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A SIMPLE PROCEDURE FOR THE COMBINED DETERMINATION OF PLASMA ESTROGEN AND ANDROGEN CONCENTRATIONS BY COMPETITIVE PROTEIN BINDING ANALYSIS*

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SUMMARY

A simple method for the measurement of estradiol-17β, estrone, testosterone and androstenedione in 4–6 ml of human male and female plasma is described.

By ether extraction of alkalinized plasma followed by a single celite column chromatographic step or by two column chromatographic steps on Sephadex LH 20, purified extracts are obtained for competitive protein binding radioassay. Cytosol from six-day pregnant rabbit uteri (according to Korenman) was used for estrogen assays, and human plasma from the third trimester of pregnancy (according to Sciarra) was used for androgen assays.

The plasma values of testosterone and androstenedione were comparable with those obtained after t.l.c. and competitive protein binding. The specificity and sensitivity of the method enabled determinations of plasma estrogen to be made not only in normal adults, but also in hypogonadal patients where estradiol-17β values are at very low levels. The accuracy of these determinations was confirmed by repeated measurements of blanks which range from 0 to 5 pg/ml, and by recovery experiments of known amounts of added estradiol-17β.

INTRODUCTION

IN RECENT years competitive protein binding (CPB) assays have been developed for the successful measurement of plasma concentrations of corticosteroids [1, 2], progesterone [1, 3], androgens [4–6] and estrogens [7–10].

Most of the papers deal with reliability criteria of the CPB methods used to obtain absolute accuracy. As is well known, these criteria are the sensitivity (with its resolving power), precision, specificity and accuracy. With CPB methods accuracy is higher than that obtained with other techniques and is dependent on the sensitivity and specificity. There is nothing further to add to the practicability of these methods which are now widely employed.

Our recent studies have been concerned with the simultaneous determination of androgens and estrogens in plasma. We therefore utilized the CPB methods and developed some practical procedures for the simultaneous determination of androgens and estrogens using ether extraction, column chromatography and different binding proteins for radioassays.

EXPERIMENTAL

Tritiated radioactive tracers were purchased from The New England Nuclear Corporation (Frankfurt-Main, Germany) and purified before use. The specific activity ranged from 40 to 50 Ci/mmol. Spectrograde diethyl ether, methylene

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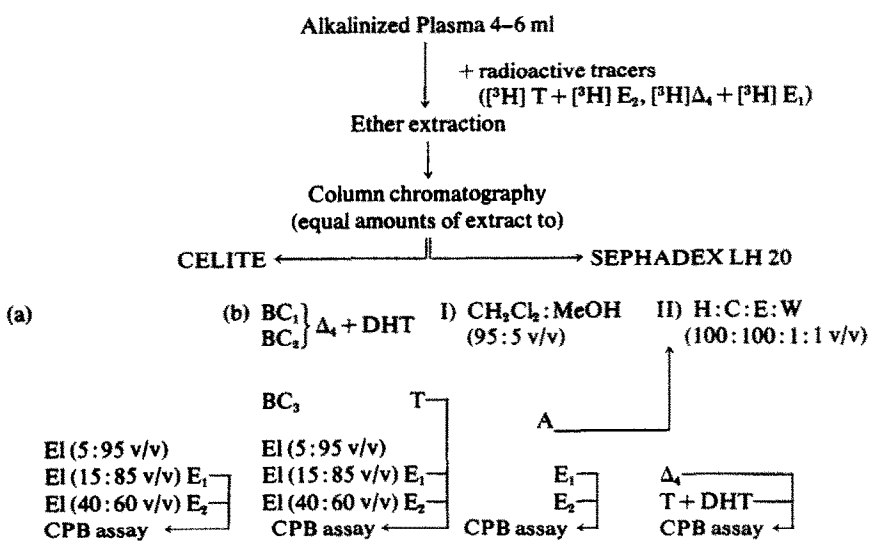
chloride, methanol, toluene, cyclohexane, isooctane, ethyl acetate, chloroform, heptane and ethanol were obtained from Carlo Erba (Milan) and never distilled before use. Benzene was purchased from Fares Co. (Milan); the other chemical reagents used were: sodium borohydride, naphthalene (Carlo Erba), activated charcoal (Riedel-De Haen AG., Seelze-Hannover, Germany), dextran (Schuchhardt, Munich, Germany), celite (Johns-Manville), Sephadex LH 20 (Pharmacia, Uppsala, Sweden), ethylene glycol and dioxane (Merck, Darmstadt, Germany), PPO and dimethyl POPOP (Beckman, Fullerton, U.S.A.).

Plasma extraction and chromatography

Six ml of male and 4 ml of female human peripheral plasma (after the addition of tracers for recovery and one or two drops of NH_4OH) were extracted in test tubes three times with 3 volumes of ether and then washed twice with redistilled water. Ether extracts were dried under nitrogen and then redissolved in small amounts of methanol and divided into two samples. An appropriate volume of suitable solvent was added to each sample and then transferred to different columns.

The Sephadex LH 20 columns were prepared by the "batchwise" technique [11]. Two solvent systems were employed for elution according to Murphy [12], as outlined in Table 1, the first being methylene chloride–methanol (95:5 v/v) with a 16 cm column and a flow rate of 0·40 ml/min. Forty fractions of 1 ml each were collected in order to check the column. Three fractions of 10 ml each were collected for blank experiments. Three aliquots were collected after application

Table 1. Scheme for measurement of androgens and estrogens*



$BC_1BC_2BC_3$ = equal amounts of benzene–cyclohexane (30:70 v/v)
El = ethylacetate–isooctane
H = heptane
C = chloroform
E = ethanol
W = water
A = androgens.

*From *J. steroid Biochem.* 3 (1972) 441.

of the sample, the androgens being eluted with the first 12 ml of effluent, estrone with the next 9 ml and estradiol-17β with the next 11 ml (Fig. 1). The system for androgens was heptane–chloroform–ethanol–water (100:100:1:1, by vol.). The height of the column was 39 cm and the flow rate 0·66 ml/min. Sixty fractions of 2 ml each were collected (Fig. 1). After the blanks were taken from the column and the sample applied, two main aliquots of effluent were taken: 12–20 ml for androstenedione and 30–48 ml for testosterone. The columns were then rinsed with 400 ml of eluant and were ready to be used again. For chromatography on celite columns we found it more advisable to use micro columns (8 cm high and 0·5 cm dia.) as suggested by Korenman [9] since no difference was found with a 16 cm column.

Discontinuous elutions were performed with 6 ml of benzene–cyclohexane (30:70 v/v) divided into three aliquots ($BC_1 + BC_2$ for elution of androstenedione and dihydrotestosterone, BC_3 for elution of testosterone), 10 ml of ethyl acetate-isooctane (5:95 v/v), 10 ml of ethyl acetate-isooctane (15:85 v/v) for elution of estrone, 10 ml of ethyl acetate-isooctane (40:60 v/v) for elution of estradiol-17β (Fig. 2). Eluted androstenedione was converted to testosterone by means of sodium borohydride for 15 min at 5°C; the reaction product was rechromatographed either on columns (System II) or on t.l.c. (alumina sheets, benzene–ether 60:40 v/v) and eluted in the testosterone area.

Aliquots from isolated compounds were counted in a Tri-Carb liquid scintillation spectrometer Model 3380 for calculations of recoveries. These ranged from 65 to 95% for estrone, estradiol-17β and testosterone and from 45 to 60% for androstenedione.

Aliquots were transferred to test tubes for CPB assays.

Preparation of binding proteins

Plasma from women in the third trimester of pregnancy was used for the CPB assay of testosterone and androstenedione according to Sciarra [6]. Although this sex steroid binding plasma protein (SBP) has a high affinity not only for

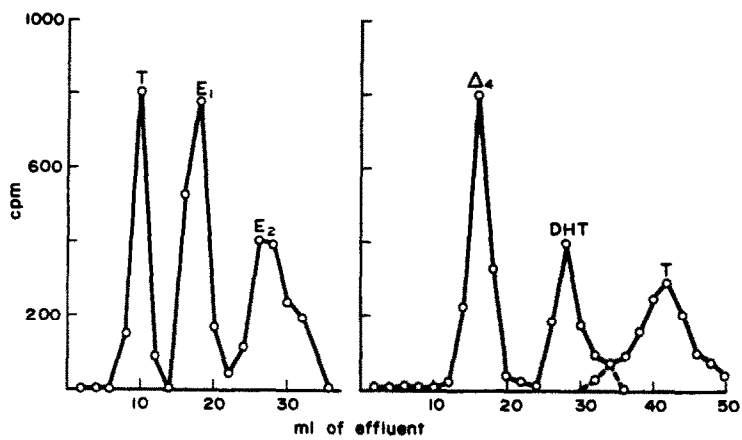


Fig. 1. Column chromatography on Sephadex LH 20. Androgens and estrogens were isolated using CH_2Cl_2–MeOH (95:5 v/v) as effluent. Column height: 16 cm; flow rate: 0·40 ml/min. Androstenedione, dihydrotestosterone and testosterone were isolated by chromatography with heptane–chloroform–ethanol–water (100:100:1:1, by vol.) as eluant. Column height: 39 cm; flow rate: 0·66 ml/min.

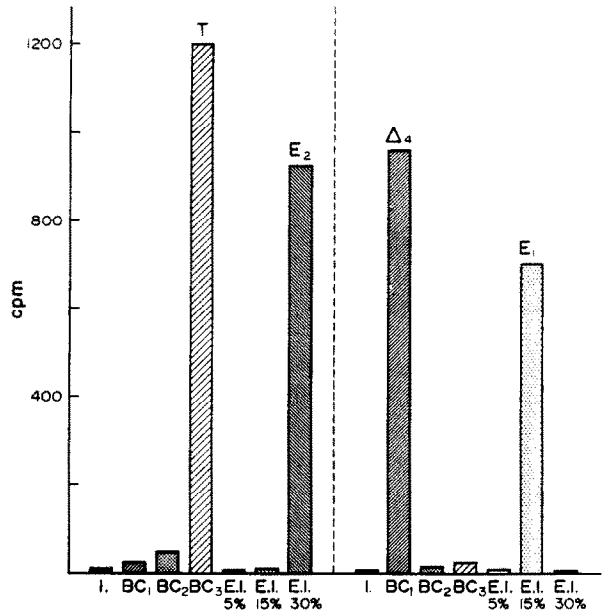


Fig. 2. Typical elution pattern from celite column. Column height: 8 cm; dia.: 0·5 cm; I = Isooctane; BC = Benzene–cyclohexane (30:70 v/v); EI = ethylacetate–isooctane.

testosterone but also for estradiol-17β[13–16], for the CPB assay of phenolic steroids a protein present in the cytosol of 6-day pregnant rabbit uteri was used according to Korenman[7]. An attempt to employ uterine cytosol from immature rabbits was unsuccessful. Aliquots of bound steroids from each sample were transferred into vials with 10 ml of Bray's solution (toluene 1000 ml, dioxane 1000 ml, methanol 600 ml, naphthalene 208 g, PPO 13 g, dimethyl POPOP 260 mg), and the radioactivity was counted in a Tri-Carb scintillation spectrometer (efficiency for tritium 45%) for 50 min.

Calibration curves of estradiol-17β (Fig. 3) and estrone (Fig. 4) were constructed from the results of duplicate standards. The unknown amounts of estradiol-17β and estrone were calculated from these curves, corrected for recovery and expressed as picograms per ml of plasma.

Details on the plasma androgen determinations can be found in the paper of Sciarra *et al.*[6], who measured the androgen concentrations reported in this work.

RESULTS

Blank values for the present methods are presented in Table 4, and recovery experiments of known amounts of steroids added to water or to plasma are shown in Table 2. Results of duplicate analyses for estradiol-17β and estrone obtained from different runs of column chromatography are presented in Table 3. All these results prove the reliability criteria of our method.

The accuracy of the method has been ascertained by addition of a known amount of estrone or estradiol-17β to one aliquot of the sample (Table 2).

The sensitivity and specificity of the method seem to be already well established. Standard curves of estradiol-17β and estrone are shown in Figs. 3 and 4. These curves were constructed using the mean values of 24 estradiol-17β and 10

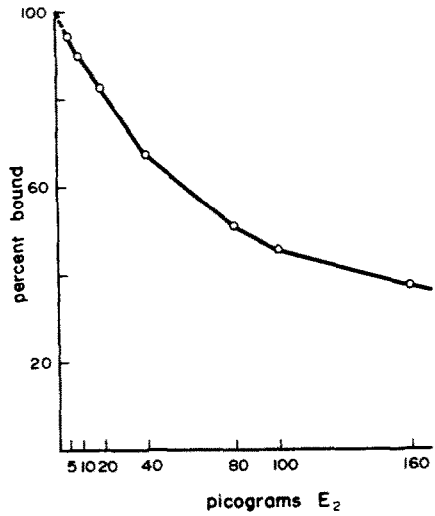


Fig. 3. Standard curve of estradiol-17β. Each point represents the mean value of 25 determinations (S.D. = ±5).

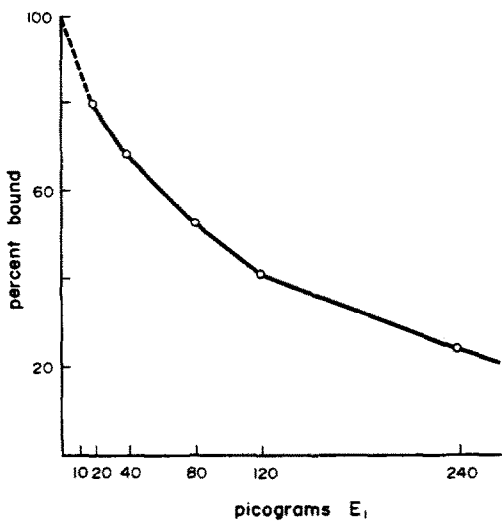


Fig. 4. Standard curve of estrone. Each point represents the mean value of 10 determinations (S.D. = ± 5).

estrone determinations. The standard deviation calculated for each point of the curves was ± 5 for both estrone and estradiol-17β.

Assay of blanks was repeated before and after cleaning of glassware (which was kept from contact with polluted air) and using different types of solvents and solvents of different brands.

Mean blank values of water samples, which were extracted, chromatographed on column and quantified using cytosol from 6-day-pregnant rabbit uteri, were 5 ± 2 pg/ml for estradiol-17β and 10 ± 5 pg/ml for estrone. These blank values enabled us to measure the androgens with CPB after one or two column chromatographic steps. No substantial difference was found using celite

Table 2. Recovery of added estradiol and estrone

Sample	Estradiol added (pg/ml)	Found (pg/ml)	Expected (pg/ml)
Water	12·5	14·8	12·5
	20·0	23·7	20·0
	20·0	19·7	20·0
	30·0	27·4	30·0
	30·0	36·4	30·0
	40·0	45·0	40·0
	50·0	49·0	50·0
Plasma	Control	99·0	—
	5·0	106·0	104·0
	Control	23·3	—
	11·6	35·6	34·9
	Control	23·2	—
	20·0	37·0	43·2
	Control	7·7	—
	20·0	20·0	27·7
	Control	28·9	—
	22·5	60·5	51·4
	Control	41·1	—
	40·0	72·1	81·1
	Control	50·4	—
	50·0	100·4	104·0
	Control	26·1	—
	12·5	36·5	38·6
	33·3	57·7	59·4
	Estrone added (pg/ml)		
	Control	86·4	—
	30·0	124·2	116·4
	Control	166·0	—
	30·0	210·0	196·0

or Sephadex LH 20. The results obtained are also comparable with those obtained after t.l.c. (Fig. 5).

Results of plasma levels of estradiol-17β and of estrone in normal women and normal men, as well as in some pathological cases, are shown in Table 4.

DISCUSSION

Korenman's method [7–9] for the determination of plasma estrogens with cytosol from 6-day pregnant rabbit uteri provides a simple and an accurate technique which gave us good results. Different cytosol preparations produced only minimal differences in the standard curves, as long as the percentage of labelled steroid bound to the protein was constant. The uterine cytosol was found to lose its activity in frozen aliquots 10–15 days after preparation. There was a direct correlation between the amounts of cytosol used and the percent of

Table 3. Plasma concentrations of estradiol and estrone: duplicate values obtained from chromatography on different columns

Estradiol		Estrone	
Celite (pg/ml)	Sephadex LH 20 (pg/ml)	Celite (pg/ml)	Sephadex LH 20 (pg/ml)
20·8	23·2	98·2	110·4
31·1	37·0	85·8	113·6
14·6	19·7	60·8	45·9
10·2	12·9	92·0	106·0
26·0	25·5	113·0	132·0
97·0	103·0	54·0	62·0
89·0	107·0	200·0	215·0
12·2	16·0	77·5	58·9
39·0	44·2	90·8	73·0
91·2	99·2	73·0	55·1

Table 4. Plasma estrogen concentrations

Case	*n*	Estradiol (pg/ml)	S.D. (pg/ml)	Estrone (pg/ml)	S.D. (pg/ml)
Blanks	25	5·0	2·2	10·0	5·0
Ovariectomized					
women: (1) 30 yr		15·3		50·0	
(2) 33 yr		18·5		—	
Normal women:	18				
follicular phase I		50·4	15·2	90·0	30·0
follicular phase II		177·0	50·0	—	
luteal phase		150·0	30·4	130·0	20·0
Normal men	8	20·6	10·7	71·4	5·8
Hypogonadal men	6				
	(1)	17·0		—	
	(2)	9·1		68·1	
	(3)	7·2		59·9	
	(4)	4·5		64·0	
	(5)	6·2		—	
	(6)	8·5		34·0	
Gynecomastic men	3				
	(1)	18·6		49·6	
	(2)	28·0		66·0	
	(3)	49·2		71·6	

n = number of assays.

labelled steroid bound. Whereas the sensitivity of the method could be increased using a smaller amount of cytosol, the accuracy decreased.

Estimation of other competitive steroids and of the diurnal variation of estrogens throughout the entire menstrual cycle was not performed.

The results we obtained are in agreement with those reported by Korenman, but not with those reported by Shutt[17]. who, using a macromolecular fraction from uteri of ovariectomized ewes, found higher values.

In comparison with the fluorimetric[18], gaschromatographic[19] and double

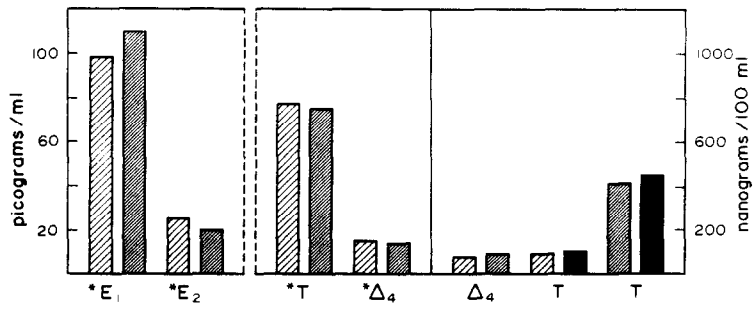


Fig. 5. Comparison of the results obtained with different chromatographic procedures (on celite ▨, on Sephadex LH 20 ▨ and by t.l.c ■). Steroids with asterisks are from the same subject. Values of estrone and estradiol-17β are expressed as pg/ml: values of androstenedione and testosterone as ng/100 ml of plasma.

isotopic derivative[20] methods, the determination of estradiol-17β and estrone by CPB appears to be a simple one. Standard curves were always reproducible and duplicate samples could be measured with an error of less than 5%.

Special care was taken to isolate the steroids in cases where androgens were to be measured. In these cases the addition of tritiated androstenedione and estradiol-17β as internal standards was found to be more suitable. Testosterone levels could be corrected for procedural losses after the first or second chromatographic step, and those of androstenedione after borohydride reduction to testosterone and further chromatography.

Estradiol-17β tracer was used for recovery of both estrone and estradiol-17β. No significant difference was found when labelled estrone was used for recovery.

As depicted in Fig. 1, column chromatography on Sephadex LH 20 according to Murphy[12] was useful for the separation of androgens from estrogens: with these types of columns androgens must be subsequently rechromatographed in the second system, as indicated in Table 1. A single celite column, however, provided a reasonable separation of both androstenedione and testosterone as well as of estrone and estradiol-17β (Fig. 2).

The combined use of the two columns (celite followed by Sephadex LH 20 in system II) was shown to be useful when dihydrotestosterone had to be isolated from androstenedione and testosterone. In fact on celite columns it was possible to separate dihydrotestosterone from testosterone, while with the Sephadex LH 20 columns androstenedione could be separated from dihydrotestosterone.

Good agreement was obtained with duplicate samples using chromatography on different columns (Table 3), as well as when the sample was measured with different cytosol preparations.

The close correlation of the results obtained with the two types of columns is also illustrated in Fig. 5: no marked differences were found between the values obtained by the two different procedures. This was true for estrogens as well as for androgens. Duplicate results for testosterone and androstenedione obtained after celite or Sephadex LH 20 and t.l.c. are also compared in Fig. 5.

The sensitivity of the Korenman method allowed us to measure plasma estrogen concentrations not only in normal male subjects, but also in hypogonadal conditions, where extremely low values of estradiol-17β can be detected. The low values were checked each time against parallel blank values which, however, were not subtracted from the values obtained. When the value of the blank was

higher than expected the results obtained were discarded and the determination repeated. Furthermore, to ascertain the accuracy of the method, each experiment was performed with a parallel sample to which a known amount of estradiol-17β was added.

Values of estradiol-17β and estrone in three gynecomastic patients are reported in Table 4. In only one case was estradiol-17β found to be higher than in normal men.

Estrone values were always within the normal range in hypogonadal and in gynecomastic patients probably on account of an adrenal origin of this compound.

Further studies on these pathological cases are in progress in our laboratory, indicating that the method we propose for the combined determination of plasma androgens and estrogens appears to be suitable for such investigations.

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