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DETERMINATION OF THE 2,4-DINITROPHENYLHYDRAZONES OF URINE KETOSTEROIDS BY THIN LAYER DENSITOMETRY

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SUMMARY

A double beam scanning spectrodensitometer is used for the determination of DHEA (dehydroepiandrosterone = 3β-hydroxyandrost-5-en-17-one), androsterone (3α-hydroxy-5α-androstan-17-one) and etiocholanolone (3α-hydroxy-5β-androstan-17-one) from urine. After ether solvolysis, purification and reaction with 2,4-dinitrophenylhydrazine, aliquots are developed on thin layer plates and quantitated directly.

The colorimetric determination of the 2,4-dinitrophenylhydrazones of ketosteroids from biological material has been reported recently[1–3]. The excellent separation behavior of these derivatives on thin layer plates and their high molar extinction (ε for 17-ketosteroids = 23,600) made a further simplification of the method possible. Instead of eluting the hydrazones and quantitating them in a spectrophotometer, they could be determined directly on the plate with a new instrument, a double beam scanning spectrodensitometer.

METHODS

Performance of the reaction

Five ml urine is acidified with 5 *N* HCl to pH 1, saturated with ammonium sulfate and extracted with 2 × 20 ml ethylacetate. The extract is neutralized with 0.5 ml ammonia and evaporated. The residue is shaken in 40 ml of 1% perchloric acid in ether and incubated for at least 15 h at 39–40°. A short boiling should precede the tight stoppering of the tube. The ether is then washed with 5 ml of 5 *N* NaOH and 3 × 5 ml water, dried over sodium sulfate and evaporated.

For the reaction itself, 0.1 ml of 0.2% (w/v) 2,4-dinitrophenylhydrazine in ethylacetate is added and the solvent is evaporated. The residue is dissolved in 1 ml of 0.03% (w/v) trichloroacetic acid in absolute benzene and kept for 40 min at 40°. After this the solution is filled up with benzene to the original urine volume, so that certain aliquots can be withdrawn easily.

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Chromatography

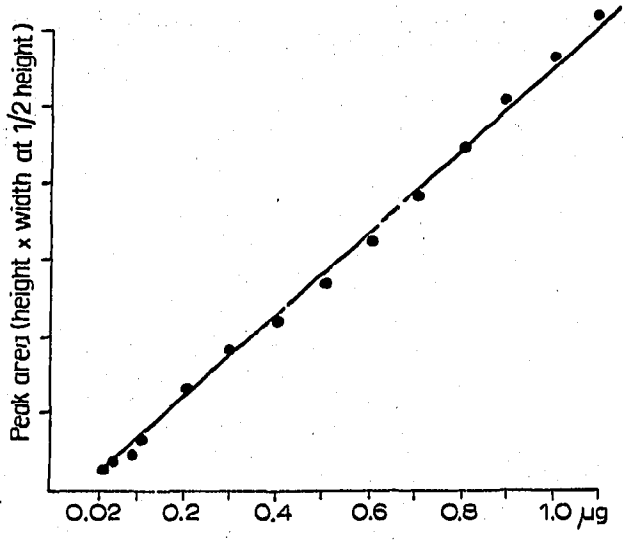
For chromatography Silica Gel G plates of 250 μ thickness without binder are used (Analtech, Wilmington, Del.). With a special scoring device (Schoeffel Instrument Corp., Westwood, N.J.) the thin layer surface is divided into bands of 1 cm width. For duplicate determination dried 0.1 and 0.2 ml aliquots of the reacted urine are transferred with 3 × 0.03 ml benzene to the plate using a 50 μl syringe. A 0.8 cm starting line is formed. Because of the double beam operating principle of the densitometer, only alternate bands are loaded. The blanks serve as reference for the instrument. On each plate standard steroid hydrazones are run as reference.

A rapid separation of dehydroepiandrosterone (DHEA), androsterone and etiocholanolone hydrazones can be achieved by chromatography of the aliquots in system A (chloroform–carbon tetrachloride, 2:1 v/v) followed by unidimensional chromatography of the same plate in system B (chloroform–dioxane, 94:6 v/v) (Fig. 5). A better purification and separation requires chromatography of the total urine residue in system B on a 5 cm plate. The zones of the steroid hydrazones are eluted with 3 × 3 ml chloroform. Aliquots corresponding to 0.5 and 1.0 ml urine are rechromatographed in system B on plates prepared for densitometry. Combined DHEA and androsterone hydrazones can run in one band (Fig. 6). Etiocholanolone is run in another band (Fig. 7).

Fig. 1. The densitometer (Schoeffel Instr. Co., Westwood, N.J., U.S.A.). Model SD 3000 (about $^1/_{10}$ of original size).

Quantitation

Direct quantitation of the plates (optimal wavelength = 367 mμ) is performed in a Schoeffel spectrodensitometer, Model SD 3000 (Fig. 1). An optical density computer, Model SDC 300, gives the data to an integrating 10″ strip recorder, Model SDR 303 (Schoeffel Inst. Corp., Westwood, N.J.). Peak areas are calculated by triangulation (height × width at $\frac{1}{2}$ height) and interpolated on standard curves (Figs. 2 and 3). A plate, once it contains 17-ketosteroid hydrazone standards, can be



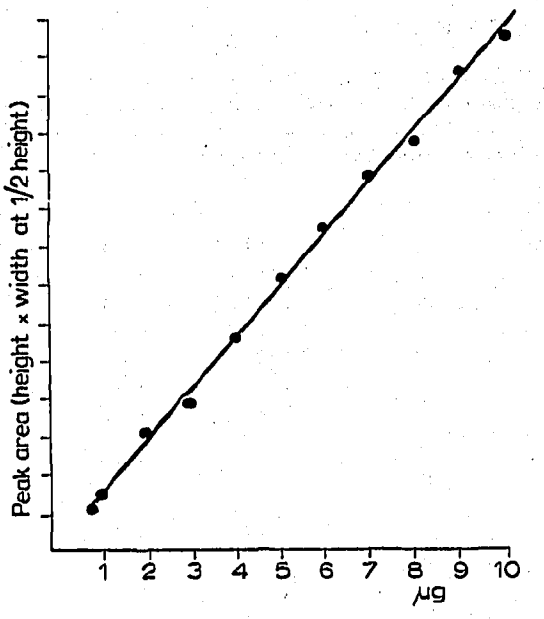


Fig. 2. Calibration curve of the densitometer (λ = 367 mμ) with recorder full scale at optical density = 0.5.

Fig. 3. Calibration curve of the densitometer with recorder full scale at optical density = 1.5.

used at any time for calibration of the instrument settings. The scanning of a 20-cm band by the densitometer takes about 20 sec.

Reliability

The reliability for the reaction of urine ketosteroids with 2,4-dinitrophenylhydrazone has been published in detail elsewhere[3]. The accuracy for DHEA sulfate, androsterone sulfate and androsterone glucuronoside from 10 ml urine was 91–95%. The precision ranged between $\pm$3.8 and $\pm$7.2%. Calculated from 6 blank determinations the sensitivity of the densitometric method ($= 6 \times S_{bl}$) lay near 0.05 μg 17-ketosteroid/sample. The sensitivity of the instrument ranged between 0.02 and 0.04 μg. Setting the recorder full scale at different optical density values, a good linearity was achieved between 0.02 and 10.0 μg (Figs. 2 and 3). The precision for one

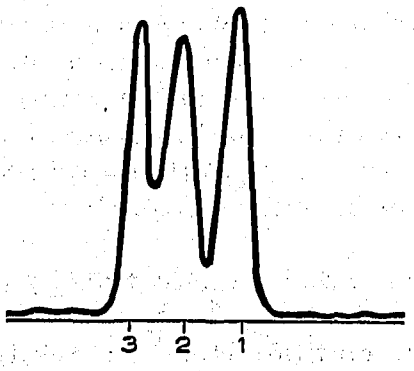


Fig. 4. Separation of about 2.5 μg each (1) DHEA, (2) etiocholanolone and (3) androsterone hydrazones in system B.

TABLE I

URINARY 17-KETOSTEROID 2,4-DINITROPHENYLHYDRAZONES DETERMINED BY EITHER (a) CONVENTIONAL SPECTROPHOTOMETRY OR (b) SPECTRODENSITOMETRY (μg STEROID/ml)

Urine No.	*Steroid*	*(a) 5 ml*[a]	*(a) 5 ml*[a]	*(a) mean*	*(b) 0.1 ml*[b]	*(b) 0.5 ml*[c]	*(b) 1.0 ml*[c]	*(b) mean*
1	D	2.78	2.70	2.74	2.20	2.50	3.05	2.58
	A	5.93	6.58	6.25	5.50	6.40	5.85	5.91
	E	3.60	4.83	4.21	3.85	4.00	4.10	3.98
2	D	3.78	4.16	3.97	3.55	3.70	3.85	3.70
	A	4.48	5.02	4.75	4.70	4.60	4.90	4.73
	E	4.36	5.04	4.70	4.05	4.45	4.55	4.35
3	D	6.20	6.28	6.24	6.55	5.95	6.10	6.20
	A	4.52	4.23	4.37	4.20	3.80	3.40	3.80
	E	2.87	2.88	2.87	3.20	2.18	2.60	2.66
4	D	3.45	3.28	3.36	2.95	3.35	3.20	3.20
	A	4.07	3.72	3.89	4.40	3.95	4.05	4.13
	E	2.58	2.41	2.49	2.80	2.70	2.65	2.71
5	D	3.37	3.69	3.53	3.10	3.25	3.45	3.26
	A	2.07	1.64	1.85	1.45	1.85	1.80	1.70
	E	2.89	2.17	2.53	1.95	2.60	2.45	2.33
6	D	2.40	2.95	2.67	2.05	2.70	2.85	2.53
	A	5.20	5.78	5.49	4.80	5.40	5.55	5.25
	E	4.28	4.07	4.17	3.80	4.35	4.25	4.13

[a] One chromatography in system B.
[b] Two unidimensional chromatographies on one plate in systems A and B.
[c] Chromatography in system B, elution and rechromatography in system B. D = dehydroepiandrosterone; A = androsterone; E = etiocholanolone.

peak in 4–8 replicate determinations was less than 0.5%. The specificity is ensured by solvent partition, at least two fold chromatography and by visualization of a proper curve.

RESULTS AND DISCUSSION

Table I gives total DHEA, androsterone and etiocholanolone (μg/ml) from urines of 6 healthy males (29–35 years of age). Two 5 ml aliquots were measured in a Beckman DK-2 spectrophotometer (a). A third 5 ml sample (b) was carried through the 2,4-dinitrophenylhydrazone reaction. Three aliquots (0.1, 0.5 and 1.0 ml) were quantitated in the spectrodensitometer, either after twofold unidimensional chromatography in systems A and B or after purification in system B, elution and rechromatography in system B. Figs. 5, 6 and 7 show corresponding curves (from urine No. 1 in Table I).

The values obtained by the densitometer corresponded well to those from a colorimetric method. For routine clinical analysis this could also be achieved when an 0.1 ml aliquot was developed on one plate without elution. Hence, a relatively simple and rapid method is offered. Ten urine samples can be finished easily in 24 h if they are incubated over night for solvolysis.

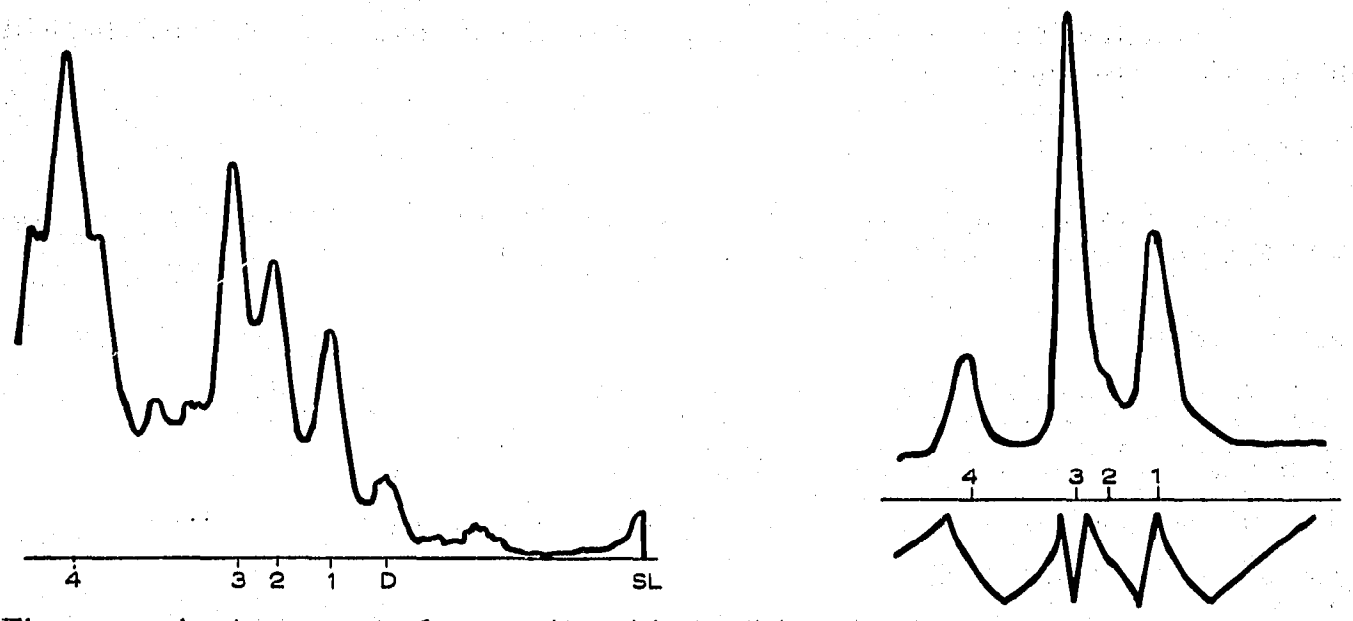


Fig. 5. 0.2 ml urine extract after reaction with 2,4-dinitrophenylhydrazone and unidimensional chromatography in systems A and B. (SL = starting line; D = reagents excess; 1 = DHEA; 2 = etiocholanolone; 3 = androsterone; 4 = nonpolar compounds).

Fig. 6. (1) DHEA, (2) rest of etiocholanolone and (3) androsterone hydrazones from 0.5 ml urine after chromatography in system B, elution and rechromatography in system B (lower curve = disc integrator curve).

A further decrease in time can be achieved when acid hydrolysis is introduced. For this purpose 5 ml of crude urine plus 0.5 ml of concentrated sulfuric acid are incubated for 1 h at 80°. The free steroids are then extracted with ether. Most of DHEA will be destroyed, yet androsterone and etiocholanolone from 5 urine samples can be determined in 4 h. The solvolysis of the steroid conjugates of urine in perchloric acid–ethyl acetate was found to be unreliable.

Several recent papers deal with the densitometry of steroids on sprayed thin layer plates[4–6]. In the present method stable color derivatives of ketosteroids are separated and quantitated as such by a practical and highly efficient and reliable instrument.

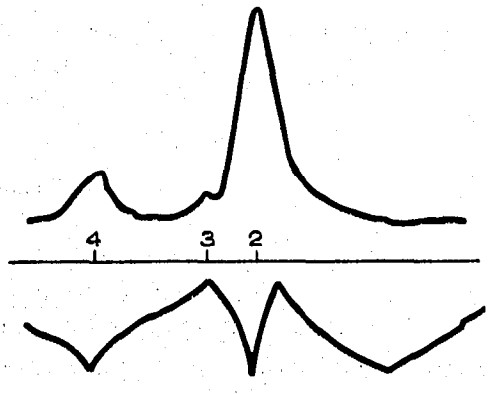


Fig. 7. (2) Etiocholanolone and (3) rest of antrosterone hydrazones from 0.5 ml of the same urine as in Fig. 6 after the same treatment (lower curve = disc integrator curve).

The method was also adapted for analysis of ketosteroids from plasma. This will be described elsewhere.

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