Journal of Chromatography, 431 (1988) 249–257
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4333

DETERMINATION OF OESTROGENS IN PREGNANCY URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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(First received April 21st, 1988; revised manuscript received May 30th, 1988)

SUMMARY

A simple and sensitive high-performance liquid chromatographic method is described for the determination of three oestrogens (oestriol, oestrone and oestradiol) in pregnancy urine. Free oestrogens are extracted with chloroform from the urine sample. The phenolic group of each oestrogen in chloroform is formylated in the presence of an alkaline aqueous solution, and the resulting aldehyde is converted into a fluorescent derivative by reaction with 1,2-diamino-4,5-dimethoxybenzene. In order to determine free and conjugated oestrogens, conjugated oestrogens are hydrolysed by heating in hydrochloric acid before the extraction and then treated in the same way as free oestrogens. The fluorescent derivatives are separated on a reversed-phase column, TSKgel ODS-120T, with stepwise gradient elution using an aqueous methanol-containing phosphate buffer (pH 2.2). The lower limit of detection for each oestrogen is ca. 200 fmol per 100-μl injection volume.

INTRODUCTION

Oestrogens [oestriol (oestra-1,3,5(10)-trien-3,16α,16β-triol), oestrone (3-hydroxyoestra-1,3,5(10)-trien-17-one) and oestradiol (oestra-1,3,5(10)-trien-3,17β-diol)] are well known as female sex hormones, which have a phenolic group in their molecules. During pregnancy, oestrogens are synthesized in large amounts by the placenta instead of the ovaries [1]. The concentrations of oestrogens in the body fluids of normal women increase during pregnancy. On the other hand, it has been reported that their urinary concentrations in patients with gestosis are lower than those in normal pregnant subjects [2]. Therefore, their determination is important to evaluate placental and foetal functions.

A significant proportion of the oestrogens in urine is in the form of glucuronide and sulphate conjugates [3]. The conjugated oestrogens also have physiological

0378-4347/88/$03.50

activities [4,5], so the determination of the conjugated oestrogens is also important in clinical and biological investigations.

Many methods, involving bioassay [6], colorimetry [7], gas chromatography (GC) [8], GC–mass spectrometry (MS) [9,10], radioimmunoassay (RIA) [11,12] and high-performance liquid chromatography (HPLC) [13–17], have been proposed for the determination of oestrogens. Bioassays based on the determination of the increased mass of the uterus after administration of oestrogens to mice or rats cannot provide good precision and specificity in quantification [6]. The colorimetric methods based on the reaction of hydroquinone and sulphuric acid have been widely used for the determination in pregnancy urine, because of the simplicity of operation. However, three oestrogens cannot be determined simultaneously [7]. RIA methods offer sufficient sensitivity to measure the oestrogens at the 20-pg level, but it is difficult to obtain constantly specific antibodies for all three oestrogens; thus column chromatography is required to prevent cross-reaction [11,12]. GC methods are required for complicated procedures [8]. GC–MS methods can determine free oestrogens and various metabolites of the oestrogens, including catechol oestrogens and conjugated oestrogens [9,10], though the procedure is relatively complicated. HPLC methods can simultaneously separate the three oestrogens. For the quantification of oestriol in pregnancy urine by HPLC, fluorescence detection [13] (detection limit, 0.35–1.40 pmol per injection volume), electrochemical detection [14–16] (detection limit, 0.3–0.5 pmol per injection volume) and ultraviolet absorption detection [17] (detection limit, 180 pmol per injection volume) have been reported.

We previously developed a precolumn fluorescence derivatization method for the selective and sensitive HPLC determination of bioactive compounds with a phenolic group in their molecules, such as *p*-hydroxybestatin [18,19] and enkephalins [20,21]. This derivatization is based on formylation of the *ortho* position of a phenolic group in alkaline medium in the presence of chloroform [22] and then conversion of the resulting aldehyde into a highly fluorescent derivative with 1,2-diamino-4,5-dimethoxybenzene (DDB) [23,24], which is a fluorogenic reagent for aromatic aldehydes.

We have recently found that oestrogens can also form fluorescent derivatives by this derivatization method. Our present objective was to develop a selective and sensitive HPLC method involving a simple pre-treatment of urine samples by precolumn fluorescence derivatization with DDB for the quantification of oestrogens in pregnancy urine. The present derivatization method does not provide fluorescent derivatives for the conjugated oestrogens. Therefore, hydrolysis is necessary for the determination of the conjugated oestrogens. Equilin (3-hydroxyoestra-1,3,5(10),7-tetraen-17-one), which has a similar structure to human oestrogens, was used as an internal standard for the determination of endogenous oestrogens.

EXPERIMENTAL

Reagents and solutions

Deionized and distilled water was used throughout. Oestriol, oestrone, oestradiol and equilin were obtained from Sigma (St. Louis, MO, U.S.A.). DDB mo-

nohydrochloride was purchased from Dojindo (Kumamoto, Japan). All other chemicals were of the highest purity available and were used as received. Pregnancy urine was stored at −20°C and used within a month.

Apparatus and HPLC conditions

Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (optical path-length, 10 mm). The HPLC system consisted of a Toyo Soda 803D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector (100 μl) and a Hitachi F1000 fluorescence spectrophotometer fitted with a 12-μl flow-cell operating at an emission wavelength of 415 nm and an excitation wavelength of 350 nm. The column was TSKgel ODS-120T (250×4.6 mm I.D., particle size, 5 μm; Tosoh, Tokyo, Japan). Two mixtures of methanol and 50 m*M* phosphate buffer (pH 2.2) (57:43 and 63:37) were used for stepwise gradient elution. The column was first eluted with the 57% methanol eluent for 15 min, followed by the 63% methanol for the next 25 min; then the column was equilibrated successively with the 57% methanol eluent for 10 min before the analysis of the following sample. The flow-rate was 0.8 ml/min. The column temperature was ambient (20–27°C).

Procedure

To hydrolyse conjugated oestrogens, the urine samples were treated as follows. A 20-μl portion of urine was mixed with 200 μl of 0.5 *M* hydrochloric acid in a screw-capped reaction vial (4 ml), and heated with a heating module (Pierce, Rockford, IL, U.S.A.) at 100°C for 100 min. To determine only the free oestrogens, a 100- or 200-μl portion of urine was used. In both cases, 20 μl of 1–4 nmol/ml equilin and 500 μl of chloroform were added to the urine samples. The mixture was shaken on a vortex mixer for 1 min to extract oestrogens and equilin. The chloroform layer (200 μl) was transferred to a test-tube. To the test-tube was added a 200-μl portion of 1.5 *M* potassium hydroxide. The mixture was heated at 60°C for 10 min to formylate the oestrogens and equilin, and then cooled in ice-water for ca. 1 min. To the mixture, 100 μl of 14 *M* acetic acid and 100 μl of 2.6 m*M* DDB aqueous solution were successively added. The mixture was heated at 60°C for 20 min to form the fluorescent derivatives. A 100-μl portion of the final reaction mixture was injected into the chromatograph.

RESULTS AND DISCUSSION

HPLC separation

Complete separation of the DDB derivatives of oestriol, oestrone, oestradiol and equilin was achieved on a reversed-phase column, TSKgel ODS-120T, within 40 min with a stepwise gradient elution (Fig. 1). The retention times for oestriol, oestrone, oestradiol and equilin were 17.5, 29.5, 35.0 and 28.5 min, respectively. The peaks 1–4 showed the same excitation and emission maxima (350 and 415 nm, respectively) as those obtained from each standard reaction mixture.

The concentration of methanol in the mobile phase affected the separation of

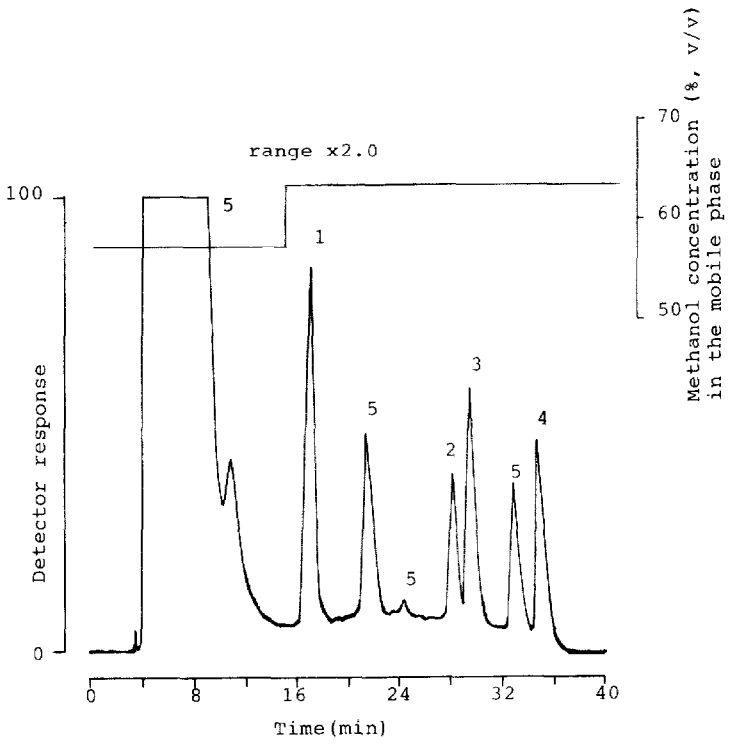

Fig. 1. Chromatogram of a mixture of oestriol, oestrone, oestradiol and equilin. A portion (100 μl) of a standard mixture (1 nmol/ml each) was treated according to the procedure. Peaks: 1 = oestriol; 2 = equilin; 3 = oestrone; 4 = oestradiol; 5 = reagent blank.

the peaks. At a concentration greater than 65%, the peak for oestriol partially overlapped with the reagent peaks, whereas a concentration of 60% or less caused delay in elution with broadening of the peaks, especially for oestrone, oestradiol and equilin Therefore, the mobile phase containing 57% methanol was used for the first 15 min, followed by the 63% methanol eluent for the next 25 min. The column was then equilibrated with the 57% methanol eluent for the following sample.

The DDB derivatives of oestriol, oestrone, oestradiol and equilin fluoresced most intensely at pH 1.3–3.5. The concentrations in the range 10–100 m*M* phosphate buffer (pH 2.2) added to the mobile phase did not have any significant effect on the separation and fluorescence intensity; 50 m*M* phosphate buffer (pH 2.2) was employed as a component of the mobile phase.

Formylation and derivatization

Oestrogens and equilin were formylated rapidly at temperatures higher than 50 °C. The peak heights from oestrogens and equilin gave maximum and constant values, respectively, after heating at 60 °C or 75 °C for 7.5 min or longer; 10 min heating at 60 °C was employed in the recommended procedure.

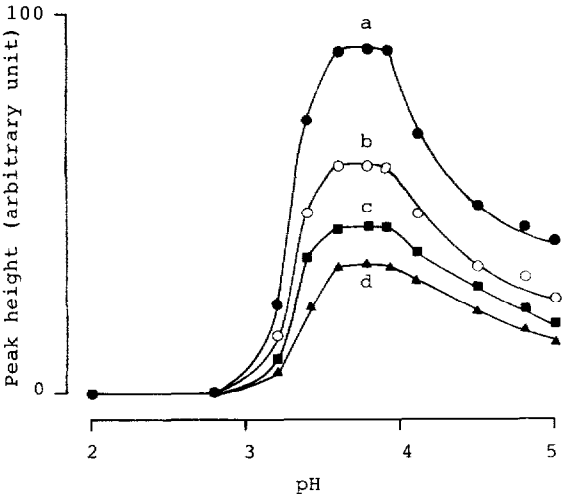

Fig. 2. Effect of pH on the fluorescence derivatization. Portions (100 μl) of a standard mixture (1 nmol/ml each) were treated as in the recommended procedure, and the pH values of the fluorescence reaction mixtures were adjusted with 5–17 *M* acetic acid (and concentrated hydrochloric acid if necessary). Curves: a = oestriol; b = oestrone; c = oestradiol; d = equilin.

peak heights. No peaks due to degradation products from oestrogens and equilin during the formylation were observed in the chromatogram.

Chloroform (100–250 μl) in the formylation mixture gave maximum and constant peak heights for oestrogens and equilin; a 200-μl volume was selected for the recommended procedure.

The resulting aldehydes should be derivatized with DDB in a weakly acidic solution. The fluorescence derivatization of oestrogens and equilin with DDB occurred most effectively at pH 3.8 in aqueous acetic acid (Fig. 2). Acetic acid (14.0 *M*) was added to adjust the pH to 3.8 in the recommended procedure.

The peak heights of the fluorescent derivatives reached maximum and constant values after heating at 60°C for 15 min or longer; 20 min heating at 60°C was employed as optimum. The DDB solution at a concentration greater than 2.3 m*M* gives the highest peaks for oestrogens and equilin; a concentration 2.6 m*M* was used.

The fluorescence in the final reaction mixture was stable for more than 3 h at room temperature.

Determination of oestrogens in pregnancy urine

Fig. 3A shows a typical chromatogram of free oestrogens in urine obtained with a normal pregnant subject. The fluorescent peaks of oestriol, oestrone, oestradiol and the internal standard in the sample were successfully separated from other peaks by the present reversed-phase HPLC method. These fluorescent peaks of oestrogens and internal standard were not observed in the chromatogram when the formylation was not performed (Fig. 3B). The oestrogen peaks were identified on the basis of retention times, co-chromatography with the standard com-

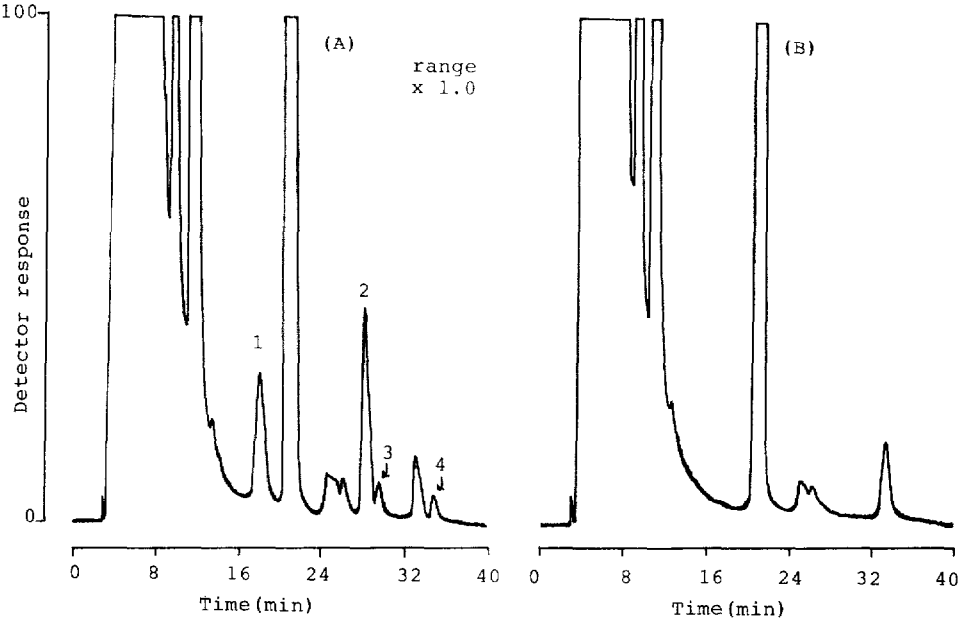

Fig. 3. Chromatograms obtained with urine from a normal pregnant woman (33 weeks) according to (A) the recommended procedure for free oestrogens and (B) the procedure in the absence of chloroform. Peaks (concentrations in urine, nmol/ml): 1 = oestriol (1.10); 2 = internal standard; 3 = oestrone (0.21); 4 = oestradiol (0.25); other peaks are endogenous substances and reagent blank.

pounds, and fluorescence excitation and emission spectra of the eluates in comparison with the standards.

Under the conditions of the fluorescence derivatization of the formylated oestrogens with DDB, tyrosine-containing peptides (e.g. leucine and methionine enkephalins), α-keto acids (e.g. pyruvic acid, α-ketoglutaric acid and phenylpyruvic acid) and sialic acids (e.g. N-acetylneuramic acid) gave fluorescent derivatives [20,21,25,26]. However, these compounds were all eluted within 8 min. In addition, tyrosine-containing peptides and sialic acids were not extracted with chloroform. Thus these endogenous compounds in urine did not interfere with the determination of oestrogens.

Fig. 4 shows a typical chromatogram of total (free plus conjugated) oestrogens in urine obtained with a normal pregnant subject. The oestrogen peaks were identified by the same techniques as for free oestrogens alone.

Several conjugated oestrogens, such as monoglucuronides, monosulphates and doubly conjugated oestrogens, are present in urine [9,10]. Acid hydrolysis used in the present method was carried out under the conditions described in the literature [14,16,17]. Oestriol-16α-glucuronide, a major conjugate of oestrogens in pregnancy urine [27], was used as a model compound in the evaluation of the hydrolysis conditions. The maximum amount of oestriol was liberated in 0.45 *M* hydrochloric acid after incubation for 90–120 min at 100°C; 100 min incubation at 100°C in the acid solution was selected. The recovery of oestriol from oestriol-16α-glucuronide (250 nmol/ml in urine) under the conditions of the acid hy-

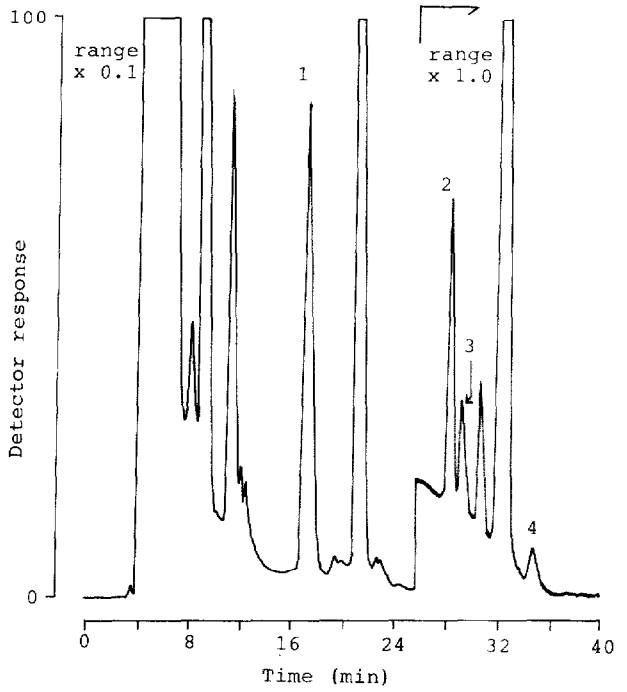

Fig. 4. Chromatogram of a urine sample from a normal pregnant woman (33 weeks) prepared according to the recommended procedure for total oestrogens. Peaks (concentrations in urine, nmol/ml): 1 = oestriol (39.5); 2 = internal standard; 3 = oestrone (0.78); 4 = oestradiol (0.32); others peaks are endogenous substances and reagent blank.

drolysis was 91 ± 2.7% (mean ± S.D., $n = 5$). When the enzymic hydrolysis was carried out using β-glucuronidase (60 U per test tube) for 24 h at 37°C in 0.3 *M* acetate–hydrochloric acid buffer (pH 5.0), the recovery (ca. 80%) was lower than that obtained by acid hydrolysis.

Oestrogens and equilin were effectively extracted with chloroform from the urine samples. The extraction recoveries of oestriol, oestrone, oestradiol and equilin (added at 100 nmol/ml each to urine) were 97.0 ± 2.2%, 101.2 ± 1.9%, 98.5 ± 2.8% and 98.0 ± 2.5% (mean ± S.D., $n = 5$), respectively.

A linear relationship was observed between the ratio of the peak heights of oestrogens to that of the internal standard and the amounts of oestrogens added to urine in the range 0–100 μg/ml each. The correlation coefficients (r) of all the calibration curves were greater than 0.999. The lower limits of detection for oestriol, oestrone and oestradiol were 100, 170 and 260 fmol per 100-μl injection, respectively, at a signal-to-noise ratio of 2. This sensitivity is ca. 2–600 times higher than those of other HPLC methods with fluorescence [13], electrochemical [14–16] and ultraviolet absorption [17] detections. The precision of the method was established by repeated determination ($n = 5$) using urine from a 33-weeks pregnant subject. The relative standard deviations were 2.5%, 3.4% and 4.7% for 95.5 nmol/ml oestriol, 2.3 nmol/ml oestrone and 0.85 nmol/ml oestradiol, respectively.

TABLE I

CONCENTRATIONS OF FREE AND TOTAL OESTROGENS IN PREGNANCY URINE

Subject	Weeks of gestation		Concentration (mg/day)		
			Oestriol	Oestrone	Oestradiol
A	38	Free	0.317	0.090	0.036
		Total	24.20	0.546	0.203
B	36	Free	0.665	0.171	0.047
		Total	14.70	1.220	0.220
C	33	Free	0.348	0.062	0.075
		Total	12.50	0.230	0.094
D	20	Free	0.423	0.154	0.082
		Total	8.420	0.581	0.161
E	13	Free	0.117	0.042	0.076
		Total	1.250	0.117	0.095
F	7	Free	0.066	0.013	0.049
		Total	2.420	0.130	0.093

The concentrations of free and total oestrogens in urine from six pregnant women were determined by the present method (Table I). The concentrations of oestriol increased with the period of pregnancy. The concentration values for the oestrogens in the samples are in good agreement with the published data [14,16]. The concentrations of free oestrogens in the same sample were unchanged for at least 4 weeks when the urine was stored at $-20\,^{\circ}C$ in order to prevent spontaneous hydrolysis of the conjugated oestrogens.

The present HPLC method provides a sensitive and simultaneous determination of oestriol, oestrone and oestradiol in pregnancy urine. It needs only simple procedures for the extraction of oestrogens with chloroform and the pre-column fluorescence derivatization, and should be useful for clinical and biomedical investigations.

ACKNOWLEDGEMENTS

The authors thank Dr. T. Higashijima, Ohmura Hospital, for the supply of urine from pregnant women. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. We are also grateful to Mr. H. Nakamura and Miss U. Taguchi for their skillful assistance.

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