allein durch die individuellen Unterschiede der Enzymaktivitäten geklärt werden, da z. B. im Falle der Benzoylcholinhydrolyse durch Vollserum (Abb. 7) der Fehler mit TRIS-Essigsäure $\pm 10\%$ beträgt, während er mit anderen Puffern ein Mehrfaches davon ausmacht. Ähnlich sind die Verhältnisse z. B. bei der endogenen Lipoproteinlipase (Abb. 3). Auch hier beträgt der Fehler beim TRIS-Essigsäurepuffer $\pm 10\%$; derselbe Wert wurde bei verminderter Absoluthöhe mit Veronalpuffer gefunden, während der Fehler mit Citronensäure-Phosphatpuffer 20% beträgt.

Damit ist erwiesen, daß sowohl die Absoluthöhe der Enzymaktivität als auch die Fehlerbreite der jeweiligen

Bestimmungsmethode von der chemischen Zusammensetzung des Puffers abhängt, sofern nicht mit Reinfärmenten gearbeitet wird. Es wäre daher wünschenswert, daß bei Beschreibungen von Enzymen und der Bekanntgabe von Methoden der enzymatischen Analyse, größeres Augenmerk auf die chemische Zusammensetzung der Puffer gelegt würde, als dies bisher der Fall war. Speziell für die klinische Chemie ist zu fordern, daß bei allen Methoden außer pH-Wert und Ionenstärke auch die genaue chemische Zusammensetzung des verwendeten Puffers sowie die Fehlergrenze der Methode bei den gewählten Bedingungen anzugeben ist.

Literatur

1. PILZ, W., Hoppe-Seyler's Z. physiol. Chem. 345, 80 (1966). —
2. PILZ, W. und I. JOHANN, Hoppe-Seyler's Z. physiol. Chem., im Druck. —
3. PILZ, W., H. HÖRLEIN und E. STELZL, Hoppe-Seyler's Z. physiol. Chem. 345, 65 (1966). —
4. PILZ, W., I. JOHANN und E. STELZL, Z. analyt. Chem. 215, 260 (1966). —
5. PILZ, W., diese Z. 3, 89 (1965). —
6. EUCKEN, A. und E. WICKE, Grundriß der physikalischen Chemie, S. 547, Verlag Geest u. Portig, Leipzig (1958). —
7. HÖRLEIN, H. und W. PILZ, Hoppe-Seyler's Z. physiol. Chem. 327, 256 (1962). —
8. PILZ, W. und I. JOHANN, Z. analyt. Chem. 218, 426 (1966). —
9. PILZ, W., Hoppe-Seyler's Z. physiol. Chem. 328, 1 (1962). —
10. PILZ, W., Hoppe-Seyler's Z. physiol. Chem. 328, 247 (1962). —
11. PILZ, W., Mikrochim. Acta (Wien) 1961, S. 614. —
12. PILZ, W. und I. JOHANN, Z. analyt. Chem. 212, 410 (1965). —
13. PILZ, W., Methoden der Enzymatischen Analyse, S. 765, Verlag Chemie GmbH, Weinheim/Bergstr. (1962). —
14. PILZ, W. und H. HÖRLEIN, Hoppe-Seyler's Z. physiol. Chem. 339, 157 (1964). —
15. PILZ, W., Zschr. exper. Med. 132, 310 (1959). —
16. PILZ, W. und I. JOHANN, Z. analyt. Chem. 210, 113 (1965). —
17. PILZ, W. und H. HÖRLEIN, Hoppe-Seyler's Z. physiol. Chem. 330, 212 (1963). —
18. PILZ, W. und I. JOHANN, Z. analyt. Chem. 215, 105 (1965).

Dr. W. Pilz
Physiol.-chem. u. analyt. Labor
der ärztl. Abteilung
559 Leverkusen-Bayerwerk

Fractionation and determination of 17-ketosteroids by means of column and thin layer chromatography

By L. E. BÖTTIGER and B. P. LISBOA

From King Gustaf Vth Research Institute, (Head: G. Birke, MD), Stockholm, Sweden

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A method is described for the fractionation of urinary 17-ketosteroids by use of gradient elution chromatography on aluminium oxide followed by one-dimensional multiple thin layer chromatography on silica gel G.

Androsterone, etiocholanolone, 11β -hydroxy-androsterone, 11-oxoetiocholanolone, 11β -hydroxyetiocholanolone, 11-oxo-androsterone and a fraction containing dehydroepiandrosterone together with its rearranged product, *i*-androstanolone, were determined by means of the ZIMMERMANN reaction. The individual steroids were characterized by means of one- and two-dimensional thin layer chromatography using single and multiple runs, and by several "in situ" developed colour reactions. The method has been developed for sulphuric acid hydrolysis followed by continuous ether extraction as well as for enzymatic hydrolysis; the results with the different hydrolysis procedures have been discussed. Results are presented for the fractionation of fifteen normal urines after sulphuric acid hydrolysis. The ratio between the amounts of etiocholanolone and androsterone obtained by this method has been compared with that found after additional oxidation of their $\Delta^9(11)$ dehydro-derivatives.

Es wird eine Methode zur Fraktionierung der 17-Ketosteroide des Harnes durch Gradienten-Elutions-Chromatographie an Aluminiumoxyd mit anschließender mehrfacher eindimensionaler Dünnschichtchromatographie auf Silikagel G beschrieben. Mittels ZIMMERMANN-Reaktion wurden folgende Substanzen bestimmt: Androsteron, Ätiocholanolon, 11β -Hydroxyandrosteron, 11-Oxyätiocholanolon, 11β -Hydroxyätiocholanolon, 11-Oxyandrosteron und eine Fraktion enthaltend Dehydroepiandrosteron zusammen mit seinem Umwandlungsprodukt *i*-Androstanolon. Die einzelnen Steroide wurden charakterisiert durch ein- und zweidimensionale Dünnschichtchromatographie — und zwar einfach und mehrfach — und durch einige „in situ“ entwickelte Farbreaktionen. — Die Methode wurde sowohl für schwefelsäure Hydrolyse mit kontinuierlicher Äther-Extraktion wie auch für enzymatische Hydrolyse entwickelt. Die Ergebnisse mit den verschiedenen Hydrolyse-Verfahren werden diskutiert. Es werden die Ergebnisse der Fraktionierung von 15 Normalharnen nach Schwefelsäure-Hydrolyse mitgeteilt. Das nach dieser Methode ermittelte Verhältnis Ätiocholanolon/Androsteron wird mit demjenigen verglichen, das nach zusätzlicher Oxydation ihrer $\Delta^9(11)$ -Dehydro-Derivate gefunden wird.

As the urinary 17-ketosteroids (17-KS) have different origins, a knowledge of the elimination of each metabolite and the ratio between some of them is necessary for a clinical investigation of the gonadal and adrenal functions. — The first fractionation procedure of DINGEMANSE et al. (1), may be followed by methods using different hydrolysis and separation procedures; some of these use complicated techniques, are time consuming and expensive, or give only a partial separation of the most important 17-ketosteroids. Thin-layer chromatography techniques provide an adequate separation of closely related 17-ketosteroids, with high reproducibility and recovery (2, 3, 4). The present work we investigate the possibility of combining column adsorption chromatography with gradient elution and a multiple one-dimensional thin-layer chromatography, in order to give complete separation of the principal six urinary ketosteroids, and thus make possible the study of the individual values and their ratios. As acid hydrolysis, in spite of some inconveniences, is the most widely employed hydrolysis procedure, sulphuric acid was used for the hydrolysis of the steroid conjugates. For comparison, studies with enzymatic preparations were also performed.

Methods

Collection of urine

24 hrs. urine specimens were obtained from 8 men and 8 women of various age, hospitalized for various benign conditions, all with normal liver function and without signs or symptoms of endocrine disorders. The urines were examined in a fresh condition without the use of preservatives, in 24 hour collections.

Hydrolysis

Most of the urines were submitted to acid hydrolysis using 1/10 (v/v) of 40% sulphuric acid, extracted with ether continuously for 16 hours, and purified as described by PLANTIN and BIRKE (5). In two cases, aliquots were submitted to enzymatic hydrolysis using a *Helix pomatia* preparation (L'Industrie Biologique Francaise S. A., Gennevilliers/Seine, France): 250 ml of urine were diluted to 500 ml with water, adjusted to pH 5.2 with acetate buffer (end of molarity: 0.02M) and submitted to hydrolysis with 8.000 units sulphatase (ROY) and 1.000 units β -glucuronidase (FISHMAN)/ml for 48 hours at +37°. The hydrolyzed steroids were extracted and purified as described above.

Ketone separation

After hydrolysis the extracts were treated with Girard's reagent T (6), and determination of total 17-KS was performed on an aliquot of the ketonic fraction.

Total 17-KS-determination

It was performed using the ZIMMERMANN reaction with stabilized alcoholic potassium hydroxide, according to HAMBURGER (7). The corrections of ALLEN (8) and of GIBSON and EVELYN (9) were employed on total determinations, but the corrected values differ only slightly from uncorrected values after column chromatography, indicating that the eluted fractions are pure.

Column chromatography

It has been performed with a linear gradient on aluminium oxide (alumina) with a column according to ZYGUNTOWICZ et al. (10); for particulars, see PLANTIN and BIRKE (5). The gradient concentration was obtained by using four identical cylinders situated on the same level, connected — just above the bottoms — by semi-capillary tubes with valves (Fig. 1). The first cylinder (a) contains

Fig. 1

Apparatus utilized for gradient elution chromatography

The bottles contain the same volume of solvent: benzene (b) and (d); 0.2% methanol in benzene (c) and 2% methanol in benzene (a)

2% and the third 0.2% methanol in benzene, the second and the fourth only benzene. For the theoretical basis of the linear gradient, see LEBRETON (11).

The eluates were collected with a 50 tube fraction collector, 5 ml in each tube. Total 17-KS determination was performed on an aliquot (2/5) from each tube. The rest of the samples were pooled in four fractions, consisting generally of the contents of the following tubes:

- I: tubes 1—15
- II: tubes 16—28
- III: tubes 29—41
- IV: tubes 42—50.

In each case, the exact number of tubes belonging to each fraction was decided from the position of the peaks in the curve obtained with the column chromatography. The number of tubes in each fraction may vary slightly from one sample to another, depending upon variations in the alumina activity.

Thin layer chromatography (TLC)

It has been used both for the characterisation of the steroids found in the different peaks and for the further separation of fractions III and IV. In this study, one-dimensional and two-dimensional TLC on silica gel G was performed according to the experimental conditions described by LISBOA (12, 13, 14). For complete separation of the steroids in fractions III and IV multiple developments were also employed.

The following solvent systems were used:

Cyclohexane/ethylacetate	50 : 50 (System C)
Chloroform/ethanol	95 : 5 (System L)
Benzene/ethanol	49 : 1 (System P)
n-hexane/ethylacetate	75 : 25 (System O)
Cyclohexane/ethylacetate	40 : 60

For quantitative determinations, aliquots of fractions III and IV were carefully put on the plates in a row, 2.5 cm above the lower edge, using a Pasteur pipette and without damaging the silica gel layer (Fig. 2). On the both sides of the test extracts, standard mixtures of the steroids to be analysed were added. After chromatography the central area containing the urinary extracts was protected by a glass plate, and the standards on both sides of the chromatoplate were developed by means of anisaldehyde/sulphuric acid reagent (15). Thereafter, the silica gel areas situated between the standards (shaded areas in the fig. 2) were carefully scraped from the plates and transferred to special columns (diameter 10 mm, length 5 cm) with coarse sintered glass disks in the bottom, similar to those used for pregnandiol determinations (16). The preparation of the column was done in the following way: one layer of washed quartz, one layer of silica gel, the silica gel from the chromatoplate and finally quartz again. The steroids were eluted with 3 x 5 ml of ethanol. The eluates, after evaporation, were submitted directly to the ZIMMERMANN reaction.

Separation of $\Delta^9(11)$ -dehydroandrosterone and $\Delta^9(11)$ -dehydroetiobolanolone

Aliquots of the samples containing a mixture of the four steroids (fraction III) were submitted to treatment with osmium oxide and sodium permanganate, respectively; in both cases $\Delta^9(11)$ -compounds give dihydroxy-derivatives, which easily may be separated from the corresponding saturated compounds by means of TLC.

Fig. 2

One-dimensional multiple chromatography of aliquots of eluent-fraction III on silica gel G (3 developments), using solvent system C (ethylacetate/cyclohexane 50:50)

The eluate of the urinary extract is deposited on the central starting line (b), a mixture of androsterone and etiocholanolone standards (1:2) is deposited on each side at the same starting line (a). After development of the plate, the areas containing the standards are stained (the central area with the test sample is covered during the staining procedure) by using the anisaldehyde/sulphuric acid reaction

Treatment with osmium-VI-oxide (17)

Two-three drops of pyridine and 3 ml of a 0.5% ether solution of osmium-VI-oxide was added to an aliquot of the steroid sample, dissolved in 2 ml of ether. The tube is then sealed in a N_2 -atmosphere and left at room temperature for 7 days. The mixture is then saturated with hydrogen sulphide and filtered over glass filter. Each sample is deposited on the starting line of a chromatoplate, which is submitted to a three times repeated one-dimensional TLC, using solvent system C. The regions containing androsterone and etiocholanolone are eluted as described above and the ratio between them was determined after the ZIMMERMANN reaction had been performed.

Treatment with sodium permanganate (18)

The hydroxylation with permanganate is carried out by a modification to micro scale of the method employed by SAVARY and DESNUELLE (18) for the hydroxylation of unsaturated fatty acids. An amount of steroids (50–100 micrograms), dissolved in cold acetone is treated by a few drops of a 0.15% sodium permanganate solution in aqueous 0.1% potassium hydroxide solution. The mixture is shaken and after 5 minutes a few drops of a saturated aqueous sodium bisulphite solution is added until the permanganate colour disappears. The solution is then extracted three times with ethylacetate; the three organic extracts are pooled, evaporated and submitted to TLC for the separation of androsterone and etiocholanolone as described above. — In order to investigate the possibilities of adapting this method for quantitative determinations, aliquots of standards containing 50 micrograms of androsterone and etiocholanolone, respectively, were submitted to the same procedure for the calculation of the recovery.

Reliability of the method

The reliability of the method employed has been tested with regard to specificity, recovery and reproducibility.

Specificity: To investigate the specificity of the gradient elution chromatography under the conditions employed in this study, experiments have been carried out with the following standards: androstane-3,17-dione, etiocholane-3,17-dione, dehydroepiandrosterone, iso-androsterone, iso-etiocholanolone, androsterone, etiocholanolone, 11 β -hydroxyandrosterone, 11 β -hydroxyetiocholanolone and 11-oxo-etiocholanolone. Furthermore, the eluates of the four fractions obtained from pooled normal urines after gradient elution chromatography were further submitted to fractionation using TLC. The different steroids were characterized by their chromatographic mobilities on silica-gel layers and by colour reactions, such as the anisaldehyde/sulphuric acid- and the ZIMMERMANN reactions, developed "in situ" (2, 15).

Recovery: The recovery of the method depends upon those of both chromatographic techniques. The recovery of 17-KS after gradient column chromatography has been studied previously for free steroids (19, 20) and is higher than 95%. Using TLC the recovery varies from 86 to 94 (mean 89.2) % for the different 17-KS investigated here. The calculation of the absolute quantities of the steroids of the eluted fractions III and IV according to their relative values in the corresponding fractions makes it unnecessary to correct for losses during the thin-layer chromatography procedure. — The recovery has also been studied after treatment of the steroids of fraction III with sodium permanganate under alkaline conditions. The results obtained with 5 aliquots containing androsterone and etiocholanolone (50 microgram each) give a recovery of 87.8 (range 80.9–96.5) %, measured as dehydroepiandrosterone.

Reproducibility: As for the recovery, the reproducibility depends on those of the two chromatographic procedures used. The coefficient of variation of the results obtained after gradient elution chromatography of 17-KS has been shown to be in the order of 6% (5,20). The reproducibility of TLC depends upon sufficient resolution of steroids with close polarity and of the reproducibility of their R_F -values; both are possible to obtain under the standardized conditions which have been used. The values used for the calculation of the ratio E/A were averages of two determinations for both androsterone (A) and etiocholanolone (E), in order to avoid errors resulting from elution procedures.

Results

Qualitative analysis of the four fractions

Experiments carried out with known standards, investigation of the four eluate fractions by means of thin-layer chromatography, and colour reactions developed "in situ" have shown the following composition of the eluates.

Fraction I: Using solvent systems C, O and P it was possible to detect androstan-17-one, androstane-3,17-dione and etiocholane-3,17-dione. However, most of the material in this fraction remained unidentified and probably consists of artefacts of hydrolysis. Gradient elution chromatography of androstane-3,17-dione and etiocholane-3,17-dione standards show that these two steroids are completely eluted in this fraction.

Fraction II: All the dehydroepiandrosterone, iso-androsterone and iso-etiocholanolone submitted to gradient elution chromatography have been recovered in this fraction. TLC of this fraction, obtained from pooled extracts from normal urines, has shown that a great part of the ZIMMERMANN chromogens contained in this eluate was no longer dehydroepiandrosterone but a less polar steroid. This steroid was also obtained when dehydroepiandrosterone standard was submitted to the same hydrolysis procedure. This suggests that this steroid was *i*-androstan-6-ol-17-one, resulting from rearrangement of dehydroepiandrosterone. This eluate fraction includes all the so-called β -ketosteroids-dehydroepiandrosterone, iso-androsterone and iso-etiocholanolone. As the amount of the two latter compounds is very small, most of the ZIMMERMANN chromogen of this fraction consists of dehydroepiandrosterone (or its rearranged *i*-androstanolone).

Fraction III: Gradient column chromatography of androsterone and etiocholanolone together with results from

TLC have shown that this fraction includes these two 17-KS. As 11-hydroxylated steroids to some extent have been transformed during hydrolysis into $\Delta^9(11)$ -dehydro-androsterone and $\Delta^9(11)$ -dehydro-etiocholanolone, which could not be separated from the corresponding saturated steroids, the values for androsterone and etiocholanolone are probably slightly high.

Fraction IV: The four 11-oxygenated derivatives of androsterone and etiocholanolone were recovered after gradient column chromatography. Qualitative studies of pooled eluates of this fraction have permitted the detection — by means of multiple two dimensional thin layer chromatography (Fig. 3) — of all these four steroids.

Fractionation of 17-KS from normal urines

Table 1 summarizes the results obtained for the four eluate fractions after gradient elution chromatography of 15 normal urines (7 men and 8 women), in mg/24 hrs. and in % of total 17-KS. In the table 1 are also given the results obtained by further separation of the steroids of fraction III by means of one-dimensional multiple thin-layer chromatography on silica gel, together with the ratios etiocholanolone/androsterone and 11-deoxy-/11-oxygenated steroids. Of the four fractions obtained after column chromatography the fraction containing the 11-oxygenated steroids (fraction IV) was submitted to one-dimensional, multiple chromatography using the system ethylacetate/cyclohexane (60:40); the results are given in table 2. — In two cases, equal aliquots of the urine were submitted to acid and enzymatic hydrolysis respectively. The results obtained for the different fractions with the two hydrolysis procedures have been summarized in table 3. — In 4 cases, the ratio etiocholanolone: androsterone was determined after treatment of equal aliquots of fraction III with osmium oxide and

Fig. 3

Characterization of the steroids of fraction IV by means of a combination of two-dimensional and multiple thin-layer chromatography on silica gel G

First, the eluate of fraction IV obtained from the gradient-elution chromatography, is deposited to the left (a) on the starting line of the plate and a mixture of the four standards to the right (b). The chromatoplate is submitted to 3 runs, using solvent system C (ethylacetate cyclohexane 50:50). Thereafter mixtures of standards are deposited on a second starting line perpendicular to the first (a—b) line: 11 β -hydroxyetiocholanolone and 11-ketoandrosterone in point (c) and 11 β -hydroxyandrosterone and 11-keto-etiocholanolone in point (d). The chromatoplate is submitted to a fourth run, now in a second direction and with another solvent system, L, (chloroform/methanol, 19:1). The chromatoplate is stained with the anisaldehyde/sulphuric acid reaction

Steroid standards (Arabic numbers) and separated urinary steroids (Roman numbers) are shown as follows:

- 11 β -hydroxyandrosterone (1.II)
- 11 β -hydroxyetiocholanolone (2.II)
- 11-ketoandrosterone (3.III)
- 11-keto-etiocholanolone (4.IV)

sodium permanganate in alkaline solution, respectively. The results obtained with these two treatments and those obtained with the general methods described here, are compared in table 4.

Tab. 1

Total 17-KS-excretion in normal individuals and the results of column and thin layer chromatographic separation. Individual values, mean values $\pm$ SD range (A $\pm$ SD) are given

Men Age	Total 17 KS/ mg/24 hrs.	I		II		III		IV		Andro- sterone		Etiocho- lanolone		E/A	11-deoxy 11-oxy
		mg	%	mg	%	mg	%	mg	%	%	mg	%	mg		
23	6.10	1.672	27.4	0.598	9.8	3.404	55.8	0.427	7.0	34.7	2.114	21.1	1.290	0.61	9.37
29	8.60	0.645	7.5	0.576	6.7	6.846	79.6	0.533	6.2	33.6	2.821	43.0	4.025	1.43	13.92
37	6.50	0.364	5.6	0.475	7.3	4.615	71.0	1.047	16.1	34.0	2.210	37.0	2.405	1.09	4.86
37	5.90	0.496	8.4	0.507	8.6	4.095	69.4	0.808	13.7	43.4	2.561	26.0	1.534	0.60	5.70
42	6.10	0.409	6.7	1.305	21.4	3.794	62.2	0.592	9.7	19.4	1.183	42.7	2.605	2.20	8.61
67	3.00	0.270	9.0	0.252	8.4	2.208	73.6	0.270	9.0	30.4	0.913	43.2	1.295	1.42	8.11
79	4.80	0.470	9.8	0.494	10.3	3.029	63.1	0.806	16.8	25.3	1.214	37.8	1.814	1.49	4.37
A $\pm$ SD	5.80 $\pm$ 0.16	0.573 $\pm$ 0.461		0.674 $\pm$ 0.368		3.872 $\pm$ 0.141		0.707 $\pm$ 0.308		1.812 $\pm$ 0.707		2.059 $\pm$ 0.934		1.23 $\pm$ 0.53	7.44 $\pm$ 3.46
S _x	0.06	0.163		0.130		0.050		0.109		0.251		0.331		0.19	1.23
Range	3.00—8.60	0.26—1.67		0.25—1.31		2.21—6.85		0.27—1.17		0.91—2.82		1.29—4.03		0.60—1.49	3.56—13.92
Women															
Age															
15	1.80	0.140	7.8	0.137	7.6	1.303	72.4	0.220	12.2	28.7	0.517	43.7	0.786	1.52	6.55
23	8.80	0.774	8.8	2.534	28.8	4.891	49.9	1.100	12.5	26.2	2.305	23.7	2.086	0.91	6.75
23	8.50	1.284	15.1	0.782	9.2	5.134	60.4	1.292	15.2	27.6	2.346	32.8	2.788	1.19	4.58
26	3.70	0.388	10.5	0.185	5.0	2.597	70.2	0.529	14.3	33.1	1.225	37.1	1.373	1.12	5.26
40	5.10	0.507	9.9	0.298	5.9	3.864	75.8	0.431	8.4	42.7	2.179	33.1	1.686	0.77	9.66
52	3.00	0.225	7.5	0.369	12.3	2.022	67.4	0.384	12.8	31.0	0.930	36.4	1.092	1.17	6.23
60	9.10	0.427	4.7	1.156	12.7	5.615	61.7	1.902	20.9	20.9	1.898	40.5	3.716	1.94	3.56
61	2.60	0.179	6.9	0.309	11.9	1.518	58.4	0.590	22.7	25.5	0.663	32.9	0.855	1.29	3.10
A $\pm$ SD	5.30 $\pm$ 0.32	0.491 $\pm$ 0.381		0.721 $\pm$ 0.773		3.368 $\pm$ 1.725		0.806 $\pm$ 0.574		1.508 $\pm$ 0.761		1.798 $\pm$ 1.024		1.24 $\pm$ 0.36	5.71 $\pm$ 2.09
S _x	0.11	0.135		0.274		0.612		0.204		0.270		0.363		0.13	0.74
Range	1.89—9.10	0.14—1.28		0.14—2.53		1.30—5.62		0.22—1.90		0.52—2.35		0.79—3.72		0.77—1.94	3.10—9.66

Tab. 2

Results of thin-layer chromatographic separation of fraction IV, obtained after column chromatography. Individual values, mean values $\pm$ SD ($A \pm SD$), SEM and range are given

Men Age	11-oxygenated 17-KS mg/24 hrs.	11 β -hydroxy-androsterone per cent		mg/24 hrs.	11 β -hydroxy-etiocholanolone and 11-oxo-androsterone per cent		mg/24 hrs.	11-oxo-etiocholanolone per cent		mg/24 hrs.
		of 11-oxy- genated 17-KS	of total 17-KS		of 11-oxy- genated 17-KS	of total 17-KS		of 11-oxy- genated 17-KS	of total 17-KS	
23	0.43	26.1	1.8	0.110	45.9	3.2	0.195	27.9	2.0	0.122
29	0.15	30.3	1.9	0.161	27.7	1.7	0.147	41.9	2.3	0.222
37	0.81	37.0	5.0	0.295	27.1	3.7	0.218	36.9	5.0	0.295
37	1.05	31.6	5.1	0.332	46.6	7.5	0.439	21.8	3.5	0.229
42	0.59	4.8	0.5	0.030	36.2	3.5	0.214	59.0	5.7	0.348
48	1.17	18.0	3.8	0.210	48.5	10.1	0.567	33.5	7.0	0.391
67	0.27	37.4	3.4	0.112	18.1	1.6	0.054	44.5	4.0	0.314
A $\pm$ SD	0.69 $\pm$ 0.33			0.179 $\pm$ 0.108			0.269 $\pm$ 0.187			0.249 $\pm$ 0.102
S _x	0.12			0.041			0.071			0.039
Range	0.27—1.17			0.03—0.35			0.05—0.57			0.12—0.39
Women										
Age										
15	0.22	13.1	1.6	0.030	26.1	3.2	0.057	60.8	7.2	0.134
23	1.10	10.0	1.2	0.110	33.0	4.2	0.363	57.0	7.1	0.627
23	1.29	12.6	1.9	0.163	28.9	4.5	0.373	58.5	8.8	0.756
26	0.53	18.6	2.7	0.079	26.7	3.8	0.141	54.7	7.8	0.287
40	0.43	36.5	3.5	0.157	15.8	1.9	0.068	47.6	4.0	0.205
52	0.38	18.9	2.4	0.072	39.0	5.0	0.150	42.1	5.4	0.162
60	1.90	11.3	2.4	0.218	51.8	10.8	0.983	36.9	7.7	0.701
61	0.59	10.6	2.4	0.062	47.2	10.7	0.278	42.2	9.6	0.250
A $\pm$ SD	0.81 $\pm$ 0.57			0.111 $\pm$ 0.063			0.302 $\pm$ 0.302			0.390 $\pm$ 0.259
S _x	0.20			0.022			0.107			0.092
Range	0.22—1.90			0.03—0.22			0.06—0.98			0.13—0.76

Tab. 3

Comparison of the values obtained for the fractionation of 17-KS and the ratio E/A after acid and enzymatic hydrolysis respectively, of urines from two normal men

Total 17-KS (mg)	Urine I (37 yrs.)				Urine II (29 yrs.)			
	Acid hydrolysis		Enzymatic hydrolysis		Acid hydrolysis		Enzymatic hydrolysis	
	%	mg/24 hrs.	%	mg/24 hrs.	%	mg/24 hrs.	%	mg/24 hrs.
Fractions I	5.6	0.364	1.8	0.142	7.5	0.645	3.1	0.357
Fractions II	7.3	0.475	5.0	0.392	6.7	0.576	6.6	0.759
Fractions III	71.0	4.615	66.1	5.222	79.6	6.846	80.8	9.292
Fractions IV	16.0	1.040	27.1	2.140	6.2	0.533	9.5	1.093
	Per cent of Fract. III	Total KS	Per cent of Fract. III	Total KS	Per cent of Fract. III	Total KS	Per cent of Fract. III	Total KS
	mg/24 hrs.	mg/24 hrs.	mg/24 hrs.	mg/24 hrs.	mg/24 hrs.	mg/24 hrs.	mg/24 hrs.	mg/24 hrs.
Androsterone	47.8	34.0	53.0	35.1	41.2	32.8	46.0	37.2
Etiocholanolone	52.2	37.0	47.0	31.1	58.8	46.8	54.0	43.6
Ratio E/A		1.09		0.89		1.43		1.17
11-hydroxy-androsterone	31.6	5.1	30.8	8.3	30.3	1.9	44.5	4.2
11-hydroxy-etiocholanolone and 11-oxo-androsterone	46.6	7.5	15.5	4.2	27.7	1.7	12.0	1.1
11-oxo-etiocholanolone	21.8	3.5	53.7	14.5	41.9	2.3	43.4	4.1

Tab. 4

Ratio Etiocholanolone/androsterone obtained after
(a) treatment with osmium-VI-tetraoxide
(b) sodium permanganate/potassium hydroxide-treatment
(c) TLC of fraction III without further treatment

Age	Ratio Etiocholanolone/Androsterone		
	(a)	(b)	(c)
23	1.12	1.17	0.91
23	0.85	0.95	1.19
23	1.01	0.65	0.61
40	1.40	0.90	0.77
	(1.09)	(0.92)	(0.87)

Discussion

Hydrolysis

The optimal method for the study of the urinary 17-KS is the separation of the different conjugates of each steroid and their determination. However, such a method involves a series of separation procedures which makes it applicable only for studies of isolated cases. For routine purposes it is necessary to develop methods which permit the analysis of a large number of samples, and a

comparison between values obtained in different laboratories. The most common hydrolysis procedure is still that using strong acids such as hydrochloric or sulphuric acid. — It is generally accepted that enzymatic hydrolysis is the best way of avoiding destruction of the urinary 17-KS and the formation of artefacts. However, the use of enzyme preparations is costly and the hydrolysis is slow; besides, some sulphates — such as those with 5 α /3 α , 5 β /3 α and 5 β /3 β -structures (22) are not hydrolysed at all or only to a very small degree by sulphatase preparations of *Helix Pomatia* or *Patella vulgata* and therefore require an additional solvolysis for their liberation. Furthermore, the presence in the urine of non-competitive or competitive inhibitors, e. g. sulphates, phosphates, chlorides and glucuronides of administered drugs makes a pre-treatment of the urines necessary or an extraction of the conjugates before hydrolysis (23). The presence of artefacts after hydrolysis with bacterial β -glucuronidase has been demonstrated by VESTERGAARD (24). After

incubation of DHA-sulphate with this enzyme preparation, he could identify i-androstanolone by means of infrared spectroscopy, together with a second less important artefact. The re-investigation of the artefacts formed by hydrochloric acid hydrolysis, using gas-chromatographic techniques (25) and the influence of both hydrochloric and sulphuric acid procedures on the gradient elution chromatography of 17-KS (26) showed that of the two procedures, sulphuric acid hydrolysis caused less destruction and transformation of the different urinary 17-KS. By means of gas-chromatographic techniques. CREECH and ABBOTT (27) could not find artefacts after employing sulphuric acid hydrolysis with simultaneous extraction. Simultaneous extraction has however not been employed in the present investigation. This was due to the fact that urine collections and steroid extractions in some pathological cases (27 A) were started several years ago, and that we wanted to have comparable results all through the study.

Fractionation

The results shown in table 1 for dehydroepiandrosterone, androsterone and etiocholanolone are in agreement with those which have been obtained by others (28—31) by using different fractionation methods and hydrolysis procedures. — The values for the ratio etiocholanolone/androsterone are identical with those given by other authors (29, 32, 33) and are the same in both sexes. For the ratio between the 11-deoxy-17-ketosteroids (androsterone, etiocholanolone and DHA) and the 11-oxygenated 17-KS we found values slightly higher than those given by DINGEMANSE et al. (1) using hydrochloric acid hydrolysis and column adsorption chromatography, or those given by KAPPAS and GALLAGHER (31) obtained by means of column and paper chromatography after β -glucuronidase and solvolysis. They are, however, much higher than those described by BROOKSBANK and SALOKANGAS (34) and VESTERGAARD (21) obtained after acid hydrolysis with simultaneous extraction. This difference could be explained by the fact

that these authors have corrected their results for the different chromogenicity of the analyzed steroids, and that by the use of acid hydrolysis with continuous extraction the transformation of 11-oxygenated steroids is decreased.

Our results show that the ratio between 11-desoxy- and 11-oxy-17-KS decreases with age, as has been shown by BROOKSBANK and SALOKANGAS (34). — A comparison between the two hydrolysis procedures used in this investigation shows that the values obtained for total 17-KS, as well as for the steroids of the fractions III and IV are higher if enzymatic hydrolysis is used. The opposite has been found, as might be expected, in elution-fraction I, depending upon the higher formation of artefacts when acid hydrolysis is used. The ratio etiocholanolone/androsterone is smaller after enzymatic hydrolysis, but the difference is not significant.

The figures in table 3 also show that the differences between acid and enzymatic hydrolysis are most marked for the steroids in fraction IV. This means that when the main interest is focused upon the steroids in fraction IV, enzymatic hydrolysis should be carried out. — In order to determine to which degree the conversion of 11-hydroxylated steroids to $\Delta^9(11)$ dehydroandrosterone and $\Delta^9(11)$ -dehydroetiocholanolone could affect the calculation of the ratio etiocholanolone/androsterone, this ratio has been determined also after hydroxylation of two $\Delta^9(11)$ unsaturated steroids. The results of table 4 show that no great differences were obtained in normal individuals when this ratio was calculated with or without the oxidation treatment. However, as we were unable to obtain the $\Delta^9(11)$ -unsaturated compounds as standards, it was impossible to investigate whether the hydroxylation of the double bonds at $\Delta^9(11)$ by the two oxidation procedures was complete.

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References

1. Dingemanse, E., L. G. HUIS IN'T VELD and S. L. HARTONGH-KATZ, *J. Clin. Endocrin.* 12, 66 (1952). — 2. LISBOA, B. P., *J. Chromatogr.* 13, 391 (1964). — 3. LISBOA, B. P., *J. Pharm. Belgique* 1965, 435. — 4. FEHÉR, T., *Mikrochimica Acta* 1965, 105. — 5. PLANTIN, L. O. and G. BIRKE, *Acta med. scand. suppl.* 291, 1954. — 6. PINCUS, G. and W. H. PEARLMAN, *Endocrinology* 29, 413 (1941). — 7. HAMBURGER, C., *Acta endocr., K'hvn* 9, 129 (1952). — 8. ALLEN, W. H., S. J. HAYWARD and A. PINTO, *J. Clin. Endocrin.* 10, 54 (1950). — 9. GIBSON, J. G. and W. A. EVANS, *J. Clin. Invest.* 16, 301 (1937). — 10. ZYGMUNTOWICZ, A. S., M. WOOD, E. CHISTO and N. B. TALBOT, *J. Clin. Endocrinology* 11, 578 (1951). — 11. LEBRETON, P., *Bull. Soc. Chim. France* 1960, 2188. — 12. LISBOA, B. P., *Steroids* 6, 506 (1965). — 13. LISBOA, B. P., *Steroids* 7, 41 (1966). — 14. LISBOA, B. P., *Systemic analysis of Steroids*. Thesis, Karolinska institutet, Stockholm 1965. — 15. LISBOA, B. P. and E. DICZFALUSY, *Acta endocr., K'hvn* 43, 545 (1963). — 16. KLOPPER, A. I., E. R. MICHIE and J. B. BROWN, *J. Endocr.* 12, 209 (1955). — 17. ZDERIC, J. A., H. CARPIO and C. DJERASSI, *J. Org. Chem.* 24, 909 (1959). — 18. SAVARY, P. and P. DESNUELLE, *Soc. Chem., France* 20, 939 (1953). — 19. VESTERGAARD, P., *J. Chromatogr.* 3, 560 (1960). — 20. WIRIOT, G., *Recherches sur l'emploi du fractionnement chromatographique avec gradient de polarité pour l'étude de l'élimination des 17-ceto-stéroïdes urinaires*. R. Foulon 3 Cie, Pa is (1963). — 21. VESTERGAARD, P., *Acta endocr., K'hvn suppl.* 64, 3 (1962). — 22. JARRIGE, P., J. YON and M. F. JAYLE, *Bull. Soc. Chim. biol.* 45, 783 (1963). — 23. HENRY, R., *Annals Biol. Clin.* 23, 211 (1965). — 24. VESTERGAARD, P., *Acta Endocrin. (Kbh), suppl.* 64, 50 (1962). — 25. CAWLEY, L. P., B. O. MUSSER, W. FAUCETTE, S. BECKLOFF and H. LEARNED, *Clin. Chem.* 11, 1009 (1965). — 27A. BÖTTIGER, L. E. and LISBOA, B. P., *Clin. chim. Acta* 16, 109 (1967). — 26. VESTERGAARD, P. and B. CLAUSSEN, *Acta endocr., K'hvn suppl.* 64, 35 (1962). — 27. CREECH, B. G. and J. P. ABBOTT, *Amer. J. Clin. Path.* 42, 514 (1964). — 28' JAMES, V.

- H. T., J. Endocrin. 22, 195 (1961). — 29. MAKESH, V. B., R. B. GREENBLATT, C. K. AYDAR, S. ROY, R. A. PUEBLA and J. O. ELLEGOOD, J. Clin. Endocrin. 24, 1283 (1964). — 30. HAMMAN, B. L. and M. M. MARTIN, J. Clin. Endocrin. 24, 1195 (1964). — 31. KAPPAS, A. and T. F. GALLAGHER, J. Clin. Invest. 34, 1566 (1955). — 32. BEAS, F., R. P. ZURBRUGG, J. CARA and L. I. GARDNER, J. Clin. Endocrin. 22, 1090 (1962). — 33. STARNES, W. R., T. F. PARTLOW, M. C. GRAMMER, L. KORNEL and S. R. HILL, Anal. Biochem. 6, 82 (1963). — 34. BROOKSBANK, B. W. L. and A. SALOKANGAS, Acta endocr., K'hvn 30, 231 (1959).

Doz. Dr. L. E. Böttiger
King Gustaf Vth Research Institute
Stockholm, Schweden

Dr. B. T. Lisboa
Karolinska Sjukhuset
Stockholm 60/Schweden

Die Problematik der GORDON'schen Testsubstanz

Von H.-J. LIESCHKE, H. HARTMANN, J. LÖBE, H. KOCH und K. SEIGE

Aus der Medizinischen Universitätsklinik Leipzig (Direktor: Prof. Dr. R. Emmrich), der Radiologischen Universitätsklinik Leipzig (Direktor: Prof. Dr. W. Oelfner) und dem Institut für angewandte Radioaktivität (elem. Direktor: Prof. Dr. C. F. Weiss) der Deutschen Akademie der Wissenschaften zu Berlin

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Es wird auf die Instabilität der GORDON'schen Testverbindung („PVP-T- ^{131}J “) zum Nachweis der Exsudativen Enteropathie hingewiesen und über die Ergebnisse von Stuhl- und Urindialysen berichtet.

Danach besteht die Aktivität des Urins zu 90% aus abgespaltenem ionogenen ^{131}J od, die fäkale Aktivität zu mindestens 30–50% aus freiem ionogenen ^{131}J od. Als Modellsubstanz für den enteralen Eiweißverlust ist die Substanz daher *nicht* geeignet. — Die Ursache der erhöhten Abspaltrate in vivo sowie der Alterung des Präparates in vitro dürfte in der einfachen Anlagerung des ^{131}J ods an PVP-Doppelbindungen zu suchen sein. Die von GORDON angenommene aromatische Bindung des ^{131}J od am PVP-T wird abgelehnt.

The instability of GORDON's test compound, „PVP-T- ^{131}I “, for the diagnosis of exudative enteropathy was studied, and results from the dialysis of faeces and urine are reported.

Free, ionisable ^{131}I is responsible for up to 90% of the activity of the urine and at least 30–50% of the faeces. Thus the compound *cannot* be used for the measurement of the enteric loss of protein. — The increased rate of cleavage in vivo and the ageing of the preparation in vitro is caused by the simple transfer of ^{131}I to the unsaturated bonds of PVP. GORDON was therefore wrong in assuming that ^{131}I is bound by an aromatic linkage in PVP-T.

Die Verwendung von jodmarkiertem, toluidinhaltigem Polyvinylpyrrolidon („PVP-T- ^{131}J “) beim GORDON-Test (1, 2) zum Nachweis der *Exsudativen Enteropathie* (3–7) stellt gewisse Stabilitätsanforderungen an dieses Präparat, um überhaupt annähernde Aussagen über einen enteralen Verlust treffen zu können. Diesen Stabilitätsanforderungen hält das Präparat nicht stand. Die Aussagekraft des GORDON-Testes mindern zwei nicht abzuschätzende Fehlermöglichkeiten; nämlich die erwähnte Instabilität des PVP-T- ^{131}J in vivo und die problematische Korrelation zwischen enteralem Verlust der Testsubstanz und enteralem Proteinverlust (8).

Der Einbau von ^{131}J od in das PVP: Aromatischer Einbau oder Additionsreaktion?

Die Abhängigkeit der ^{131}J odeinbauraten in das PVP von der Markierungsart ist erwiesen (9, 10). VON GUNTEN (9) und MEDENWALD (10) konnten durch UV-Bestrahlung einen höheren ^{131}J odeinbau in das PVP-T bzw. in das PVP-Molekül nachweisen, als mit der klassischen GORDON'schen Markierungsmethodik möglich ist. Die erste Beobachtung des höheren Aktivitätseinbaues unter UV-Bestrahlung geht dabei auf GORDON zurück. Das GORDON'sche Markierungsverfahren basiert auf der Überlegung, daß unter Anwesenheit von p-Toluidin polymerisiertes PVP noch freie Aminogruppen enthalten müßte. Diese sollen sich mit Natriumnitrit in mineral-saurer Lösung diazotieren und sich ähnlich der *Sandmeyer-Reaktion* bei Bromiden und Chloriden gegen

trägerfreies ^{131}J od austauschen lassen (11). GORDON (11) nahm daher folgende Formel für sein PVP-T- ^{131}J -Produkt an (Abb. 1).

Die Instabilität des GORDON'schen Markierungsproduktes läßt aber berechnete Zweifel an der aromatischen Bindung des ^{131}J od aufkommen. Offenbar kommt es

Abb. 1

Die hypothetische Formel für das PVP-T- ^{131}J nach GORDON