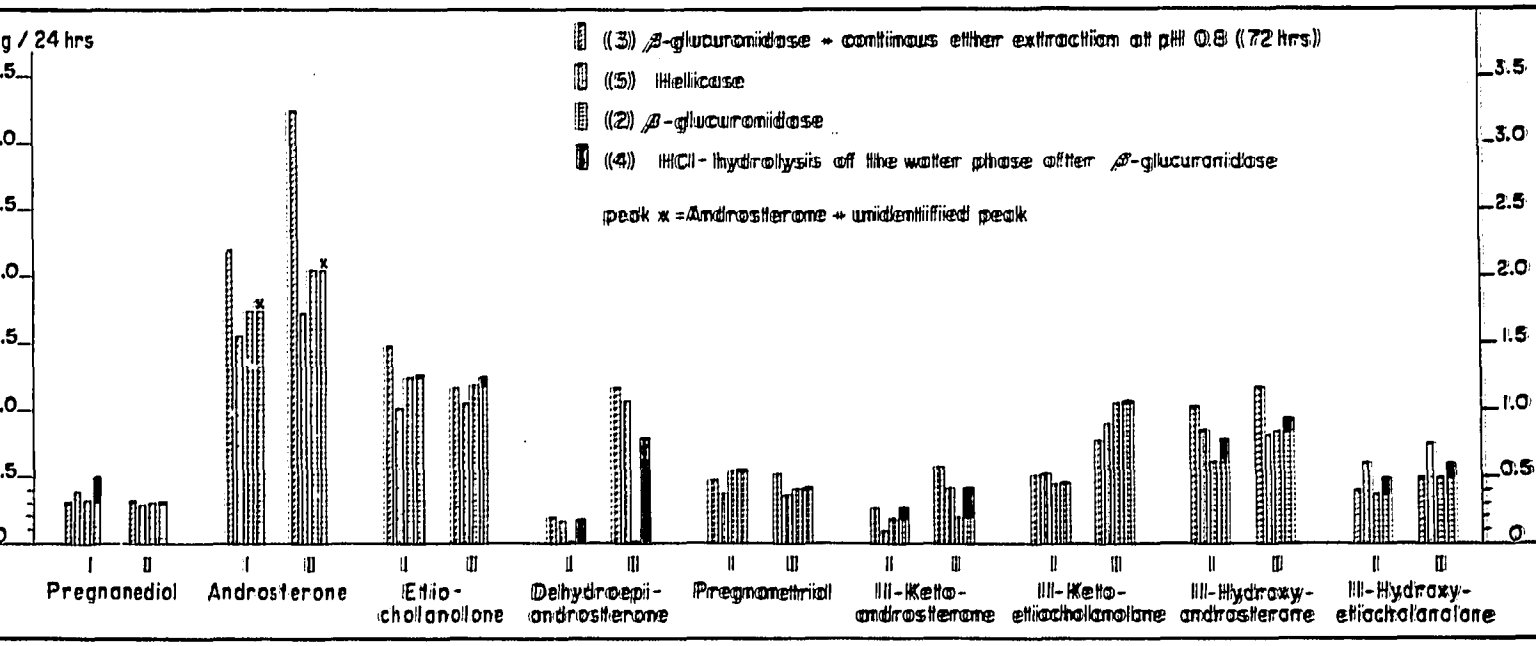



Fig. 11. Results of analysis of urinary steroids using four different methods of hydrolysis. Numbers in brackets identify the methods of hydrolysis. Samples I and II were 24 h urine portions of two healthy male adults.

TABLE V

SAME DATA AS REPRESENTED IN FIG. 11

| | *Method 3* | | *Method 5* | | *Method 2* | | *Method 4*** | |
|---|---|---|---|---|---|---|---|---|
| | *I* | *II* | *I* | *II* | *I* | *II* | *I* | *II* |
| Pregnanediol | 0.31 | 0.32 | 0.38 | 0.28 | 0.32 | 0.30 | 0.17 | — |
| Androsterone | 2.19 | 3.24 | 1.55 | 1.72 | 1.73 | 2.04 | * | * |
| Etiocholanolone | 1.47 | 1.16 | 1.01 | 1.04 | 1.24 | 1.18 | — | — |
| Dehydroepiandrosterone | 0.19 | 1.16 | 0.16 | 1.06 | — | — | 0.17 | 0.78 |
| Pregnanetriol | 0.47 | 0.52 | 0.37 | 0.36 | 0.53 | 0.40 | — | — |
| 11-Ketoandrosterone | 0.25 | 0.56 | 0.08 | 0.30 | 0.18 | 0.18 | 0.08 | 0.12 |
| 11-Ketoetiocholanolone | 0.50 | 0.76 | 0.51 | 0.89 | 0.43 | 1.04 | — | — |
| 11β-Hydroxyandrosterone | 1.01 | 1.16 | 0.74 | 0.80 | 0.60 | 0.83 | 0.17 | 0.10 |
| 11β-Hydroxyetiocholanolone | 0.39 | 0.48 | 0.59 | 0.73 | 0.36 | 0.48 | 0.12 | 0.10 |

* Results cannot be calculated as two substances are present.
** Under Method 4 only the values of the aqueous phase are listed. — Not detectable amount.

remains lipid-insoluble by this method. Consequently the yield of the helicase method compared to the standard method was smaller for androsterone, 11-ketoandrosterone and 11-hydroxyandrosterone. For the remaining steroids under investigation the results of the two methods were comparable.

Hydrolysis with β-glucuronidase gave good results for all steroids mainly present as glucuronides. Since dehydroepiandrosterone is excreted almost completely and androsterone in part as sulfate, this method is not suitable for the estimation of these two compounds (see Fig. 11).

HCl hydrolysis, following the application of β-glucuronidase gave satisfactory results for all steroids with the exception of androsterone. Because of an interfering

peak overlapping the peak of androsterone (see above) an evaluation of the sulfate fraction of this steroid was impossible. The black columns in Fig. 11 indicate the proportions of steroids not split by β-glucuronidase. These consist mainly of sulfates. As expected, the largest proportion of sulfate was found in dehydroepiandrosterone. 11-Ketoandrosterone, 11-hydroxyandrosterone and 11-hydroxyetiocholanolone also had measurable fractions not hydrolyzed by β-glucuronidase, which are possibly sulfates as well.

Among the solvolysis methods only the method using β-glucuronidase with sulfuric acid in tetrahydrofuran can be recommended and only if dehydroepiandrosterone is not to be determined.

## CONCLUSIONS

From our results, it may be concluded that for the gas–liquid chromatography of urinary 17-ketosteroids and progesterone metabolites the standard method (No. 3, β-glucuronidase/continuous ether extraction at pH 0.8) can be recommended as being superior to all other procedures tested.

Some other methods, *e.g.* helicase or glucuronidase, give satisfactory results. Method 4 as well as method 7 can also be applied but not for the analysis of androsterone or dehydroepiandrosterone, respectively. In our opinion, the solvolysis methods should not be used for the gas–liquid chromatographic estimation of urinary steroids; method 7 is the only exception.

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## SUMMARY

A method using gas–liquid chromatography for the routine determination of 17-ketosteroids and progesterone metabolites in human urine is described. The method was used to evaluate ten different hydrolysis and solvolysis procedures. Hydrolysis with β-glucuronidase combined with ether extraction of the water phase at pH 0.8 was found to be the most suitable technique. Hydrolysis with helicase was also acceptable.

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