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QUANTITATIVE ESTIMATION OF URINARY ESTROGENS DURING THE MENSTRUAL CYCLE BY GAS LIQUID CHROMATOGRAPHY WITH A GLASS CAPILLARY COLUMN

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SUMMARY

A method is described for the measurement of estrone, estradiol-17β and estriol in nonpregnant women, using 5 ml urinary samples. It requires only one purification step subsequent to extraction. This simplification is made possible by the use of anion-exchange chromatography on AG1-X2 and especially, by high resolution gas liquid chromatography with a glass capillary column. Data on specificity, accuracy and precision, demonstrate the reliability of this method.

INTRODUCTION

The quantitative analysis of urinary estrogens during the menstrual cycle belongs to the group of difficult hormone assays [1]. This is due to the low steroid concentrations and to the abundance of various contaminants [2].

Gas liquid chromatography (g.l.c.) has been used frequently to increase specificity of a quantitative estimation. Purification procedures, preliminary to g.l.c. of the urine sample, consist mainly of:

(i) selective extraction of estrogens from an organic phase into soda lime solution [3–10]; (ii) thin layer chromatography (t.l.c.) [3, 5, 7–9, 11, 12] or adsorption chromatography on an alumina column [4, 6, 10]; (iii) formation of methylethers or saponification [3–5, 8]. More recently was introduced the ion exchange chromatography on AG1-X2 anionic resin, which simplified the purification procedures [11–13]; (iv) extraction with Amberlite XAD-2 resin and liquid-gel ion exchange chromatography on modified Sephadex LH-20 [14, 15].

Gas chromatographic columns packed with supports, coated with stationary phases of various polarity (OV-1, XE-60, OV-225), have been used in conjunction with a flame ionisation detector, or sometimes with an electron capture detector [8, 9, 12].

Progress made by the use of glass capillary columns and by the coupling of these columns with a mass spectrometer, have drastically increased the specificity and simplified the purification of extracts prior to g.l.c. analyses of steroids [16]. Consequently, we started to develop the use of glass open tubular columns for the analysis of estrogens [10]. The present paper describes a simple and specific method for the quantitative estimation of urinary estrone (E_1), estradiol-17β (E_2) and estriol (E_3) during the menstrual cycle.

The following trivial names, not listed in *J. steroid Biochem.* have been used: androstenediol: 5-androstene-3β, 17β diol; pregnenediol: 5-pregnene-3β, 20-diol.

EXPERIMENTAL

Steroids. [2, 4, 6, 7-^{3}H]-estrone ([^{3}H]-E_1; 100 Ci/mmol), [2, 4, 6, 7-^{3}H]-estradiol-17β ([^{3}H]-E_2; 100 Ci/mmol), [2, 4, 6, 7-^{3}H]-estriol ([^{3}H]-E_3; 77 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England and non radioactive steroids were supplied by Steraloids Inc., Wilton N.H., U.S.A.

Solvents and reagents. Diethylether was freshly distilled and peroxide free; water was bidistilled. Absolute ethanol and *n*-hexane for U.V. spectroscopy were used without further purification; benzene was made anhydrous on sodium wires; pyridine was refluxed on KOH and distilled. All these solvents were obtained from E. Merck, Darmstadt, G.F.R., AG1-X2 ion exchange resin (200–400 mesh) was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. The silylation reagents were: N, O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA; Supelco Inc., Bellefonte, Pen., U.S.A.), trimethylchlorosilane (TMCS; Pierce Chemical Co., Rockford, Ill., U.S.A.).

Hydrolysis. Helix pomatia digestive juice, with a β-glucuronidase titre of 140,000 Fishman units and an arylsulfatase titre of 55,000 Roy units per ml was used for hydrolysis.

Preparation of micro-columns. The AG1-X2 resin was prepared under the HCO_3^- form, prior to pouring into the columns (55 × 5 mm). This small bed volume assured a good reproducibility of the separation.

An amount (12.5 g) of resin, required for the packing of six columns was weighed, washed eight times with 20 ml bidistilled water and treated three times with 20 ml $NaHCO_3$ 0.5 M and twice with 20 ml ethanol–water (8:2, v/v). The resin was then suspended

twice in 5 ml pure ethanol and the final suspension was degassed under vacuum at −20°C. The micro-columns were poured in four or five portions while sufficient solvent remained on top. The eluents were successively: pure ethanol and ethanol–water (8:2), v/v); the flow rate through the six micro-columns was maintained between 20 and 25 ml/h with a peristaltic pump.

Formation of trimethylsilylethers (*TMSi*): The urinary extract was dissolved in 100 μl benzene–pyridine (50:50, v/v), containing 6-oxo-estradiol as an internal standard. Silylation was performed with 100 μl BSTFA containing 5% TMCS at 60°C overnight.

Gas liquid chromatography. g.l.c. was performed on a Carlo Erba chromatograph (model G1) equipped with an all glass solid injector [17] and with a flame ionisation detector. The column was mounted in such a way as to exclude all contact of the sample with hot metal surfaces. The open tubular column was drawn with Pyrex glass and statically coated with SE-30 stationary phase, according to Bouche and Verzele[18].

Column characteristics were:

40 m length, 0.3 mm internal diameter; number of effective plates, measured on the E_3-$(TMSi)_3$ peak, N = 80,000; partition ratio for E_3-$(TMSi)_3$, k = 14.1.

Column temperature was isothermal at 215°C; carrier gas was hydrogen with a flow rate, measured at column outlet and ambient temperature, of 2.7 ml/min.

Method. The 24-h urines were kept at 4°C in presence of sodium mercurothiolate when they were stored for a period of less than 6 months, otherwise they were frozen and kept at −20°C.

A 5 ml aliquot of urine was buffered with 0.5 ml of sodium acetate buffer (2 M, pH 5.2) and incubated for 18 h at 37°C with *Helix pomatia* digestive juice (750 Fishman units per ml urine). After addition of a fresh ethanolic solution of radioactive tracers, the hydrolysate was extracted with 15 ml ether. The organic phase was washed, first with 5 ml Brown buffer (150 ml NaOH 20% and 100 ml $NaHCO_3$ 8%) [19], then twice with 3 ml water and subsequently evaporated under nitrogen at 50°C. The dry extract was quantitatively transferred on the AG1-X2 column with 3 times 500 μl ethanol and allowed to penetrate slowly into the resin. Elution was started immediately; the first fraction contained 11 ml pure ethanol and 1.5 ml ethanol–water (8:2, v/v). The next fraction (5.5 ml ethanol–water, 8:2, v/v) containing the estrogens, was evaporated under nitrogen at 60°C and the TMSi derivatives were formed. After evaporation of the silylation reaction mixture under nitrogen at approximately 55°C, the extract was dissolved in *n*-hexane or in a hexane solution containing the *n*-alcanes (*n*-pentacosane, *n*-heptacosane and *n*-nonacosane) required for the calculation of the retention indices. A 4 μl aliquot was deposited on the needle of the solid injector in order to perform quantitative estimation, another aliquot was used for the determination of the recovery.

A standard curve was plotted for each series of estimations. A 50% variation of the peak height of the internal standard for one day was considered to be due to a significant modification of the chromatographic conditions and was thus incompatible with correct quantitative determinations. Care was also taken to run sufficiently diluted solutions as to avoid saturation of the capillary column, which occurred for quantities equal or higher than 50 ng per compound.

RESULTS AND DISCUSSION

Ion exchange chromatography on AG1-X2

The separation of estrogens on highly basic resins was introduced by Sjöström and Nykänen[20]. Eberlein[11] has demonstrated the interest of the purification on the AG1-X2 resin, in order to avoid extraction by strong alkali. With our procedure, we obtain a very efficient separation between neutral and phenolic steroids (Table 1). The mechanism of this separation depends primarily on the presence of the phenol group. This is shown by the considerable decrease of the elution volume of the estrogens after methylation of the phenolic hydroxyl group. This resin also has a high enough capacity, because no modification of the elution volumes was observed when a mixture of 50 μg of each estrogen E_1, E_2, E_3 was run on our columns.

Specificity

It is generally admitted that pure compounds can be accurately identified by their retention indices [21] on a high resolution capillary column. In the case of urinary extracts, the usefulness of retention indices for identification purposes is limited by the risk of interferences due to contaminating drugs or drug

Table 1. Neutral steroids eluted in the first fraction of chromatography on AG1-X2

Steroids	GLC Solvent systems	R_f
Androsterone	II	0.35
11-hydroxyandrosterone	II	0.15
Dehydroepiandrosterone	II	0.34
Androstenediol	II	0.23
Pregnenediol*	—	—
Pregnanetriol	III	0.37
Pregnanediol	I	0.15
Tetrahydrocortisol	III	0.41
Cholesterol	II	0.44

Detection: aqueous sulfuric acid solution (10% H_2SO_4 in water) with 2,4-dinitrophenylhydrazine (1%)
Solvent systems:
I: chloroform–ethyl acetate (8:2, v/v)
II: chloroform–ethyl acetate (8:4, v/v)
III: ethyl acetate.
* Detection by radioactivity measurement of labelled steroid.

metabolites. This can be avoided by having a high enough resolution on the capillary column, or checking the identity by g.l.c.-mass spectrometry. Nevertheless, the addition of a solution of *n*-alcanes with 25, 27 and 29 carbon atoms to the urinary extract just before g.l.c., is always advisable in cases where drug contamination is suspected.

Recovery

A systematic control of recoveries increases the accuracy of the method, but as there are different possibilities of conjugation for the three classical estrogens, a method using total internal standards is unfeasible and we were thus obliged to add tracers after hydrolysis. In order to correct variations occurring in the g.l.c. system, a second internal standard (6-oxo-estradiol) is needed. This compound has a chemical structure very close to that of the classical estrogens, it has an intermediate polarity, it is absent in urine and it is well separated on the g.l.c. column from other compounds present in the extract.

Reliability criteria

The urine of a male impuber child has been used as a blank. The gas chromatogram of this urinary extract demonstrated the absence of any measurable signal at the retention times corresponding to the estrogens and at the most sensitive electrometer setting (8×10^{-12} A for a full scale pen deviation on a 1 mV potentiometric recorder).

Accuracy was determined with 48 samples of the urinary blank. After hydrolysis, these samples were distributed in four equal groups, to which were added estrogen amounts equivalent to 10, 25, 50 and 75 μg/l of the initial urine and in the mean ratios encountered during the menstrual cycle (1/3 for E_1, 1/6 for E_2, 1/2 for E_3). The data obtained after analysis of these samples allowed the calculation of the equations of the linear regression lines, according to the method of the least squares (22). These equations are ($n = 48$, $P = 0.05$)

for $E_1: y = (0.87 \pm 0.03)x + (0.02 \pm 0.04)$
for $E_2: y = (0.86 \pm 0.03)x + (0.00 \pm 0.02)$
for $E_3: y = (0.74 \pm 0.04)x + (0.01 \pm 0.07)$

The intercepts to zero are not significantly different from zero and the slope of the regression lines indicates the recovery, which is 87% for E_1, 86% for E_2 and 74% for E_3.

Another procedure has also been used for the calculation of recoveries in urine of the menstrual cycle (7–50 μg/l total estrogens). A tracer amount of E_1, E_2 and E_3 was added to the urine after hydrolysis. The total recovery, measured by liquid scintillation counting of an aliquot, prior to g.l.c., was 83.4% ($n = 62$, $s = 3.7$). The latter control has been performed systematically on all the samples.

Duplicate measurements were performed on 33 urines from normal females. The values are classified in three groups, according to the concentration of E_1,

Table 2. Precision of estrogen determinations

Concentration range (C) (μg/l)	Number of duplicate measurements	$\bar{x}$*	s	cv(%)**
	Estrone			
$C \leq 5$	12	3.95	0.31	8.0
$5 < C \leq 10$	11	7.06	0.19	2.8
$C > 10$	10	15.30	0.49	3.2
	Estradiol			
$C \leq 2$	11	1.51	0.15	10.2
$2 < C \leq 4$	12	2.70	0.16	5.9
$C > 4$	10	5.60	0.14	2.4
	Estriol			
$C \leq 7$	12	5.36	0.20	3.7
$7 < C \leq 15$	12	10.90	0.42	3.8
$C > 15$	9	26	0.64	2.5

* Mean value of C

** $cv(\%) = \frac{100s}{\bar{x}}$

E_2 and E_3 (Table 2). As the time needed for a single g.l.c. analysis took 20–30 min, these measurements were made in several series of analyses delayed on several days. However, each duplicate of a given series was assayed on the same day. The differences d_i between the N duplicate measurements enables the estimation of the standard deviation which characterizes the repeatability

$$s = \sqrt{\frac{\Sigma d_i^2}{2N}}$$

This estimation has been made for concentration ranges where the standard deviation is supposed to remain constant. The results indicate generally, as could be expected, a decrease of the precision with decreasing estrogen concentration.

An *a priori* detection limit, $L_D = 3.29\ s_0$, where s_0 was the standard deviation of precision for the lowest concentration, was obtained with blank values equal to zero (23–24). The values of L_D are:

for E_1: 1.0 μg/l;
for E_2: 0.5 μg/l;
for E_3: 0.7 μg/l.

Application to the study of the menstrual cycle and correlation with a fluorometric method

The urinary excretion levels, which were measured in the follicular and in the luteal phase, are mostly comparable to those quoted in the literature (Table 3), excepted the very low values obtained by Takano[13]. Figure 1 illustrates the study of an entire menstrual cycle. Follicular and luteal phases have been determined by radioimmunoassay of urinary LH

Table 3. Urinary concentrations during the menstrual cycle (μg/24 h), measured by different methods

Author	Phase of cycle	E_1	E_2	E_3
Brown [25]	F	5* (4–7)†	2 (0–3)	6 (0–15)
	L	14 (10–23)	7 (4–10)	22 (8–72)
Knorr [8]	F	3.7 (0.9–5.8)	1 (0.2–1.5)	
	L	12.4 (5.1–24.8)	3.3 (2.7–4.1)	
Kaplan	F	6	2.7	7.3
[9]	L	10.6	4.5	15.3
Morreal	F	11.9	3.5	9
[12]	L	14.6	6.1	19.7
Takano	F	2.3	0.3	2.2
[13]	L	5.2	1.0	7
Present‡ method	F	4.2 (2.6–7)	1.8 (0.8–4.5)	5.6 (3.5–13.3)
	L	7.7 (6.7–11.3)	3.4 (1.1–5.6)	19.4 (11.6–32.6)

* Mean values
† Minimal and maximal values
‡ $n = 10$ for each phase of the menstrual cycle
F = Follicular phase, L = Luteal phase.

and FSH. The chromatograms corresponding to the different periods of the cycle, indicate that the risk for contamination by interfering compounds is higher for the lower estrogen concentrations (5th day of the cycle; 12 μg/l total estrogens) than for the higher concentrations (13th day of the cycle; 62 μg/l total estrogens).

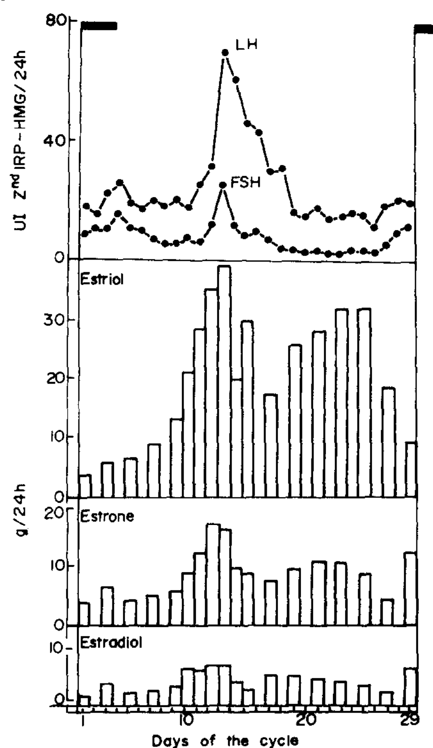

Fig. 1. Urinary estrogen and gonadotropin patterns during the cycle (each point is the mean of duplicate measurements).

The characteristic pattern of the ovulatory menstrual cycle (Fig. 1) shows two maxima for the estrogen excretion during the cycle, the first and generally the most important one is located close to ovulation. The menstrual cycle which we studied, had a 24 h delay between the excretion maxima of estrone and gonadotropins. As it has been noticed by Burger *et al.*[26], this time sequence is compatible with the establishment of a feedback mechanism between the ovary and the hypothalamo-pituitary axis.

Figure 2 illustrates the correlation made between the values obtained by our method (sum of the E_1, E_2 and E_3 concentrations) and those of a routine fluorometric method [27] for the estimation of total estrogens. The confidence interval of the correlation

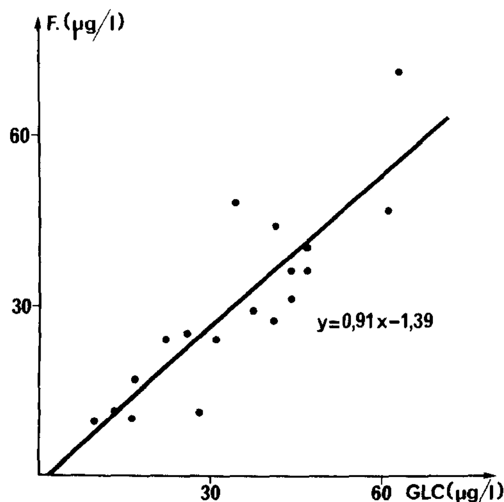

Fig. 2. Correlation between estrogen concentrations obtained by fluorometry (F) and by g.l.c.

coefficient (r) of these two methods is situated between 0.68 and 0.95 ($P = 0.95$). It must be noticed that the higher the concentration range, the better will be r [28]. If we consider the regression line, we observe a discrepancy higher than 40% in two cases. Nevertheless the slope of the regression line is not significantly different from 1 ($P = 0.5$) and the intercept is not different from zero ($P > 0.8$). Thus the agreement between these two methods is acceptable in the concentration range which was studied.

The urinary extracts used for this comparative study had no anomalous fluorescent coloration and they were obtained from subjects who did not absorb drugs.

CONCLUSION

Very few satisfactory methods have been proposed for the control of routine determinations of low level concentration of estrogens. A highly specific technique, such as g.l.c. coupled to mass spectrometry, allows efficient quality control of routine analyses, but our aim here was to devise a less sophisticated method, which is accessible to more laboratories.

The specificity of the various procedures of our method, as well as the controls made at the different stages of the preliminary purification, have contributed to the reliability. The whole procedure can also be applied to the study of estrogens other than E_1, E_2 and E_3. Thus, chemically labile estrogens, such as α-ketolic and catechol estrogens, may be analysed by an analogous method, because no concentrated acids or concentrated bases are used. It may also be mentioned, that the separations obtained on AG1-X2 column, allow the use of the fraction containing the neutral steroids, for further g.l.c. analysis.

Capillary columns are most interesting in two respects, one is the specificity of the quantitative estimations which may be done, and the other is the wide variety of metabolites which may be analysed on a single run. It is our opinion that despite minor drawbacks, such as fragility of the glass capillary column and need for a suitable gas chromatograph equipped with a correct injection system [29], the use of these capillary columns represent a considerable improvement with regard to the conventional packed columns.

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