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ROUTINE DETERMINATIONS OF 17-KETOSTEROIDS AND PORTER-SILBER CHROMOGENS COMPARED WITH THE CHROMATOGRAPHIC MEASUREMENT OF GROUPED AND INDIVIDUAL STEROIDS

By

Ingrid Ernest, Britt Håkansson, Jörgen Lehmann and Björn Sjögren

ABSTRACT

The accuracy of two routine methods for the determination of urinary steroids — 17-ketosteroids (17-KS) and Porter-Silber chromogens — has been investigated by chromatographic separation and quantitative determination of individual 17-KS and Porter-Silber reacting steroids in 141 urine samples. For this purpose urine was submitted to enzyme hydrolysis and subsequent solvolysis. The pertinent steroids were separated by column chromatography into three groups, 11-deoxy-17-KS, 11-oxy-17-KS and Porter-Silber reacting steroids. The final separation was accomplished by paper chromatography.

As a mean, the urinary excretion of true 17-KS corresponded to about 40–50 per cent of the routine figures. Due to non specific chromogens,

The following trivial names are used:

3β-hydroxy-androst-5-en-17-one: dehydroepiandrosterone (DHA).
3α-hydroxy-5α-androstan-17-one: androsterone.
3α-hydroxy-5α-androstan-11,17-dione: 11-ketoandrosterone.
3α,11β-dihydroxy-5α-androstan-17-one: 11-hydroxyandrosterone.
3α-hydroxy-5β-androstan-17-one: aetiocholanolone.
3α-hydroxy-5β-androstan-11,17-dione: 11-ketoaetiocholanolone.
3α,11β-dihydroxy-5β-androstan-17-one: 11-hydroxyaetiocholanolone.
3α,11β,17α,21-tetraandroxy-5β-pregn-20-one: tetrahydrocortisol (THF).
3α,17α,21-triandroxy-5β-pregnane-11,20-dione: tetrahydrocortisone (THE).
17,21-dihydroxy-20-keto-steroids: Porter-Silber steroids.
individual routine figures were completely unreliable and probably had no more significance than showing a difference between a high and a low urinary content of 17-KS. A true figure, however, was never higher than that indicated by the routine method.

The routine method for determination of Porter-Silber chromogens also overestimated the urinary content of steroids, the true excretion of Porter-Silber steroids being, on an average, about 25% lower. Again, the significance of individual determinations was low.

The determination of 17-KS and Porter-Silber steroids by column chromatography was found to be rather simple and reliable as only minor amounts of unspecific chromogens were included in the results. Moreover with this method, the 17-KS were separated into 11-deoxy-17-KS and 11-oxy-17-KS.

Early work showed that a large and varying part of the Zimmermann chromogen in a urine extract is due to contaminants (Talbot et al. 1940; Pincus & Pearlman 1941; Dobriner et al. 1948). In modifications of routine methods for the determination of 17-ketosteroids (17-KS), evaluations of specificity were often inadequate, as has recently been pointed out by Goldzieher & Axelrod (1963). These authors found excellent correlation between the urinary excretion of total 17-KS as determined by column chromatography (after enzyme hydrolysis and subsequent solvolysis) and the sum of dehydroepiandrosterone (DHA), androsterone, aetiocholanolone and the 11-oxygenated 17-KS as measured by quantitative paper chromatography. The determination of the total 17-KS by a routine method, however, was found to be quite inadequate and they recommended the use of more precise methods even for clinical work.

The common belief that routine determinations of 17,21-dihydroxy-20-ketosteroids are more or less unspecific is inherent in the term Porter-Silber chromogens (Mason 1954). Vermeulen (1957) found a significant correlation between the urinary excretion of the four Porter-Silber reacting corticosteroids – the tetrahydroderivatives of cortisol (THF) and of cortisone (THE), cortisol (F) and cortisone (E) – and Porter-Silber chromogens as determined by a routine method. As a mean, however, less than 50% of the Porter-Silber chromogens were accounted for by the corticosteroids isolated. Similar results have been reported by Baulieu & Jayle (1957).

Baggett (1954) also found a correlation between 17-hydroxycorticosteroids as estimated in butanol extracts by the Porter-Silber reaction and the amounts of THE found in these extracts.

In routine hospital work, steroid determinations have to be reasonably simple. In spite of the development of rather rapid methods for the isolation and determination of individual steroids, such methods are available only in larger medical centres. In spite of warnings to the contrary (Mason 1953), it
is often believed that common routine methods for steroid determinations are sufficiently good for the clinical diagnosis of most endocrine diseases.

During the last few years we have performed chromatographic separations of grouped and individual urinary steroids for diagnostic purposes. In the present paper the results of two routine methods – the determination of 17-KS and of Porter-Silber chromogens – and the results of more elaborate methods, are compared.

MATERIAL AND METHODS

The material comprises 141 consecutive studies in which in addition to routine determinations of 17-KS and Porter-Silber chromogens, chromatographic separations of individual and grouped steroids were performed. Excluded from the material were only a few cases showing high urinary excretion of tetrahydro-11-deoxycortisol (THS). No data were discarded from this study because of the lack of agreement between results obtained with routine methods and chromatographic methods.

The accuracy of the routine methods was tested by performing 20 and 22 determinations in triplicate, respectively, of 17-KS and Porter-Silber chromogens. Samples from one urine were analyzed on three different occasions, each sample being included in a current series of routine determinations (Table 1).

The recovery of authentic steroids added to the column used for chromatography, was also tested in a small number of experiments (Table 2).

17-ketosteroids were determined by a routine method according to Norymberski et al. (1953). This method uses hot acid hydrolysis and extraction with dichloroethane (C₂H₅Cl₂). Porter-Silber chromogens were determined according to Silber & Porter (1957) after β-glucuronidase hydrolysis (Sigma, type 1, no. 105-8). One ml of urine + four ml of phosphate buffer (pH 6.8) was incubated with the enzyme (150 units) for 18 hours at 37°C. Extraction was performed with 25 ml of dichloromethane (CH₂Cl₂).

Chromatographic separation of grouped and individual steroids was performed according to the scheme in Fig. 1. Before column chromatography, the urine (volume corresponding to 1–3 mg of 17-KS) was incubated at pH 6.8 with β-glucuronidase (150 units per ml) and bensylpenicillin (800 units per ml urine) for 18–20 hours at 37°C. Equal volumes of urine and of phosphate buffer were taken. Glucuronides and

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>m mg</th>
<th>range mg</th>
<th>s. d.</th>
<th>v</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-KS</td>
<td>60</td>
<td>9.6</td>
<td>0.8–25.8</td>
<td>0.78</td>
<td>8.1</td>
</tr>
<tr>
<td>P-S</td>
<td>66</td>
<td>7.1</td>
<td>0.2–19.4</td>
<td>0.77</td>
<td>10.8</td>
</tr>
</tbody>
</table>
Table 2.

Recovery of individual, authentic steroids when added directly to the chromatographic column. Number of experiments within brackets. Results of duplicate determinations of the urinary content of C₁₉O₂- and C₁₉O₃-steroids as well as of Porter-Silber steroids by column chromatography.

<table>
<thead>
<tr>
<th>Steroids</th>
<th>C₁₉O₂</th>
<th>C₁₉O₃</th>
<th>Porter-Silber steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery of steroids per cent</td>
<td>84–113 (6)</td>
<td>87–107 (4)</td>
<td>83–106 (3)</td>
</tr>
<tr>
<td>Duplicate determinations mg</td>
<td>3.3; 3.1</td>
<td>1.8; 1.7</td>
<td>6.2; 5.8</td>
</tr>
<tr>
<td></td>
<td>3.2; 3.2</td>
<td>0.5; 0.5</td>
<td>5.2; 5.1</td>
</tr>
<tr>
<td></td>
<td>2.5; 2.5</td>
<td>2.6; 2.4</td>
<td>3.9; 3.6</td>
</tr>
</tbody>
</table>

Free steroids were extracted with CH₂Cl₂ and the resultant urine phase was submitted to «solvolysis» according to Burstein & Lieberman (1958).

Column chromatography was performed according to Wilson et al. (1958) using an aluminum silicate column and eluting three different fractions A, B and C (Fig. 1). In fraction B, the percentage of chloroform in hexane and the amount of eluate was altered (13% of chloroform; 100 ml of eluate). In fraction A the 11-deoxy-17-KS (C₁₉O₂-steroids, i.e. DHA, androsterone and etiocholanolone) were determined. The 11-oxy-17-KS (C₁₉O₃-steroids) were measured in fraction B. They comprised 11-hydroxyandrosterone, 11-hydroxyetiocholanolone, 11-ketoandrosterone and 11-ketoetiocholanolone. Most of the Porter-Silber reacting material was found in fraction C comprising compounds THF, THE, F and E.

The 17-KS in eluates A and B were determined against a DHA standard using m-dinitrobenzene (according to Zimmermann). Readings were made at 480, 520 and 560 μɛ using the Allen correction.

Porter-Silber steroids were measured in eluate C against a cortisol standard with the phenylhydrazine-sulphuric acid reaction (according to Porter and Silber). Readings were made at 375, 410 and 445 μɛ using the Allen correction.

Paper chromatography was performed using Bush's (1952) solvent systems A, B1 and B3. Petrol was replaced by trimethylpentane. DHA, androsterone and etiocholanolone were eluted with ethanol and determined quantitatively by the Zimmermann reaction. Readings were made at 520 μɛ against a DHA standard. The same colour reaction was also used for semiquantitative determinations on the paper by comparison with simultaneously run standards in two different concentrations.

The four different C₁₉O₃-steroids were determined only semiquantitatively on the paper by comparison with respective standards run simultaneously in two concentrations.
THF, THE, F and E were determined after elution with ethanol using the Porter-Silber reaction and readings were made at 375, 410 and 445 \( \mu m \) against a cortisol standard. All figures were corrected for an average loss of steroids of 25 per cent. Semiquantitative determinations were also made directly on the paper with standards in two different concentrations and using blue tetrazolium as colour reaction. In addition, as a further check, semiquantitative determinations were also made in most cases from a thin layer chromatographic method. In this work, however, only the results obtained from elution of steroids from the paper were used. The results obtained from semiquantitative determinations on the paper as well as from thin layer chromatography showed a good agreement with the results of quantitative paper chromatography.

Fig. 1.
Procedure followed for the determination of grouped and individual 17-KS and Porter-Silber steroids in the present work. The hydrolysis and extraction separates glucuronides (left column) and sulphates (right column).

Hydrolysis of urine with \( \beta \)-glucuronidase

\[ \text{CH}_2\text{Cl}_2 \text{ extraction} \]

extract

urine phase solvolysis

COLUMN CHROMATOGRAPHY

COLUMN CHROMATOGRAPHY

Three fractions, A, B and C, collected from each column and submitted to the following analyses:
1. DETERMINATION OF 17-KS (A and B) AND P-S STEROIDS (C)
2. PAPER CHROMATOGRAPHY
   a. semiquantitative determinations of the separated steroids (A, B and C)
   b. elution of the steroids from the paper and quantitative determinations (A and C).
RESULTS AND COMMENTS

The measurements of individual and grouped 17-KS were performed after preliminary separation of glucuronides and sulphates (Fig. 1). 17-KS from these two fractions were added to give the results in Fig. 2-3. It is a reasonable assumption that the methods used for hydrolysis and extraction do indeed measure the amounts of glucuronides and sulphates actually present. Zumoff & Bradlow (1963) recently described a procedure for the quantitative extraction of steroid conjugates from human urine and showed that the »glucuronide« fraction obtained from urine by appropriate enzyme hydrolysis represented a good approximation of the amount of glucuronides actually present. The »sulphate« fraction subsequently obtained by solvolysis agreed well with the solvolyzable fraction, obtained after chromatographic separation.

The amount of β-glucuronidase (Sigma, type 1, no. 105-8) necessary for hydrolysis was determined by experiments on 12 different urines – including specimens with a content of Porter-Silber chromogens as high as 50 mg/day and of 17-KS up to 49 mg/day – which were incubated with increasing

![Graph showing urinary content of 11-deoxy-17-KS](image)

**Fig. 2.**
Urinary content of 11-deoxy-17-KS (C₁₉O₂-stereoids) as determined by column chromatography and by paper chromatography in 56 samples. The diagonal line dotted. Y = 5.9 + 1.04 (x - 6.1), r = 0.99. In the corner similar data from 41 determinations of 11-oxy-17-KS (C₁₉O₃-stereoids). Y = 0.07 + 0.45 (x - 0.19), r = 0.77.

557

37°
Results of determinations of the urinary content of 17-KS by a routine method compared with the figures obtained from column chromatography (55 experiments) and paper chromatography (46 experiments). The former data are represented by equation \( Y = 7.4 + 0.52(x - 15.3) \) and the regression line is continuous, \( r = 0.97 \). The latter data are summarized in equation \( Y = 5.5 + 0.45(x - 14.6) \). The regression line is broken, \( r = 0.97 \).

amounts of enzyme. Concentrations higher than 150 units/ml did not significantly increase the yield of free steroids.

The recovery of authentic steroids, when added to the column, was tested in 13 experiments with different steroids. The recovery varied between 83 and 113% of the added substances (Table 2). In a few cases different samples of the same 24 hour urine were submitted to chromatographic analyses. The results showed good agreement between duplicates (Table 2).

Fig. 2 shows the excellent correlation between the \( C_{19}O_2 \)-steroids (11-deoxy-17-KS) as determined from column chromatography (eluate A) and from quantitative paper chromatography (\( r = 0.99 \)). The regression line is very close to the diagonal line which represents the locus of points, when results from column and from paper chromatographic determinations coincide. The absence of a systematic difference between the two measurements of 17-KS shows that there could not be more than a small amount of unspecific chromogens in eluate A.

The determinations of \( C_{19}O_2 \)-steroids by paper chromatography were only semiquantitative and this could partly explain the discrepancies between the results obtained from column chromatography only and from paper chromato-
graphic separation of steroids in eluate B (Fig. 2). Unspecific chromogens were present in eluate B and these also interfered with the semiquantitative determination of the C19O3-steroids on the paper.

Because the C19O3-steroids comprise the smaller fraction of the total 17-KS, the sum of the C19O3- and C19O2-steroids as determined from paper chromatography, only slightly underestimated the true amount of total 17-KS present in an extract. By the same token, the sum of steroids determined directly in eluate A and B did not unduly overestimate the true amount of 17-KS, as the total amount of unspecific chromogens in the eluates was not very large. Consequently, in Fig. 3, the true amount of 17-KS in relation to the amount of Zimmermann chromogens found by the routine procedure should be found in the area between the two regression lines based on column chromatography (x) and paper chromatography (·).

Fig. 3 also shows that there was a high degree of correlation between routine determinations of 17-KS and true 17-KS (r = 0.97 and 0.97, respectively, using column chromatography only or paper chromatography after preliminary column chromatography). On an average, the absolute amount of non specific Zimmermann chromogens increased with increasing amounts

![Graph](image-url)

**Fig. 4.**

The urinary content of Porter-Silber steroids in 181 cases as determined from column and paper chromatography. \( Y = 6.3 + 0.90 \times (x - 7.1), \ r = 0.96. \)

The diagonal line dotted.
of true steroids comprising about 60 per cent of the total amount of chromogen. The spread around the regression lines was large and a prediction of the true amount of 17-KS from a routine determination can not be very accurate. True figures for 17-KS represented from zero to 70 per cent of routine levels.

Our findings are very similar to those of Goldzieher & Axelrod (1963) who developed a chromatographic method for the determination of \( \text{C}_{19}\text{O}_2 \)- and \( \text{C}_{19}\text{O}_3 \)-steroids which gave figures closely corresponding to those obtained from quantitative paper chromatography. These authors concluded that »the unpredictable admixture of non specific chromogens reduces the significance of the clinical 'total 17-ketosteroids' determination to little more than a rough approximation«. We would agree that a routine 17-KS determination cannot be expected to accomplish much more than a separation of a low from a high steroid excretion. In our material the true amount of 17-KS found was always lower than the figure obtained from our routine method.

In Fig. 4 the Porter-Silber positive material measured in eluate C is plotted against the sum of the compounds THF, THE, F and E as determined by

![Fig. 4](image-url)

**Fig. 5.**

Results of determinations of Porter-Silber chromogens by a routine method in 128 cases compared with results obtained from paper chromatography. The diagonal line dotted. \( Y = 6.1 - 0.75 (x - 8.0) \), \( r = 0.87 \).
quantitative paper chromatography. The regression line \( r = 0.96 \) is close to the diagonal line showing that the Porter-Silber positive material in eluate C mainly comprised 17,21-dihydroxy-20-ketosteroids. In this paper we have, therefore, referred to the Porter-Silber positive material in eluate C as Porter-Silber steroids (Fig. 4–5).

The error of a single figure for Porter-Silber steroids as determined from paper chromatography was not small as the error in the determination of each component was of the order of magnitude of 15 per cent. This probably explains the rather large spread around the regression line in Fig. 4. The error in the determination of the steroids in eluate C by the Porter-Silber reaction is probably smaller than the error in the routine determination of Porter-Silber chromogens in urine (Table 1).

In Fig. 5 the Porter-Silber chromogens as determined from the routine method are plotted against the Porter-Silber steroids, measured by paper chromatography. On an average, 76 per cent of a routine figure corresponded to the true urinary content of Porter-Silber steroids. The coefficient of correlation is high \( r = 0.87 \) but the significance of an individual, routine figure is nevertheless low. Such routine determinations do not afford much more than a distinction between a high and low excretion of steroids.

The disappointing experience with routine determinations of 17-KS and Porter-Silber chromogens does not exclude the possibility that such determinations can be significant when used under special circumstances. Thus, it is a common clinical experience that clinical methods for the determination of Porter-Silber chromogens can be reliable in connection with the ACTH stimulation test. The reliability of routine methods for the evaluation of the ACTH inhibition test will be the aim of a future report from our laboratory.

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REFERENCES


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