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MEASUREMENT OF URINARY ESTRIOL GLUCURONIDES DURING THE MENSTRUAL CYCLE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple high-performance liquid chromatographic (HPLC) method for measuring estriol-3-glucuronide (E_3-3-G) and estriol-16-glucuronide (E_3-16-G) in the urine of non-pregnant women is described. Estriol conjugates were extracted from 4 ml of urine with a small cartridge of graphitized carbon black (Carbopack B). After washing, E_3-3-G and E_3-16-G were desorbed separately by a two-step elution system. After solvent removal, the two glucuronides were quantified by isocratic ion-suppression HPLC with fluorimetric detection. The analytical recovery of the two estriol metabolites was about 95%. The detection limit of the method was 0.6 ng/ml for both analytes in urine, which is well below the concentrations of clinical interest, and the method is not susceptible to substantial interferences. Data relative to urinary levels of E_3-3-G and E_3-16-G measured by this method on a daily basis in early morning samples from nine women during their menstrual cycles were compared with those reported in the literature and obtained by radioimmunoassay techniques. Moreover, the potential use of defined changes in the concentrations of the two conjugates for predicting the fertile period of women was assessed.

INTRODUCTION

The identification and assessment of the potential value of measuring estrogen metabolites in female urine to predict the occurrence of ovulation has been the aim of many investigations. Most of these studies indicated that defined changes in the concentrations of urinary estrogen glucuronides might be used as chemical indices to locate the start and finish of the probable fertile period.

Following the production of specific antisera against a wide range of steroid glucuronides, rapid and sensitive radioimmunoassays (RIA) have been developed for measuring intact, individual estrogen metabolites in urine [1–7] and

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they are now a routine procedure in many clinical laboratories for monitoring the ovarian function. However, the increasing proliferation of RIA has raised serious problems concerning health hazards associated with the handling of radiolabelled ligands and solvents necessary for liquid scintillation counting. Another problem is the waste disposal of radioactivity. Moreover, cross-reaction studies made evident that all but one of the estrogen glucuronides measured in urine by RIA are considerably overestimated [6].

To date, only the chemiluminescence immunoassay technique [8,9] has been proposed as an alternative to RIA for determining low levels of intact estrogen glucuronides. Chromatographic techniques have been extensively employed for measuring estrogens in body fluids of both pregnant and non-pregnant women. Only very few of these methods dealt with the analysis of intact estrogen glucuronides by exploiting the features of high-performance liquid chromatography (HPLC) [10–12]. None of these procedures, however, have been applied to monitoring low levels of estrogen glucuronides, such as those excreted in non-pregnant woman urine.

By use of HPLC, we recently succeeded in determining the conjugation profile of the three major estrogens in late-pregnancy body fluids [13]. Such multi-component analysis was performed by a simple and convenient three-step analytical procedure, i.e. solid-phase extraction, solvent removal and quantitation by HPLC. The simultaneous purification and class separation of the analytes mentioned above was accomplished with a cartridge containing Carbopack B, graphitized carbon black (GCB). This material has on its surface some ion-exchange sites, the nature of which was investigated earlier [14], which enable it to act as both an ion exchanger and a non-specific adsorbent.

The main object of this work was to modify suitably the above procedure in order to develop an easily practicable and reliable HPLC assay for measuring estriol-3-glucuronide (E_3-3-G) and estriol-16-glucuronide (E_3-16-G) in the urine of non-pregnant women. We assessed the daily excretions of the two estriol metabolites in early morning urine samples throughout nine menstrual cycles to obtain reference ranges suitable for clinical use and to validate their reliability as chemical indices of the fertile period of women.

EXPERIMENTAL

Chemicals and solvents

Dichloromethane and formic acid were of analytical-reagent grade and the other solvents were of HPLC grade. All were used as supplied. Authentic estriol, E_3-3-G and E_3-16-G were purchased from Sigma (St. Louis, MO, U.S.A.). Stock standards of estriol and its two conjugates were dissolved separately in methanol at 1 g/l and later diluted with methanol to give working standards of concentrations of 20 μg/l. These solutions were stable for at least three months when stored at $-20°$C. The urinary luteinizing hormone concentration was measured with an hCG HAR Kit (Mochida Pharmaceutical, Tokyo, Japan).

Urine collection

Early morning urine (EMU) samples, defined as the first urine passed after rising from bed inthe morning, were collected daily through the menstrual cycle in nine women (aged 20–38 years). Any history of liver or kidney disease or the recent use of hormonal contraceptives precluded inclusion in the study. The volunteers collected an EMU specimen every day starting three days after the first day of appreciable blood flow through the vagina. The collected samples were stored at −20°C until assayed.

Preparation of the Carbopack cartridge

The Carbopack cartridge was prepared as reported elsewhere [13], except that the GCB particles had a size range between 105 and 74 μm. The adsorbing material, plastic tubes and polyethylene frits used to prepare the cartridge were kindly supplied by Supelco (Bellefonte, PA, U.S.A.). The cartridge fitted directly into the vacuum manifold. Before use, the cartridge was washed by passing through it sequentially 2 ml of dichloromethane, 2 ml of methanol and 2 ml of water.

Procedure with urine samples

A 4-ml volume of urine was diluted with 8 ml of water and passed through the Carbopack cartridge at a flow-rate of 6–7 ml/min. Then the absorbent was washed by passing sequentially 5 ml of 10 mmol/l hydrochloric acid and 20 ml of 100 mmol/l methanolic formic acid. E_3-3-G was desorbed by passing through the Carbopack column 5 ml of dichloromethane–methanol (25:75, v/v) containing 25 mmol/l formic acid. Thereafter, E_3-16-G was eluted by passing 5 ml of 25 mmol/l formic acid in dichloromethane–methanol (60:40, v/v). These two solvent mixtures were renewed every two or three days and stored at 0°C when not in use. The two collected fractions were evaporated in a water-bath at 60°C under a stream of nitrogen, the residues were reconstituted in 60 μl of the mobile phase for HPLC (see below) and 40 μl were injected into the HPLC apparatus.

High-performance liquid chromatography

HPLC was carried out with a Perkin-Elmer Series 3B instrument equipped with a Rheodyne Model 7125 injector with a 40-μl loop and a Model 650-10S fluorescence detector. A 25 cm×4.6 mm I.D. column filled with 5-μm C_{18} reversed-phase packing and a guard column containing Pelliguard, both from Supelco, were used. Solvent A was methanol–acetonitrile (23:77, v/v) and solvent B was 1% formic acid. The fraction containing E_3-3-G was chromatographed with 22% solvent A and that containing E_3-16-G with 36% solvent A. The flow-rate was 1.5 ml/min in both instances. The two estriol glucuronides were monitored with the excitation and emission wavelengths of the detector set at 278 and 305 nm, respectively, with 13-nm, slit widths. The concentrations of estriol conjugates in standard and patients' samples were calculated by comparing the peak heights produced by the analytes in the sample with those of reference standards. These were prepared by taking appropriate volumes of working standard solutions and, after solvent removal, by reconstituting the residue with 60 μl of the HPLC mobile phase.

RESULTS AND DISCUSSION

For simplicity and rapidity we initially attempted to determine E_3-3-G and E_3-16-G simultaneously. For this purpose, the two estriol conjugates were collected together in a single fraction by eliminating from the purification scheme the intermediate step for the separation of E_3-3-G from E_3-16-G. However, no chromatographic conditions succeeded in separating E_3-16-G from some unknown urinary constituents, which, vice versa, following the complete purification procedure (see Experimental) no longer interfered in the analysis of E_3-16-G as they were removed from the Carbopack surface together with E_3-3-G.

During the follicular phase, only a few nanograms of estriol glucuronides are usually excreted per millilitre of urine. For the purpose of measuring the two conjugates with adequate precision, the maximum volume of urine that could be applied to the Carbopack B cartridge without loss of the analytes was assessed by processing increased volumes of a pooled male urine spiked with E_3-3-G and E_3-16-G at the level of 50 ng/ml. The results are reported in Table I. As can be seen, loss of E_3-3-G occurred only on processing 10 ml of urine, and no saturation effect was evident for E_3-16-G. The data relating to E_3-3-G appear to conflict with those reported in a recent paper [13] showing that some loss of E_3-3-G was evident on processing more than 1 ml of late-pregnancy urine. This discrepancy might be explained considering that the levels of estrogen conjugates in late-pregnancy urine are about 1000 times higher than those in non-pregnancy urine. Moreover, E_3-3-G, compared with the other estrogen metabolites, had the highest mobility on the Carbopack surface [13]. Consequently, when a urine sample rich in estrogen conjugates is percolated through the Carbopack column, E_3-3-G may be displaced by the other conjugates. The mean recovery of E_3-16-G at a concentration of 50 ng/ml calculated from these experiments ($n=12$) was 96.0% (S.D.=1.18%). The mean recovery of E_3-3-G calculated from nine of these experiments was 94.8% (S.D.=1.54%).

TABLE I

RECOVERIES OF E_3-3-G AND E_3-16-G BY VARYING THE VOLUME OF URINE APPLIED TO THE CARBOPACK CARTRIDGE

Volume (ml)	Compound	Recovery (%)		
		Urine effluent*	Methanol washing*	Eluent system* (mean, $n=3$)
1	E_3-3-G	—	—	93.3
	E_3-16-G	—	—	94.4
2	E_3-3-G	—	—	96.6
	E_3-16-G	—	—	97.0
4	E_3-3-G	—	—	94.9
	E_3-16-G	—	—	96.8
10	E_3-3-G	20.4	18.8	56.3
	E_3-16-G	—	—	95.9

*For details, see Experimental.

TABLE II

WITHIN-ASSAY PRECISION OF THE METHOD AT LOW, MEDIUM AND HIGH URINARY CONTENTS OF E_3-3-G AND E_3-16-G ($n=7$)

Content	Compound	Concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Low	E_3-3-G	5.8 ± 0.29	5.0
	E_3-16-G	4.9 ± 0.24	4.8
Medium	E_3-3-G	15.6 ± 0.56	3.6
	E_3-16-G	14.3 ± 0.44	3.1
High	E_3-3-G	51.4 ± 1.49	2.9
	E_3-16-G	48.7 ± 1.17	2.4

The within-assay and between assay precision of the method at low, medium and relatively high E_3-3-G and E_3-16-G concentrations infemale urine were evaluated by assaying three selected EMU samples, each one seven times, during a working day. The results are shown in Table II. Further, the medium-content urine sample was assayed six times during a month. The between-assay relative standard deviations were 3.9 and 3.5%, respectively, for E_3-3-G and E_3-16-G.

The extent of background interferences eluting at the same times as E_3-3-G and E-16-G was evaluated by analysing pooled urine from four male impuber children. In both instances the background was not higher than 0.7 ng/ml of urine. In addition to the use of a very selective detection technique, such as fluorimetry, two other reasons contributed to creating such a favourable situation. One is that estrogen conjugates have a particular affinity for the GCB surface, probably owing to the presence in these compounds of an aromatic ring. This accounts for the fact that large volumes of acidified methanol, which elutes an impressive number of urinary constituents, can be percolated through the extraction column without loss of the analytes considered. Second, with respect to our previous work [13], the subfractionation of the two fractions containing the two estriol metabolites was performed by the so-called ion-suppression HPLC technique instead of ion-pair HPLC. Although some loss of column efficiency was observed with respect to the previously used technique, the overall selectivity of the chromatographic process was improved. This was expected as the formation of derivatives, as occurs in the ion-pair technique, obscures subtle differences in the physicochemical characteristics of the solutes. From a practical point of view, another advantage of using the ion-suppression over the ion-pair technique is the prolonged column lifetime, as silica is known to be stable in a moderately acidic medium. In fact, after six months of continuous use the chromatographic characteristics of the stationary phase remained completely unaltered. The only precaution adopted to avoid the appearance of double peaks for the analytes of interest was the reversal of the column every fifteen days.

We observed that washing the Carbopack cartridge with acidic methanol was effective in eliminating many drugs in common use, such as barbiturates, antidepressants, antiarrhythmics, salicylates, phenolphthaleine, quinidine and theo-

phylline. Moreover, the solvent mixtures selected for the elution of E_3-3-G and E_3-16-G from the Carbopack cartridge are presumably ineffective for the removal of urinary constituents conjugated with sulphuric acid, as observed for estrogen sulphates [13].

We assessed whether glucuronides of estrogens other than the major three unknown compounds might affect the measurement of the two estriol glucuronides by taking through the procedure thirty urine specimens, calculating the total estriol as the sum of the two measurements. These data were then compared with those for the total estriol concentration, determined by a recently developed HPLC procedure [15], which involves a preliminary enzymatic hydrolysis of estriol conjugates. Linear regression analysis of data obtained by the present method (y) and the comparison method (x) gave the following results: slope = 0.920, intercept = −0.0134 ng/ml, r = 0.998. The mean values were: y = 20.8 ng/ml and x = 22.6 ng/ml. As measured experimentally, the mean content of total estriol is about 9% higher than that calculated from the contributions of the two glucuronides. This is probably due to the fact that the measurement of total estriol includes also estriol liberated by hydrolysis of estriol-3-sulphate and estriol-3-sulphate-16-glucuronide. No data have been reported concerning the conjugation profile of estriol in urine of non-pregnant women. By assuming that this profile is similar to that during the late pregnancy [12], the cumulative contributions of the two sulphate forms of estriol to total estriol should be approximately 12–13%. The two methods under consideration appear to be very well correlated and comparable, thus demonstrating that no unknown, endogenous compound in urine causes significant overestimation of the two analytes of interest.

The detection limit of the method (signal-to-noise ratio = 3) was found to be 0.6 ng/ml of urine for both compounds. At this concentration, the relative standard deviation was about 12%. Fig. 1 shows typical chromatograms obtained by this procedure.

The data obtained from urinary excretion on a daily basis of nine apparently healthy women were applied to the study of the menstrual cycle. The urinary excretion levels of E_3-3-G and E_3-16-G were measured and the mean values obtained both during the follicular phase and the day of maximum output were compared with those quoted in the literature and obtained by RIA (Table III). Our data for E_3-16-G appear to be fairly comparable to those obtained by direct RIA techniques. Conversely, the data for E_3-3-G as obtained by us are much lower than those obtained in the past. Our finding substantiates the studies made by Stanczyk et al. [6], which showed that, except for E_3-16-G, the patterns obtained for the other four estrogen glucuronides by RIA on unpurified urine samples during a menstrual cycle, although consistent with those obtained by classical procedures, represent changes in concentrations of incompletely identified estrogen fractions rather than actual variations in the concentration of an individual estrogen metabolite. It follows that direct RIA of urinary estrogens may be valuable in clinical practice but they are of only limited value from a biochemical point of view.

The excretory patterns in EMU samples of the two estriol glucuronides, assessed on a daily basis throughout a conceptional cycle in nine subjects, are re-

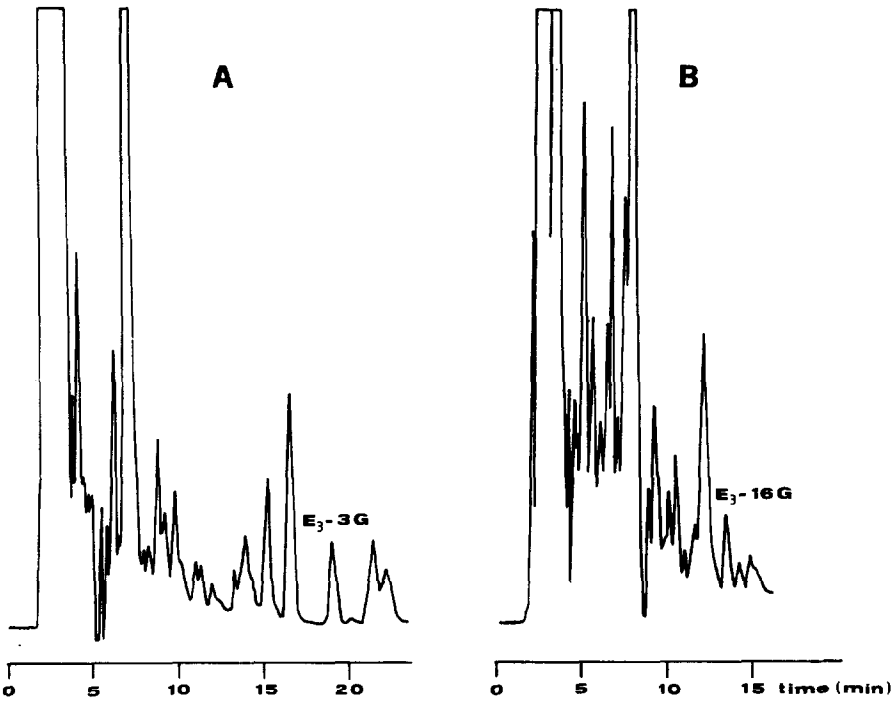

Fig. 1. Typical chromatograms obtained with the described procedure. The contents of (A) E_3-3-G and (B) E_3-16-G in urine were 6.9 and 5.3 ng/ml, respectively.

TABLE III

URINARY CONCENTRATIONS OF E_3-3-G AND E_3-16-G DURING THE MENSTRUAL CYCLE MEASURED BY DIFFERENT METHODS

Ref.	Follicular phase (ng/ml)		Peak value (ng/ml)	
	E_3-3-G	E_3-16-G	E_3-3-G	E_3-16-G
4	39.3	12.3	121	38.6
5	—	7.5	—	24.5
6	22.4*	7.7*	53.8*	23.0*
3	48.0*	12.7*	90.4*	28.9*
This work	9.2	8.1	16.8	19.8

*Original data represented total excretion of the analyte over 24 h. These data were rearranged to give concentrations by assuming a mean volume of urine of 1.3 l excreted over 24 h.

ported in Fig. 2. Mean values were calculated as the arithmetic mean on each day of the cycle for all the subjects investigated. Standard deviations about the mean are also given. The data were correlated with the luteinizing hormone (LH) peak value in urine, as a sharp rise in LH urinary concentration is the most effective index of impending ovulation [16]. The excretory patterns of the two estriol conjugates were characterized by a low and fairly constant output early in the follicular phase then, in both instances, a rise in the concentration occurred from

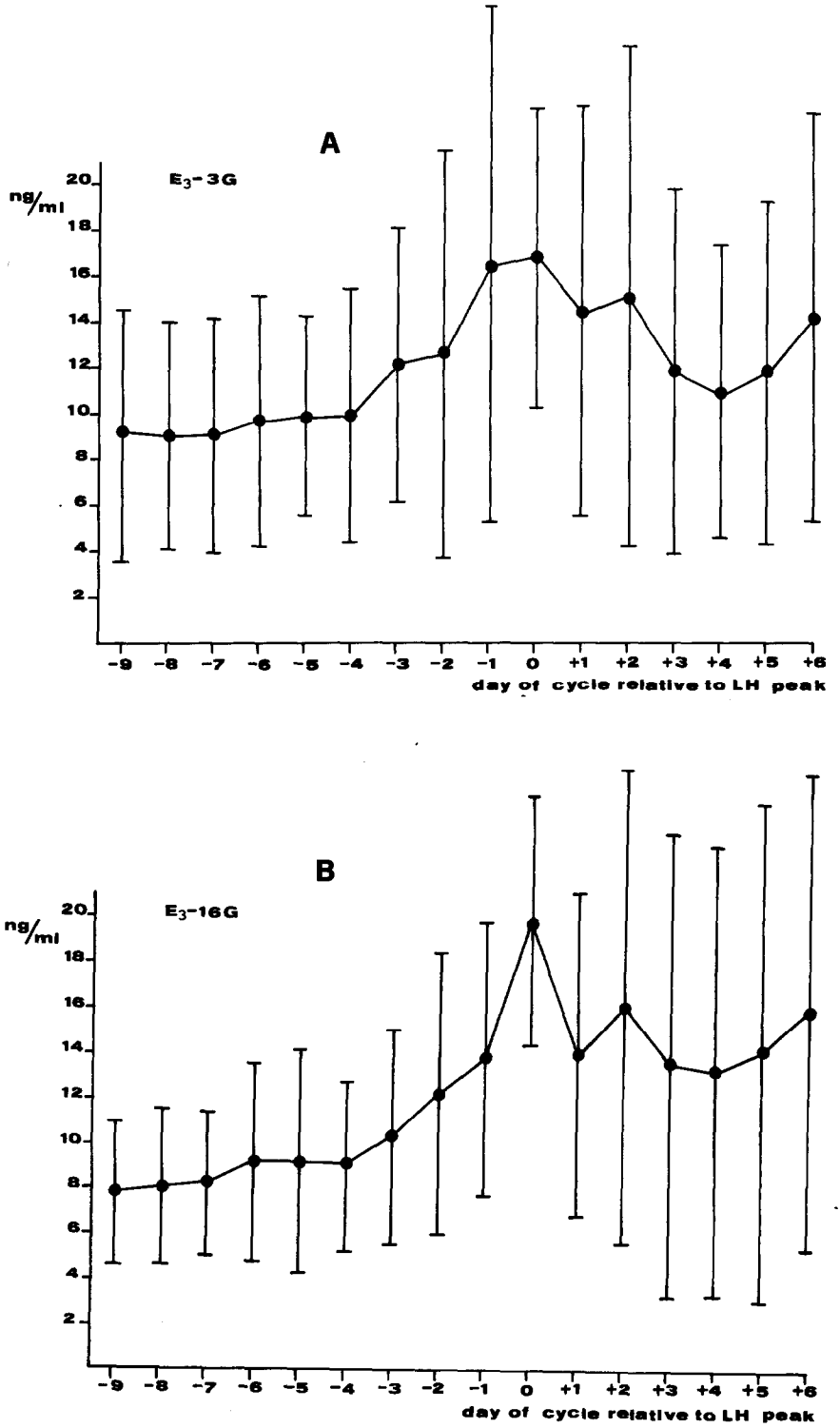

Fig. 2. Excretory patterns of (A) E_3-3-G and (B) E_3-16-G in EMU samples based on meanvalues (±S.D.).

TABLE IV

DAYS (RELATIVE TO THE LH PEAK) ON WHICH THE URINARY CONCENTRATIONS OF THE TWO ESTRIOL CONJUGATES SHOW A 50% RISE, PEAK VALUE AND 50% FALL

Subject	E_3-3-G			E_3-16-G		
	Rise	Peak	Fall	Rise	Peak	Fall
1	−1	+1	+4	−2	0	+4
2	−3	−1	0	−3	−1	0
3	−1	+2	+5	−2	+2	+5
4	−2	0	+4	−2	0	+3
5	−1	0	+1	−2	0	+2
6	0	0	+4	−1	0	+2
7	−2	0	+2	−1	0	+2
8	0	0	+3	−1	0	+3
9	−3	0	+2	−3	0	+2
Mean	−1.5	+0.21	+2.8	−1.9	+0.11	+2.5

day −3 to reach a maximum value, which was best defined for E_3-16-G, coincident with the LH day. Then, a fall in the output of both glucuronides took place over the next one to three days. Two days after the appearance of the first maximum, a second maximum occurred in the pattern of both estriol glucuronides, as observed for total estriol [17].

To establish whether the measurement of the excretion of a hormone metabolite may be useful in predicting the start and end of the fertile period, a 50% rise in concentrations over the mean of three preceding values at least 3 days before the LH peak value and a 50% decrease over the mean of three preceding values two days following ovulation is the criterion usually followed. To verify whether the excretion patterns of E_3-3-G and E_3-16-G as measured by us met these requirements, data were elaborated as reported in Table IV. As can be seen, if the criterion chosen was that of a 50% change in concentration, only in two of the nine subjects investigated could a positive signal be obtained for the start of the fertile period. The results were more encouraging if the first significant rise in the concentration of estriol conjugates was defined as the first amount that exceeded two standard deviations above the mean of values calculated during the follicular phase [3]. In such a case, positive signals were obtained from the pattern of E_3-3-G in five of the nine subjects, whereas in four instances E_3-16-G was able to indicate the start of the fertile period.

REFERENCES

1 F.Z. Stanczyk, I. Miyakawa, J.R. Soares and U. Goebelsmann, J. Steroid Biochem., 10 (1979) 443.
2 K. Wright, D.C. Collins and J.R.K. Preedy, Steroids, 34 (1979) 445.
3 T.S. Baker, K. Jennison and A.E. Kellei, J. Steroid Biochem., 12 (1980) 411.
4 H. Adlercreutz, J. Brown, W. Collins, U. Goebelsmann, A. Kellie, H. Campbell, J. Spieler and G. Braissand, J. Steroid Biochem., 17 (1982) 695.

5 C.M. Branch, P.O. Collins and W.P. Collins, J. Steroid Biochem., 16 (1982) 345.
6 F.W. Stanczyk, I. Miyakawa and U. Goebelsmann, Am. J. Obstet. Gynecol., 137 (1980) 443.
7 H. Honjo, K. Otsubo, J. Yasuda, J. Kitawaki, H. Okada, T. Ohkubo and T. Nambara, Acta Endocrinol., 106 (1984) 374.
8 F. Kohen, J.B. Kim, G. Bernard and H.R. Lindner, Steroids, 36 (1980) 405.
9 G. Barnard, W.P. Collins, F. Kohen and H.R. Lindner, J. Steroid Biochem., 14 (1981) 941.
10 P.F. Dixon, P. Lukha and N.R. Scott, Proc. Anal. Div. Chem. Soc., 16 (1979) 302.
11 F. Andreolini, C. Borra, A. Di Corcia, R. Samperi and G. Raponi, Clin. Chem., 31 (1985) 124.
12 F. Andreolini, C. Borra, F. Caccamo, A. Di Corcia, R. Samperi and F. Improta, Clin. Chem., 31 (1985) 1698.
13 F. Andreolini, C. Borra, F. Caccamo, A. Di Corcia and R. Samperi, Anal. Chem., 59 (1987) 1720.
14 L. Campanella, A. Di Corcia, A. Gambacorta and R. Samperi, Mater. Chem., 7 (1982) 429.
15 G. Carfagnini, A. Di Corcia, M. Marchetti and R. Samperi, Chromatographia, 23 (1987) 835.
16 H. Adlercrentz, T. Lehtinen and A.L. Kairento, J. Steroid Biochem., 12 (1980) 395.
17 J.P. Fels, L. Dehemin, J. Grennier and R. Scholler, J. Steroid Biochem., 11 (1979) 103.