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A PAPER CHROMATOGRAPHIC SATURATION ANALYSIS METHOD FOR MEASURING ESTRADIOL, TESTOSTERONE AND 5α-DIHYDROTESTOSTERONE FROM THE SAME SAMPLE

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SUMMARY

A new method permitting the estimation of estradiol, testosterone and 5α-dihydrotestosterone in the same plasma sample is presented. This is made possible by a 20 h overflow paper chromatography system used to purify the plasma extract prior to steroid analysis. Estradiol is well separated from estrone as is testosterone from 5α-dihydrotestosterone. The method has low blank values, good agreement between duplicates, acceptable recovery through the extraction and purification and a good recovery of steroids added to plasma prior to extraction. Plasma levels of steroids measured using this method agree well with the values obtained by other methods.

INTRODUCTION

In steroid based endocrinological investigations the tendency is towards the measurement of more and more steroids in order to more fully enlighten the problems under investigation. It is also of importance that such multisteroid analyses be possible using as small sample quantities as possible. It would be of great benefit, therefore, to be able to measure many steroids from a single sample. This is possible in the case of estradiol, testosterone and 5α-dihydrotestosterone using a radioimmunoassay and/or competitive protein binding technique after one paper chromatography and it is the development of such a method which concerns us here.

MATERIALS

The following solvents were used as purchased, diethyl ether (Merck, UVASOL), acetone (Fisher, certified A.C.S.), n-heptane and benzene (Fisher, Certified A.C.S.), toluene (M&B, analar) and absolute ethanol (AB Vin- och spritcentralen, Stockholm). Each new lot of methanol which was to be used for the elution of paper chromatograms was tested for possible residues which interfered with the binding of testosterone and/or estradiol to TeBG (testosterone–estradiol binding globulin) and antibody, respectively. No significant interference has yet been encountered. PCG (paperchromatography) was performed on Whatman no. 1 chromatography paper, each paper cut into eight 2 cm wide strips, prewashed by Soxhlet extraction, five times with double distilled water, four times with absolute ethanol and three times with redistilled n-hexane. Human γ-globulin (KABI, Stockholm), 250 mg/10 ml, in normal saline containing 0·1% sodium azide, was stored at 4°. BSA (Schwarz–Mann, lot No. 1146) 1 mg/10 ml in double distilled water was also stored at 4°. Reagent grade ammonium sulfate was dissolved in double distilled water with excess of crystals indicating saturation. Plasma was obtained from pregnant subjects during the third trimester, heparinized, and stored frozen in 1 ml lots in sealed ampoules. The estradiol antibody was a gift and was raised in rabbits against estradiol-17β-6-CMO-BSA [1]. The 5α-dihydrotestosterone antibody was obtained commercially (Endocrine Sciences Tarzana, Cal.) against testosterone-3-oxime-BSA but with satisfactory crossreaction to 5α-dihydrotestosterone [2]. Borate buffer (0·05 M, pH 8·0) was stored at 4°. Purified [1α,2α-^{3}H(n)]testosterone (NEN), S.A. 40 Ci/mmol, was dissolved in benzene to give $3{\cdot}5.10^6$ d.p.m./300 μl for assay reagent and 1500 d.p.m./50 μl for recovery reagent. Purified [6,7-^{3}H]-estradiol (NEN), S.A. 48 Ci/mmol was dissolved in benzene to give 350,000 d.p.m./10 μl for assay reagent and 2000 d.p.m./50 μl for recovery reagent. All radioactive solutions were stored at −20°.

Purified testosterone (Sigma) and estradiol (Sigma) were dissolved in absolute ethanol to give standard solutions of 0·35, 0·70, 1·0, 1·5 and 2·0 ng/100 μl for testosterone and 25, 50, 75, 100 and 150 pg/100 μl for estradiol and these were stored at 4°. Steroids used as PCG standards only, purchased from Sigma and not further purified were: estrone, 5α-dihydrotestosterone (DHT), 4-androstene-3,17-dione, 5-androstene-3β,17β-diol, 17β-hydroxy-5α-androstan-3-one, 5α-androstane-3β,17β-diol, dehydroepiandrosterone (DHA), 5α-androstane-3α,17β-diol, 3α-hydroxy-5α-androstan-17-one and 3β-hydroxy-5α-androstan-17-one.

Liquid scintillation counting was performed in polyethylene vials (NEN) using 10 ml of a solution of 27·5 g of Permablend (Packard, scintillation grade) dissolved in 5 l toluene.

Method. Twenty-six plasma samples and two distilled water blanks are conveniently processed together. 1500 d.p.m. of [^{3}H]-testosterone and 2000 d.p.m. of [^{3}H]-estradiol are dried down, under nitrogen, in extraction tubes and to these are added the plasma (2–4 ml) and distilled water blanks (2–4 ml). The samples are extracted three times with 5 ml of diethyl ether and the extracts from each sample are combined and dried down under nitrogen at 40°. The dried extracts, after concentrating at the bottom of the tubes by washing down the walls with 300 μl ether acetone (1:1 v/v), are transferred to PCG strips with three washes of 100 μl ether–acetone (1:1 v/v). Four chromatograms (eight strips each) are run in one tank, with 15 μg of testosterone, androstenedione, estrone and estradiol applied to the outer strips of two of the chromatograms to serve as markers. After equilibration for 2 h in the system, methanol (425 ml), heptane (490 ml), distilled water (75 ml), benzene (10 ml), which is a modification of an earlier described system of Bush [3]. The chromatograms were developed for 20 h with overflow.

Testosterone and androstenedione are located by photographing the marker strips using U.V. light, and subsequently estradiol and estrone with the Turnbull reagent soaking these strips in a 0·5% solution of ferric chloride and potassium ferricyanide newly mixed from 1% solutions of the two substances, followed by washes in 10% hydrochloric acid and distilled water to decolorize the background. 5α-Dihydrotestosterone was located with dinitrophenylhydrazine spray [3]. 5-Androstene-3β,17β-diol, 5α-androstane-3β,17β-diol and 3β-hydroxyandrostan-17-one were located with phosphomolybdic acid, 5% in ethanol [3]. 17β-Hydroxy-5α-androstan-3-one, DHA, 5α-androstane-3β,17β-diol and 3α-hydroxy-5α-androstan-17-one were located with the Zimmermann reagent using 1% (w/v) m-dinitrobenzene in ethanol: 3M KOH in ethanol (2:1 v/v). For routine purposes DHT was eluted from the area immediately 5 cm. above 4-androstene-3,17-dione, as located by U.V. photography on a nearby strip with standard steroid.

The areas corresponding to, and 1 cm., before and after, testosterone and estradiol are eluted with 5 ml methanol and dried down under nitrogen at 40°. The residues are then dissolved in 500 μl methanol and 100 μl taken directly into counting vials, and dried down under a stream of warm air, for the estimation of recovery. A convenient vol. of the remaining samples is then taken into glass tubes (80 × 8 mm), where they are dried down under nitrogen at 40° prior to protein binding analysis and radioimmunoassay. Standard series in duplicate of testosterone (0, 0·35, 0·70, 1·00, 1·50, 2·00 ng), estradiol (0, 25, 50, 75, 100, 150 pg) and 5α-dihydrotestosterone (0, 25, 50, 75, 100, 150 pg) are pipetted into and dried down under nitrogen in similar tubes.

Patients. Plasma samples were drawn from endocrinologically normal persons, women aged between 20–64 years (n = 17), and men 18–65 years (n = 11). Six women more than 47 years old are shown in a separate group. The rest of the women plasma samples were taken once during the menstrual cycle. No phase separation was made. Plasma 5α-DHT samples were drawn from 8 women aged between 20–45 years and 9 men aged between 23–37 years.

Protein-binding assay for testosterone. Frozen pregnancy plasma is thawed and 1 ml diluted to 100 ml with borate buffer in a flask containing 3·5. 10^6 d.p.m. of [^{3}H]-testosterone. After mixing the solution thoroughly, 500 μl is pipetted into each of the tubes containing dried eluates and standards and also into two counting vials for estimation of the total radioactivity. The tubes are then mixed gently for 5 s three times under a period of 15 min at room temperature. 500 μl of saturated ammonium sulfate is finally added to each tube after which it is mixed vigorously for 5 s on a vortex mixer, allowed to stand for 5 min and centrifuged at 4500 rev./min for 15 min. 500 μl of each supernatant is pipetted into a counting vial, 10 ml of scintillation fluid added to all vials which are then shaken vigorously for 30 s and counted 4 × 5 min. Testosterone concentrations (ng/100 ml) are calculated using a program written for a Hewlett–Packard model 9810A desk calculator.

Estradiol radioimmunoassay. Two μl of the estradiol antibody is diluted in 6 ml borate buffer (pH 8·0) containing 350,000 d.p.m. [^{3}H]-estradiol and 150 μl

Table 1. Localisation of steroids on paper after 20 h chromatography

Steroid	Maximum cm from start	Borders cm from start
Estradiol	1·5	0 2·6
Testosterone	9·2	6·9–11·1
Estrone	8·0	6·0 9·7 tail
5α-Dihydrotestosterone	23·2	27·0–27·0
Androstenedione	27·5	25·5 28·9
5-Androstene-3β,17β-diol	0·1	−0·7– 0·6
17β-Hydroxy-5α-androstan-3-one	28·0	26·3–30·7
5α-Androstene-3α,17β-diol	0	0·4 4·0 tail
DHA	21·0	19·5 24·5
5α-Androstane-3α,17β-diol	off paper	
3α-Hydroxyandrostan-17-one	off paper	
3β-Hydroxyandrostan-17-one	30·5	28·5 33·5

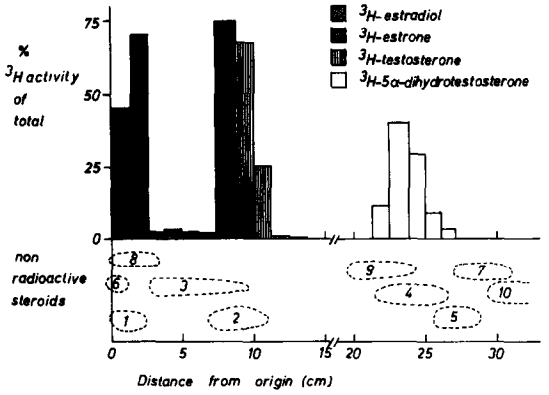

Fig. 1. Distribution of steroids on paper after chromatography. List of non-radioactive steroids shown in the figure: 1. Estradiol, 2. Testosterone, 3. Estrone, 4. 5α-Dihydrotestosterone, 5. Androstenedione, 6. 5-Androstene-3β,17β-diol, 7. 17β-Hydroxy-5α-androstan-3-one, 8. 5α-Androstane-3β,17β-diol, 9. DHA, 10. 3β-Hydroxyandrostan-17-one.

each of γ-globulin and albumin solutions. 200 μl of this antibody reagent is pipetted into the tubes containing the dried estradiol eluates and standards and into two counting vials. The samples are then allowed to stand at room temperature for 3 h with occasional shaking, after which 200 μl of saturated ammonium sulfate is pipetted into each of them, mixed vigorously, without frothing, and centrifuged at 4500 rev./min for 15 min. 200 μl of the supernatants is then pipetted into counting vials and all vials counted for 4 × 5 min with 10 ml scintillation fluid. The concentration of estradiol (pg/100 ml) is then calculated using a program written for the desk calculator.

5α-Dihydrotestosterone radioimmunoassay. 20 μl testosterone antibody is diluted in 16 ml borate buffer (pH 8·0) containing 350,000 d.p.m. ^{3}H-testosterone and 200 μl albumin and 300 μl γ-globulin solution. 250 μl of this is taken to the standards and unknown samples. 250 μl supernatants is taken to counting as described above.

Calculations. The programs for the calculation of both testosterone, estradiol and 5α-dihydrotestosterone concentrations are essentially the same. The only difference is that whereas the estradiol and 5α-dihydrotestosterone standard curve is of the form $(1/y + c) = ax + b$ over the whole range of concentrations used, that for testosterone is a straight line, $y = ax + b$, for concentrations 0 – 1·00 ng and of the form $(1/y + c) = ax + b$ from 1·00 ng upwards. The effect of the ^{3}H-steroids added to the plasma for the estimation of recovery is compensated for in the calculation of the per cent steroid bound to protein.

Deviations from the theoretical curves for each standard and regression coefficients and constants for each curve are calculated as a continual control of the reliability and reproducibility of the standard curves. Deterioriation of antibody or TeBG is soon reflected in these data. Blanks are calculated and subtracted from all samples.

RESULTS

For the distribution of the standard steroids see Table 1 and Fig. 1. Estradiol is separated from both testosterone and estrone, and testosterone is separated from 5α-dihydrotestosterone and androstenedione in the chromatography system used. Estrone shows a tendency to streaking and coincides to a great extent with testosterone. 5α-Dihydrotestosterone and androstenedione are relatively closely located to one another. None of the steroids cross-reacting to a great extent with TeBG are located near testosterone or 5α-dihydrotestosterone (Table 1, Fig. 1). DHA however coincides with DHT. Blank values of both testosterone (0·90 ± 0·02 SE ng/100 ml, n = 20) and estradiol (1·32 ± 0·23 SE pg/ml, n = 21) were low (Table 2). Recoveries were relatively high, being 65·1 ± 0·86

Table 2. Data of the analytical methods for testosterone and estradiol

	Estradiol	n	Testosterone	n
Blank values	1·32 ± 0·23 SE pg/ml	21	0·09 ± 0·02 SE ng/100 ml	20
Per cent recovery	66·68 ± 0·74 SE	133	65·11 ± 0·86 SE	174
Mean difference between duplicates:				
below 60 pg/ml	6·1 ± 1·0 SE pg/ml	49		
above 60 pg/ml	15·9 ± 3·4 SE pg/ml	25		
below 100 ng/100 ml			6·5 ± 1·2 SE ng/100 ml	33
above 100 ng/100 ml			26·4 ± 5·7 SE ng/100 ml	22
Coefficient of interassay variation, per cent:				
below 60 pg/ml	14·8	49		
above 60 pg/ml	7·2	25		
below 100 ng/100 ml			10·2	33
above 100 ng/100 ml			6·8	22
Plasma concentrations in women:				
above 47 years, Mean ± SE	42·9 ± 7·9 pg/ml	6	75·1 ± 13·7 ng/100 ml	6
Range	24·9 ± 93·6 pg/ml	6	28·1 ± 121·0 ng/100 ml	6
below 47 years, Mean ± SE	82·2 ± 14·3 pg/ml	11	48·3 ± 7·8 ng/100 ml	11
Range	22·9 ± 192·6 pg/ml	11	18·7 ± 110·0 ng/100 ml	11
Plasma concentrations in men:				
Mean ± SE	55·5 ± 3·8 pg/ml	11	550·3 ± 44·5 ng/100 ml	11
Range	35·3–71·5 pg/ml	11	339–835 ng/100 ml	11

Table 3. Recovery of testosterone and estradiol

Estradiol					
pg added	25	50	75	100	150
pg recovered, mean ±SE	26·4 ± 2·0	51·0 ± 2·7	71·9 ± 4·1	106·0 ± 4·3	150·0 ± 2·3
n	9	12	3	8	5
Testosterone					
ng added	0·35	0·70	1·00		1·50
ng recovered, mean ±SE	0·356 ± 0·36	0·730 ± 0·05	0·958 ± 0·63		1·475 ± 0·07
n	8	6	5		6

SE % (n = 174) for testosterone and 66·7 ± 0·74 SE % (n = 133) for estradiol (Table 2). The interassay variation between duplicate samples was small and the coefficient of variation was 14·8% for concentrations below 60 pg/ml estradiol and 8·6% for concentrations above this value (Table 2). In the case of testosterone the coefficient of variation was 10·2% for concentrations below 100 ng/100 ml and 6·8% for those above (Table 2). Standard quantities of steroids added to plasma prior to extraction were recovered satisfactorily in the case of both testosterone and estradiol (Table 3). The regression line for the plot of added quantities of estradiol against those quantities recovered was y = 1·106 + 1·009x with standard error 8·8 pg/ml and a regression coefficient of 0·979 ($P < 0.0005$) (see Fig. 2). In the case of testosterone the equation was y = 0·027 + 0·962x with standard error 0·11 ng/100 ml and regression coefficient 0·969 ($P < 0.0005$) (see Fig. 2).

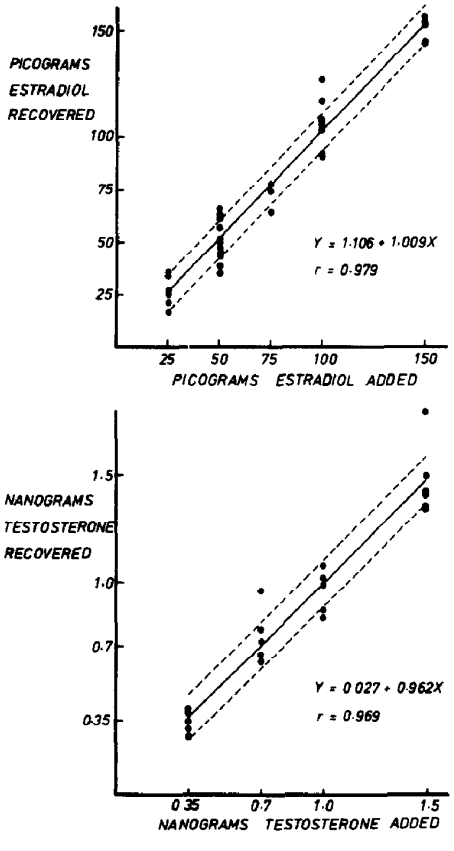

Fig. 2. Recovery of added testosterone and estradiol.

The plasma estradiol levels in samples measured with this method varied between 22·9–192·6 pg/ml in women below 47 years, between 24·9–93·6 in women above 47 years of age, and 35·3–71·5 in men. Testosterone levels varied between 18·7–121·0 ng/100 ml in women and 399–835 ng/100 ml in men (see Table 2).

In the 5α-dihydrotestosterone measurements we got low blank values (0–3 pg). DHT levels in female plasma varied between 1·43–12·4 ng/100 ml with a mean value of 5·4 ng/100 ml (n = 8). Plasma levels in males varied between 15·6–37·8 ng/100 ml, with a mean of 26·8 ng/100 ml (n = 9). (See Table 4). There was a good recovery of 5α-dihydrotestosterone added to plasma samples (Table 5).

DISCUSSION

By this 20 h overflow system we are able to separate crossreacting steroids. Using this solvent system we are able to keep the steroids on the paper, cut off their tails and thus make them analysable. By this overflow system we have also solved the problem of high water blanks, which have earlier been a problem in the radioimmunoassay after paper chromatography.

We have so far only developed the method for the specific determination of testosterone and estradiol using protein binding analysis for the testosterone determination and radioimmunoassay for estimation of estradiol. However, antisera are available for both testosterone and estradiol which also specifically bind 5α-dihydrotestosterone and estrone respectively with relatively high binding affinity. This makes possible the use of the same antibody for the determination of both estradiol and estrone and likewise testos-

Table 4. DHT plasma values ng/100 ml

	Women	Men
	7·8	15·6
	6·6	17·6
	1·4	29·7
	12·4	22·9
	9·6	34·0
	3·0	28·2
	1·6	22·9
	0·7	32·2
		37·8
Mean	5·4	26·8
SE	0·8	2·51

Table 5. Recovery of 5α-dihydrotestosterone added to plasma samples

Nothing added ng/100 ml	Added ng/100 ml	Theoretical ng/100 ml	Found ng/100 ml	Difference ng/100 ml
9·6	5	14·6	13·1	1·5
9·6	10	19·6	19·3	0·3
9·6	15	24·6	24·3	0·3
12·4	5	17·4	15·7	1·7
12·4	10	22·4	22·1	0·3
12·4	15	27·4	32·1	4·7
17·5	5	22·5	23·2	0·7
17·5	7·5	25·0	27·6	2·6

terone and 5α-dihydrostestosterone. The few preliminary estimations of 5α-dihydrotestosterone which we have carried out so far have been satisfactory and the results have, for women, agreed with those in the literature [4]. For men our values are within the range of those found by others [5]. 5α-Dihydrotestosterone measurements using competitive protein binding analysis, in female plasma, are difficult because of the low values, although the greater binding affinity of TeBG for DHT compensates this to some extent and makes the analysis feasible with a 4 ml plasma sample. The plasma estradiol and testosterone levels as measured agree well with those previously reported from this laboratory [6, 7] as well as with those reported in the literature [8, 9, 10]. The regression lines for the recovery experiments were very close to ideal and the slope of the estradiol curve was very close to 1 (1·009) and even that for testosterone was close to 1 (0·962). The lines did not show any significant parallel shift (testosterone, 0·027 ng; estradiol, 1·1 pg). The use of the 20 h system presents a time delay but the development of the method to include a greater number of steroids in the same sample compensates this to some extent, and makes the method usable.

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REFERENCES

1. Lindner T. R., Perel E., Friedlander A. and Seitlin A.: *Steroids* **19** (1972) 357–375.
2. Endocrine Sciences Inc., Tarzana, California: *Plasma testosterone radioimmunoassay procedure* (1972).
3. Bush I. E.: *The Chromatography of Steroids.* Pergamon Press, Oxford (1961) pp. 374–382.
4. Parker C. R. Jr., Ellegood J. O. and Mahesh U. B.: *J. steroid Biochem.* **6** (1975) 1–8.
5. Pirke K. M. and Doerr P.: *Acta endocr., Copenh.* **79** (1975) 357–365.
6. Bäckström T. and Carstensen H.: *J. steroid Biochem.* **5** (1974) 257–260.
7. Carstensen H., Amér B., Amér I. and Wide L.: *J. steroid Biochem.* **4** (1973) 45–55.
8. Baird D. T.: *J. clin. Endocr. Metab.* **28** (1968) 244–258.
9. Korenman S. C., Perrin L. E. and McCallum T. P.: *J. clin. Endocr. Metab.* **29** (1969) 879–883.
10. Judd H. L. and Yen S. C.: *J. clin. Endocr. Metab.* **36** (1973) 475–481.