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RAPID MEASUREMENT OF OESTRADIOL AND OESTRIOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER AUTOMATIC PRETREATMENT

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SUMMARY

A fluorometric liquid chromatographic method was developed for measurements of unconjugated oestradiol and oestriol in the serum of pregnant women. The serum samples are injected directly into the apparatus and pass to a pretreatment column, where oestrogens are adsorbed while hydrophilic components such as proteins and carbohydrates are not. The oestrogens then pass into a separation column containing a new type of polymer gel. The mobile phase consists of an acetonitrile—water mixture, and separation is achieved by a reversed-phase mechanism. The eluent is monitored for fluorescence. Data on the reproducibility and recovery by this method and the correlation of values with those obtained by radioimmunoassay are reported. Results on the increases of oestradiol and oestriol in the serum during pregnancy are also reported.

INTRODUCTION

The importance of determining serum oestrogens as indicator of foetal distress is well recognized [1—3]. Many methods have been used to measure serum oestrogens, and now high-performance liquid chromatography (HPLC) is widely used for their analysis [4—13]. However, a troublesome and time-consuming pretreatment procedure is necessary before HPLC. Therefore, we have developed a liquid chromatographic procedure that requires no such pretreatment procedure.

The serum sample is first introduced into a pretreatment column where

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deproteinization occurs and oestrogens are concentrated. Then, by turning a stop cock, the oestrogens pass through an HPLC column where they are separated. This rapid procedure for serum oestrogen determination is simple and gives excellent recovery.

MATERIALS AND METHODS

Apparatus

A flow diagram of our HPLC system is shown in Fig. 1. The system is composed essentially of a pretreatment part and an HPLC part. Two liquid chromatographic Model LC 3A pumps (Shimadzu, Kyoto, Japan) are used (pump 1 and pump 2). A Model 7125 Rheodyne injector and Model 7000 Rheodyne six-port valve, both from Kemko, Osaka, Japan, are used. The fluorescence detector, Model SK-4, equipped with a liquid chromatographic flow cell is from Sekisui (Osaka, Japan).

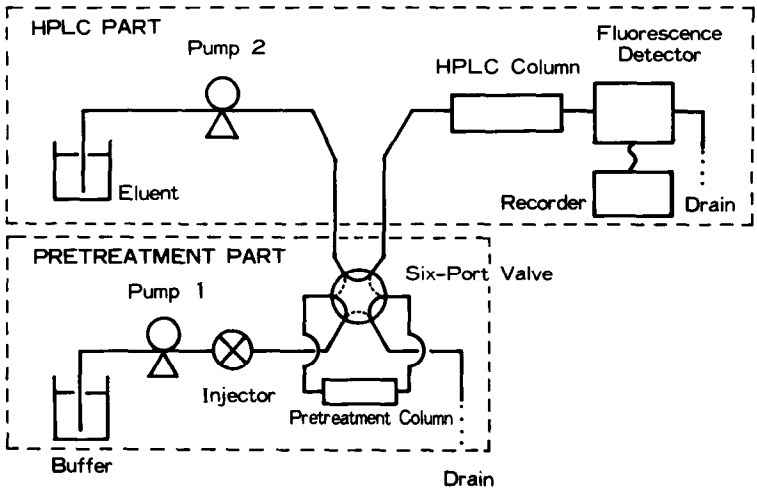


Fig. 1. Flow diagram of the HPLC system (for details, see text).

HPLC and pretreatment columns

Copolymer beads were synthesized by a standard suspension polymerization method. The aqueous portion of the polymerization mixture was prepared in a reaction flask (5 l) by adding 60 g of polyvinyl alcohol as dispersing agent to 2.5 l of distilled water. The monomer phase consisted of 250 g of tetraethyleneglycol diacrylate, 50 g of tetramethylolmethane triacrylate (both from Sin Nakamura, Wakayama, Japan), 300 g of toluene, and 3.5 g of benzoyl peroxide.

The aqueous phase was warmed to 50°C, and then the monomer—diluent—initiator solution was added with stirring and the temperature was maintained at 80°C for 10 h. When polymerization was complete, the resin beads were filtered off and washed. About 25 g of spherical porous beads about 10—15 μm in diameter were obtained by sieving. These porous beads were packed into a stainless-steel column (250 mm × 6 mm I.D.; HPLC column). Similar beads were packed into the pretreatment column (20 mm × 4 mm I.D.).

Reagents and standards

Acetonitrile and methanol, both reagent grade, were from Nakarai (Kyoto, Japan). Oestriol (E_3), oestradiol (E_2) and other oestrogenic steroids were also obtained from Nakarai.

Standard solutions of E_2 and E_3 at 0.5 μg/ml in methanol—water (1:1, v/v), and a mixture of E_2 and E_3 in the same solvent were prepared and stored at 4°C until used.

Pretreatment buffer

Phosphate buffer, 0.05 mol/l, adjusted to pH 8.5, was used.

Eluent

Acetonitrile—water (65:35, v/v) was used as eluent.

Serum samples

Samples (5 ml) of whole blood were taken from about 50 pregnant women. The samples in glass test tubes were left to stand at room temperature long enough (about 30 min) for clot retraction to occur. Then they were centrifuged (1000 *g*, 10 min) and the resulting supernatant serum was transferred to another glass tube and stored at −10°C until use.

Radioimmunoassay (RIA)

E_2 and E_3 in serum samples were measured by RIA at Teikokuzoki Clinical Laboratory (Tokyo, Japan).

Chromatographic procedure

Pretreatment. A sample of 150 μl of serum was injected into the apparatus through the injector. The flow-rate of the pretreatment buffer was maintained at 0.6 ml/min. In the pretreatment column, oestrogens and other hydrophobic components were adsorbed on the porous beads, whereas serum components such as proteins and carbohydrates were not. At 6 min after injection of the sample, when all the hydrophilic components had flowed out of the column, the six-port valve was turned and the eluent was allowed to flow (0.8 ml/min) into the pretreatment column. The oestrogens were eluted and passed into the HPLC column.

Separation and detection. A reversed-phase system was used for the separation. The flow-rate was 0.8 ml/min, and the column temperature was 25°C. The fluorescence detector was set at an excitation wavelength of 220 nm and emitted light was measured at 320 nm.

RESULTS

Chromatograms

The chromatogram of a standard mixture of E_2 and E_3 is shown in Fig. 2A. The right chromatogram (Fig. 2B) is of a serum sample from a normal pregnant woman (35 weeks of pregnancy), for which we calculated the concentrations of E_2 and E_3 to be 32.7 and 17.2 ng/ml, respectively.

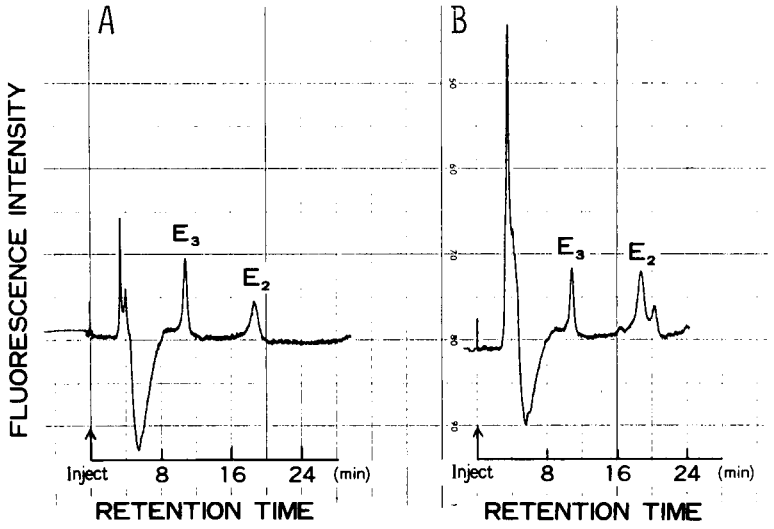


Fig. 2. (A) Chromatogram of a mixture of E_2 and E_3 (20 ng of each). (B) Chromatogram of serum E_2 and E_3 of a pregnant woman.

Interference

We injected several naturally occurring oestrogenic steroids into the chromatograph to determine their potential interference in this procedure. The glucuronides and sulphates of E_2 and E_3 were not adsorbed on the pretreatment column, and so could not be measured by this technique.

Linearity

A mixture of E_2 and E_3 was added to serum from male subjects that had previously been shown to contain no E_2 or E_3. Standard samples of serum

TABLE I

PERCENTAGE RECOVERIES OF E_2 and E_3, DETERMINED BY ADDING DIFFERENT AMOUNTS OF OESTROGENS TO THREE SERUM SAMPLES WHICH CONTAINED DIFFERENT AMOUNTS OF ENDOGENOUS OESTROGENS

E_2				E_3			
Amount added (ng/ml)	Amount expected (ng/ml)	Amount determined (ng/ml)	Recovery* (%)	Amount added (ng/ml)	Amount expected (ng/ml)	Amount determined (ng/ml)	Recovery** (%)
0	—	17.0	—	0	—	4.0	—
5	22.0	20.8	95	5	9.0	9.2	102
10	27.0	27.7	102	10	14.0	16.9	121
20	37.0	38.9	105	20	24.0	28.2	117
0	—	25.4	—	0	—	11.2	—
5	30.4	31.2	103	5	16.2	15.6	96
10	35.4	34.7	98	10	21.2	20.5	97
20	45.4	45.7	101	20	31.2	31.8	102
0	—	31.3	—	0	—	20.2	—
5	36.3	35.9	99	5	25.2	24.6	98
10	41.3	39.3	95	10	30.2	29.0	96
20	51.3	51.6	101	20	40.2	40.8	101

*Mean ± S.D. = 99.9 ± 3.4%.
**Mean ± S.D. = 103 ± 9.0%.

containing E_2 and E_3 at concentrations of 5, 10, 20, 40 ng/ml were prepared and chromatographed. The peak heights of E_2 and E_3 were linearly proportional to their concentrations in the range 0–40 ng/ml. The standard curves obtained are $Y = 0.5539X - 0.2609$ ($r = 0.9996$) for E_2, and $Y = 1.086X - 0.2391$ ($r = 0.9998$) for E_3, where Y is the peak height (mm) and X is the oestrogen concentration (ng/ml sample).

Recovery

Mixtures of E_2 and E_3 were added to sera from pregnant women, and the peak heights of oestrogens in these sera were converted into oestrogens concentrations with the aid of the standard curves. The recoveries thus obtained are shown in Table I.

Reproducibility

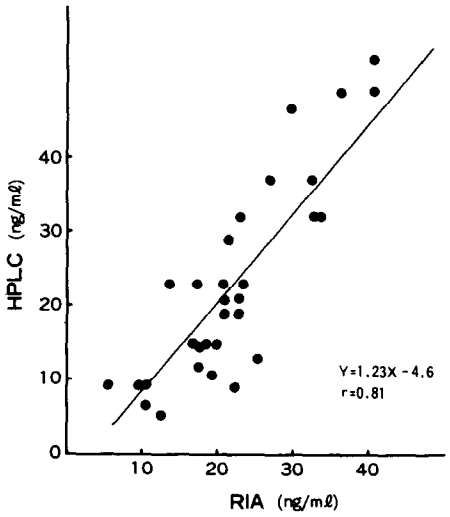
Intra-assay reproducibility was examined by measuring the oestrogen concentration in the same sample (serum of a woman in week 38 of pregnancy) six times in one day. The coefficient of variation (C.V., %) was calculated from the mean and standard deviation of the values. Table II shows the results.

TABLE II

INTRA-ASSAY VARIABILITY OF OESTROGEN VALUES

Six determinations were made on serum of a woman in week 38 of pregnancy.

Oestrogen	Average (ng/ml)	S.D.	C.V. (%)
E_2	40.2	1.62	4.0
E_3	16.6	0.59	3.5



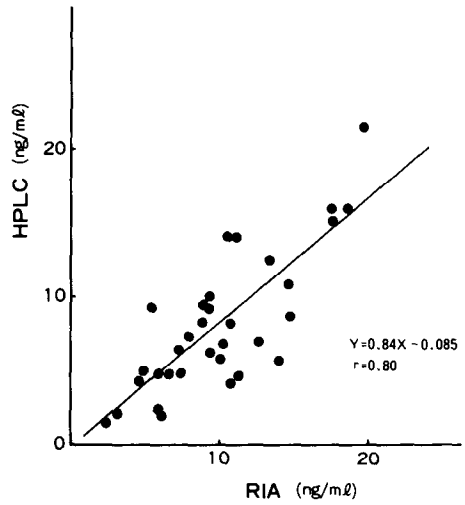


Fig. 3. Correlation between E_2 values determined by HPLC and RIA.

Fig. 4. Correlation between E_3 values determined by HPLC and RIA.

Correlation between values obtained by HPLC and RIA

Samples were divided into two portions and assayed by HPLC and RIA. The values for E_2 and E_3 obtained by HPLC correlated well with those obtained by RIA (r = 0.81 and r = 0.80, respectively), as shown in Figs. 3 and 4, respectively.

Changes in concentrations during pregnancy

Figs. 5 and 6 show plots of E_2 and E_3 concentrations against the duration of pregnancy. Both E_2 and E_3 showed increases in median values and in the distribution of values, which were skewed toward higher values with progress of pregnancy. These tendencies are similar to those observed previously by RIA [1, 14].

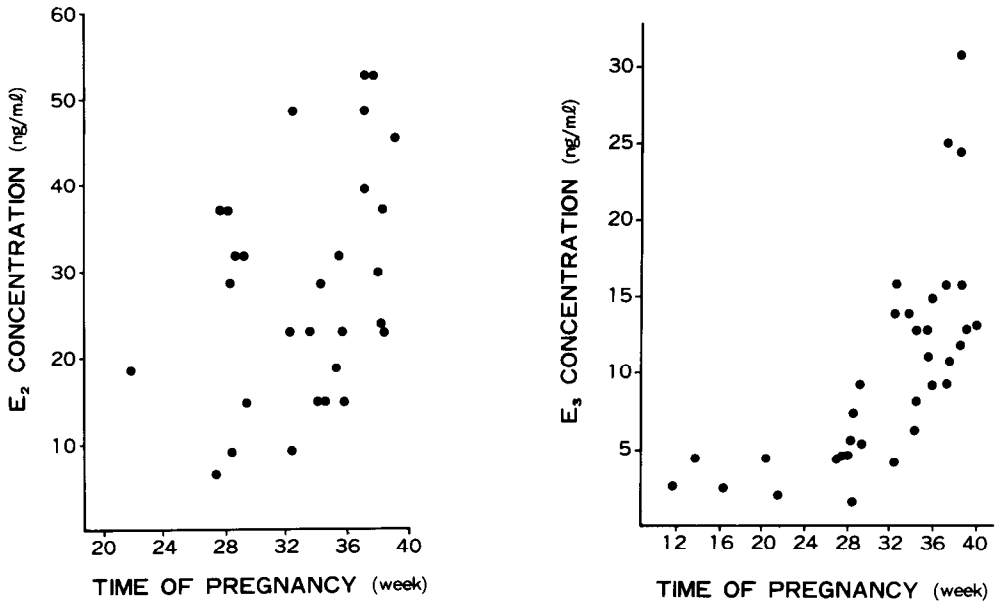


Fig. 5. Serum E_2 concentrations as a function of the duration of pregnancy.

Fig. 6. Serum E_3 concentrations as a function of the duration of pregnancy.

DISCUSSION

The present procedure is highly specific in that it is virtually unaffected by compounds such as oestrogen derivatives and other steroids that might interfere with the accurate determination of serum unconjugated oestrogens by RIA. Many liquid chromatographic methods for the measurement of serum oestrogens have been reported, but these all involve a time-consuming pretreatment consisting of solvent extraction, evaporation, and reconstitution, and thus are not suitable for routine use. Our procedure does not involve a complicated pretreatment and so the total time required for one measurement is only about 25 min. With this procedure it is possible to measure 1.0 ng/ml E_2 or E_3 using a sample of 100 μl of serum. The recovery, reproducibility, and correlation with RIA values are also sufficient for clinical purposes.

In summary, the present procedure for determination of serum E_2 and E_3 is simpler and more rapid than previous chromatographic methods, and seems suitable for use in routine measurements of foetoplacental function.

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