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Note

Thin-layer chromatographic determination of urinary testosterone, epitestosterone and androstenedione

NADA JAGARINEC* and GORDANA PARAG

Institute of Clinical Chemistry, Research Unit, Clinical Hospital "Dr Ozren Novosel" Zajčeva 19, 41000 Zagreb (Yugoslavia)

and

MELITA TAJIČ

Department of Endocrinology, University Hospital, Zagreb (Yugoslavia)

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A thin-layer chromatographic (TLC) method for the separation of C_{19} steroids was combined with a spectrophotometric method for the determination of urinary testosterone, epitestosterone and androstenedione, instead of the previously used paper chromatography. A reproducible separation was obtained on silica gel F-254 precoated plates in the solvent system chloroform—diethyl ether, as well as easy identification due to the UV absorption of the Δ^4-3-keto group on a fluorescent background. The reliability of the modified method was evaluated from a study of sensitivity, precision, accuracy and comparison with radioimmunoassays (RIA).

MATERIALS AND METHODS

The method of acid hydrolysis, extraction, separation of ketonic from non-ketonic fraction and spectrophotometric measurement performed on a 24-h urine is adapted from that reported by Longhino et al. [1], except for the enzymatic hydrolysis and chromatographic separation of steroids. Steroids in the free form and after liberation by hydrolysis with beef liver β-glucuronidase (500 Fishman units per ml of urine; obtained from Pliva, Zagreb, Yugoslavia) and hydrochloric acid were extracted with diethyl ether. The combined ether

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extracts were evaporated and the residue was subjected to separation into ketonic and non-ketonic fractions using Girard T reagent.

TLC of testosterone (T), epitestosterone (ET) and androstenedione (A)

The ketonic fractions were dissolved in absolute ethanol (0.2 ml) and one half applied as small spots 2 cm apart on a starting line 2 cm from the lower edge of the plate. A mixture of standards T, ET and A, 2 μg of each dissolved in 0.2 ml of absolute ethanol, was applied to the middle and the sides of the plate. Before use the plates (0.25 mm thick, precoated silica gel F-254 plates, 20 × 20 cm, on glass support; obtained from Merck, Darmstadt, F.R.G.) were washed twice in ascending fashion with absolute ethanol, dried, activated at 105°C for 30 min and stored above silica gel in a desiccator. A 100-ml volume of the solvent system chloroform—diethyl ether (9:1, v/v) was mixed immediately before use and transferred to the chromatography chamber. The plate was developed to 15.0 cm from the starting line at room temperature [2].

T, ET and A standards were located and marked as UV-absorbing spots on a fluorescent background under a shortwave UV lamp in a dark room. The marked zones of silica gel (standards and corresponding zones of urinary steroids) were scraped into glass tubes and eluted by adding absolute ethanol (2 × 5 ml) and shaking vigorously. The silica gel was precipitated by centrifugation at 1500 *g* for a few minutes. The ethanol was transferred to clean tubes and evaporated under nitrogen.

The dry residues of the respective androgens produced color with sulphuric acid—ethanol reagent. The absorbance of the color read at 565, 600 and 635 nm (Gilford spectrophotometer, Model 250) against the reagent blank was corrected using Allen's formula. The concentrations of urinary T, ET and A were calculated by referring to the known amount of standard run on the same plate.

All solvents were reagent grade (Kemika, Zagreb, Yugoslavia).

RESULTS AND DISCUSSION

Separation of the steroids

The most common urinary C_{19} oxosteroids, such as androsterone (3α-hydroxy-5α-androstan-17-one, R_F = 0.42), dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one, R_F = 0.39), 11-oxoandrosterone (3α-hydroxy-5α-androstan-11,17-dione, R_F = 0.15), 11-oxoetiocholanolone (3α-hydroxy-5β-

TABLE I

R_F VALUES OF T, ET AND A OBTAINED BY TLC IN CHLOROFORM—DIETHYL ETHER (9:1)

The R_F values are the averages of ten chromatograms.

Steroid	R_F
Epitestosterone	0.21
Testosterone	0.30
Androstenedione	0.77

androstan-11,17-dione, R_F = 0.13), 11β-hydroxyandrosterone (3α,11β-dihydroxy-5α-androstan-17-one, R_F = 0.11), 11β-hydroxyethiocholanolone (3α,11β-dihydroxy-5β-androstan-17-one, R_F = 0.09) separate well from testosterone (T) (17β-hydroxy-4-androstene-3-one), epitestosterone (ET) (17α-hydroxy-4-androstene-3-one) and androstenedione (A) (4-androstene-3,17-dione) (Table I). Etiocholanolone (3α-hydroxy-5β-androstan-17-one, R_F = 0.29), which runs close to T, does not interfere, because it does not show any color reaction with the sulphuric acid—ethanol reagent.

Evaluation of the procedure

Sensitivity. The sensitivity of the modified method was evaluated by means of the recovery experiments as shown in Tables II—IV. The recovery of approximately 97.7% obtained for T (in the range 0.35—27.7 nmol/sample), 92.8% ET (in the range 0.7—8.7 nmol/sample) and 91.7% for A (in the range 0.9—24.4 nmol/sample) indicated that only negligible losses occurred during TLC. The silica gel blank carried through the procedure did not give any optical absorbance. The sensitivity is variable since it depends on the urine aliquot used. Under our working conditions, providing that we are dealing with one-third of 1 l of urine, it can be expected that reliable estimates will be obtained when the concentration of T is as low as 2.1 nmol/l (C.V. = 25.2%) and of A 5.2 nmol/l (C.V. = 28.8%), which is below the lowest values found for 24-h urine of healthy women according to Longhino et al. [1] (9.7—81.5 nmol/day for T, 11.2—86.6 nmol/day for A, and 4.2—34.5 nmol/day for ET). However, the equally high reliability of the ET estimates could be expected only at a concentration of 10.4 nmol/l, which is approximately twice as high as the lowest value indicated.

TABLE II

RECOVERY OF TESTOSTERONE AFTER TLC

Average recovery = 97.7%.

Testosterone (nmol/sample)			No. of determinations	C.V. (%)
Applied (nmol)	Recovered (mean ± S.D.)			
	nmol	%		
0.35	0.29 ± 0.073	82.9 ± 21.0	5	25.2
0.69	0.73 ± 0.17	105.8 ± 24.6	6	23.3
0.87	0.80 ± 0.17	92.0 ± 19.5	5	21.3
1.73	1.60 ± 0.31	92.5 ± 17.9	5	19.4
3.47	4.16 ± 0.76	119.9 ± 21.9	8	18.3
6.93	6.73 ± 1.21	97.1 ± 17.5	3	18.0
8.67	8.11 ± 1.42	93.5 ± 16.4	5	17.5
10.40	10.06 ± 1.25	96.7 ± 12.0	5	12.4
15.60	15.26 ± 1.84	97.8 ± 11.8	3	12.1
17.34	17.34 ± 1.95	100.0 ± 11.2	33	11.2
20.80	19.76 ± 2.32	95.0 ± 11.2	9	11.7
27.74	27.60 ± 1.84	99.5 ± 6.6	5	6.7

TABLE III

RECOVERY OF EPITESTOSTERONE AFTER TLC

Average recovery = 92.8%.

Epitestosterone (nmol/sample)			No. of determinations	C.V. (%)
Applied (nmol)	Recovered (mean ± S.D.)			
	nmol	%		
0.69	0.66 ± 0.35	95.6 ± 50.7	5	53.0
0.87	0.87 ± 0.34	87.4 ± 39.1	5	44.7
1.73	1.53 ± 0.45	88.4 ± 26.0	5	29.4
3.47	3.40 ± 0.87	98.0 ± 25.1	8	25.6
6.93	6.00 ± 1.84	86.6 ± 26.6	5	30.7
8.67	8.74 ± 2.43	100.8 ± 28.0	5	27.8

TABLE IV

RECOVERY OF ANDROSTENEDIONE AFTER TLC

Average recovery = 91.7%.

Androstenedione (nmol/sample)			No. of determinations	C.V. (%)
Applied (nmol)	Recovered (mean ± S.D.)			
	nmol	%		
0.87	0.59 ± 0.17	67.8 ± 19.5	4	28.8
1.75	1.96 ± 0.56	112.0 ± 32.0	4	28.6
3.49	2.97 ± 0.49	85.1 ± 14.0	4	16.5
7.00	6.28 ± 0.84	89.7 ± 12.0	12	13.4
12.22	11.31 ± 1.50	92.6 ± 12.3	5	13.3
17.46	17.32 ± 2.09	99.2 ± 12.0	22	12.1
20.95	18.99 ± 1.54	90.6 ± 7.4	7	8.1
24.44	23.67 ± 1.19	96.8 ± 4.9	4	5.0

Precision. The method of Snedecor [3] was used to give an estimate of the precision between the duplicate determinations obtained with a number of different urines. The coefficient of variation in the range 0.0—70.0 nmol/l is 11.0% for T, 20.9% for ET and 23.6% for A (Tables V—VII). This range covers the lowest levels of the respective androgens in 24-h urine of healthy women.

Accuracy. The accuracy of the modified method was measured by adding various amounts of T, ET and A to the ketonic fraction before TLC. A linear relationship was observed between the amounts added and the amounts recovered throughout the range examined, which is expressed by the regression equations: for T, $Y = 37.1 + 0.96X$, $r = 0.932$; for ET, $Y = 30.2 + 0.99X$, $r = 0.932$; for A, $Y = 51.0 + 0.92X$, $r = 0.918$, where Y is amount recovered, X is amount added and the intercept (a) is the endogenous amount of each respective androgen in the 24-h urine.

TABLE V

PRECISION OF DUPLICATE ESTIMATIONS OF URINARY TESTOSTERONE AT DIFFERENT RANGES

Range (nmol/l)	Mean ± S.D.	No. of determinations	C.V. (%)
0.0— 70.0	43.6 ± 4.8	32	11.0
70.0— 208.0	139.4 ± 14.6	28	10.5
138.0— 346.0	275.4 ± 18.8	24	6.8
346.0— 690.0	511.8 ± 23.6	28	4.6
1386.0—2080.0	1848.2 ± 38.8	12	2.1

TABLE VI

PRECISION OF DUPLICATE ESTIMATIONS OF URINARY EPITESTOSTERONE AT DIFFERENT RANGES

Range (nmol/l)	Mean ± S.D.	No. of determinations	C.V. (%)
0.0— 70.0	43.0 ± 9.0	26	20.9
70.0— 208.0	114.4 ± 16.0	20	14.0
208.0— 346.0	294.8 ± 18.8	26	6.4
346.0— 690.0	554.0 ± 24.2	26	4.4
1386.0—2428.0	2205.2 ± 46.4	10	2.1

TABLE VII

PRECISION OF DUPLICATE ESTIMATES OF URINARY ANDROSTENEDIONE AT DIFFERENT RANGES

Range (nmol/l)	Mean ± S.D.	No. of determinations	C.V. (%)
0.0— 70.0	47.4 ± 11.2	24	23.6
70.0— 208.0	123.6 ± 20.0	18	16.3
208.0— 416.0	322.0 ± 26.6	28	8.3
1040.0—1734.0	1410.6 ± 48.2	12	3.4

Comparison of the results obtained by TLC and RIA

In order to determine to what extent our results agree with those obtained by RIA, we carried out a comparative study on over 40 urines. One half of the ketonic fraction was subjected to the TLC separation and the other half of the same ketonic fraction was used for the determination of T (testosterone radioimmunoassay kit, from Biolab, Limal, Belgium) and A (androstenedione antiserum, from Biolab; [1,2,6,7-^{3}H] androst-4-ene-3,17-dione, from the Radiochemical Centre, Amersham) by RIA. The linear regression equations so obtained were for T (n = 43) $Y = 5.7 + 0.78X$, $r = 0.940$ ($P < 0.001$), and for A (n = 32) $Y = -5.7 + 0.81X$, $r = 0.976$ ($P < 0.001$), indicating a strong and significant correlation between the two methods (Figs. 1 and 2). RIA for ET was not available.

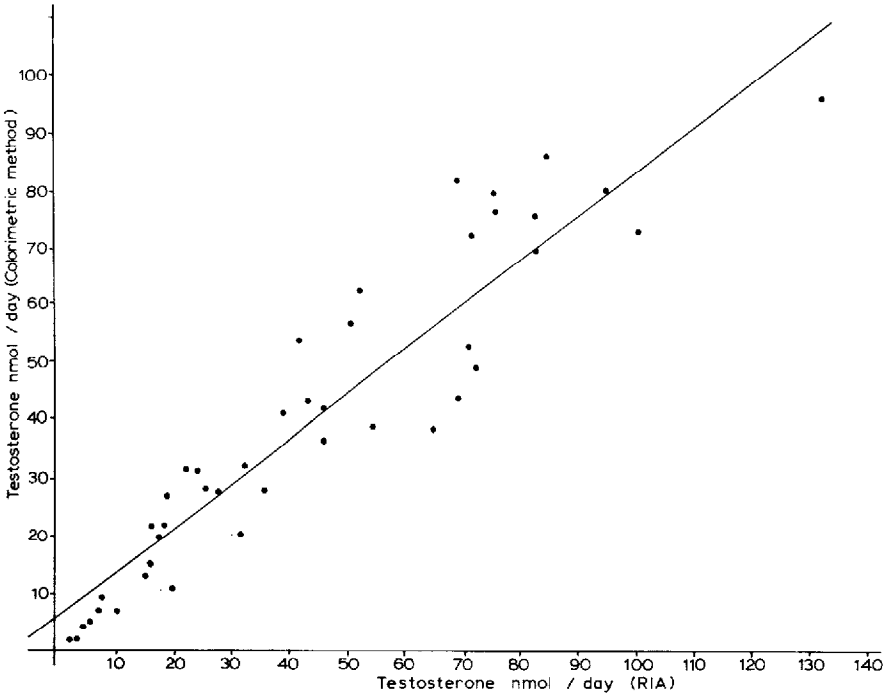

Fig. 1. Correlation between results for urinary testosterone obtained by TLC (Y) and by RIA (X). $Y = 5.7 + 0.78X$, $r = 0.940$, $P < 0.001$, $n = 43$.

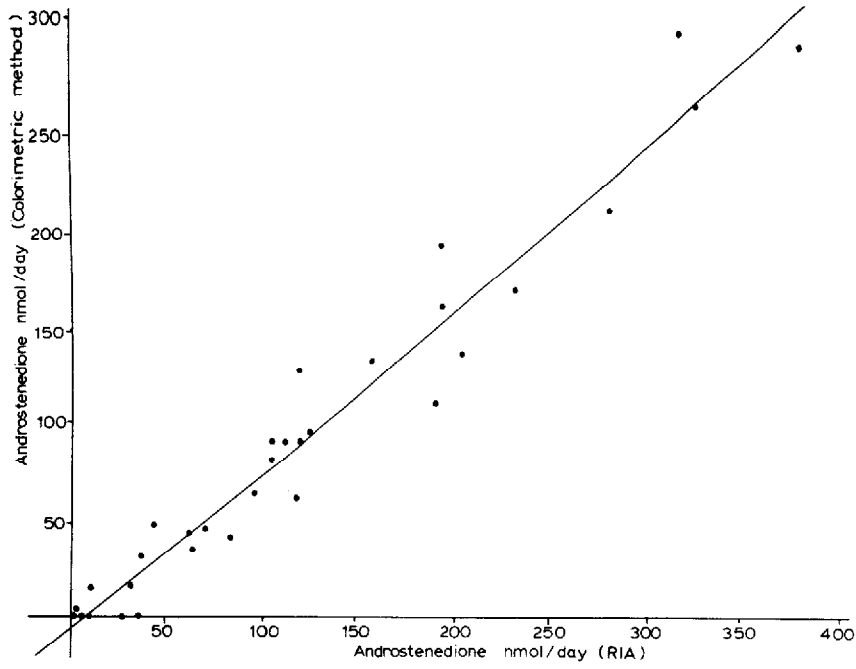

Fig. 2. Correlation between results for urinary androstenedione obtained by TLC (Y) and by RIA (X). $Y = -5.7 + 0.81X$, $r = 0.976$, $P < 0.001$, $n = 32$.

No significant average difference $P > 0.05$ was found between the results of the two methods either for T ($t = 0.641$; d.f. = 84) or for A ($t = 0.465$; d.f. = 62).

TLC shortens the already very long procedure by at least one working day. The other advantage of using TLC instead of paper chromatographhy to separate urinary T, ET and A is the relative simplicity of operation as well as reproducibility of separation, which makes it suitable for use in the routine clinical laboratory.

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