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Note

Total oestriol in maternal serum or plasma as measured by liquid chromatography

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Although several authors [1—3] consider that the level of unconjugated oestriol in serum is the most reliable biochemical indicator of foetal well-being, the assay of total oestriol in serum is still the parameter most widely used. Moreover, the assay of total oestriol can be more useful than that of unconjugated oestriol in the management of some pathological pregnancies [4].

Recently, we reported a procedure involving high-performance liquid chromatography (HPLC) with fluorimetric detection for determining unconjugated oestriol in pregnancy serum or plasma [5].

We describe here a rapid procedure for the assay of total oestriol in serum or plasma from pregnant women, based on enzymatic hydrolysis with purified *Helix pomatia* juice, extraction of oestriol with Carbopack B and quantification by HPLC with fluorimetric detection. Our method is as sensitive and accurate as the radioimmunoassay (RIA) method, and has the advantages of being more economical and avoiding manipulation of hazardous materials.

MATERIALS AND METHODS

Instrumentation

A Series 3B liquid chromatograph equipped with a Model 650 S LC

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fluorescence detector having a 20-μl flow cell, and a Rheodyne Model 7125 injector with a 20-μl loop (all from Perkin-Elmer, Norwalk, CT, U.S.A.) was used. The column was 25 cm × 4.6 mm, filled with 5-μm (average particle size) C_{18} reversed-phase packing and protected by a Pelliguard guard column (all from Supelco, Bellefonte, PA, U.S.A.). A mixture of acetonitrile—phosphate buffer, pH 6.2 (24:76, v/v) at a flow-rate of 1.5 ml/min was used as mobile phase. Fluorescence detection was used with excitation at 280 nm, emission at 308 nm, and slit widths of 12 nm.

Reagents

Stock oestriol, oestriol 16-glucuronide (E_316-G) and oestriol 3-sulphate (E_33-S) standards (all from Sigma) were dissolved in methanol to give a concentration of 1 g/l, expressed as oestriol. These standards were diluted with methanol to give 20, 10, 5, and 1 mg/l working standards. To prepare the working plasma or serum standards, 100 μl of either E_316-G or E_33-S working standards were evaporated and the residue was reconstituted in 10 ml of oestriol-free fresh serum or plasma. These standards were stable for at least fifteen days if kept at 4°C.

Working phosphate buffer, pH 6.2, was prepared by diluting a stock solution ten fold, which was prepared by dissolving 27.6 g of $NaH_2PO_4 \cdot H_2O$ in 1 l of freshly distilled water and adjusting the pH to 6.2 with 1 mol/l potassium hydroxide. The stock solution was stable at 4°C for at least one month.

Unpurified *Helix pomatia* juice containing (per ml) 120,000 Fishman units of β-glucuronidase (EC 3.2.1.31) and 3,430,000 Roy units of arylsulphatase (EC 3.1.6.1) was from Merck (Darmstadt, F.R.G.).

Acetonitrile, chromatography grade, was obtained from Carlo Erba. All other solvents (Carlo Erba) were of analytical grade and were distilled in a glass system before use. Carbopack B (80—120 mesh) was kindly supplied by Supelco.

Procedure

Enzyme purification. The Carbopack B column was prepared by pouring 0.25 g of the adsorbent into water and then introducing the suspension into a 15 × 0.6 cm glass column with a small pledget of glass wool in the bottom. One ampoule (2 ml) of crude *Helix pomatia* extract was diluted with 5 ml of 0.5 mol/l acetate buffer, pH 4.6, and passed through the column followed by 3 ml of acetate buffer. The effluent was collected and stored at 4°C. This enzyme preparation is stable at least for fifteen days.

We determined the activities of the purified enzymes relative to those of the untreated ones. Both untreated and treated enzyme preparations were diluted 1000-fold with acetate buffer and 50 μl of these solutions were used to partially hydrolyse 0.5 μg of E_316-G at 37°C for 30 min. The same procedure was used for 1 μg of E_33-S. After that, 4 ml of the mobile phase were added and 20 μl were injected into the chromatograph. In all cases, the efficiency of hydrolysis was assessed by measurement of the peak height of the free oestriol.

Hydrolysis and sample purification. A 600-μl volume of 0.5 mol/l acetate buffer, pH 4.6, and 200 μl of the buffered and purified enzyme preparation were added to 100 μl of serum or plasma. After mixing gently, the mixture was

incubated at 60°C for 30 min. The hydrolysate was diluted with 9 ml of 10 mmol/l hydrochloric acid and passed through a Carbopack column prepared in the same way as described for the enzyme purification. The vial was rinsed successively with two 2.5-ml portions of aqueous 3 mmol/l hydrochloric acid and the rinsings passed through the column. The column was washed with 10 ml of 0.3 mol/l formic acid in methanol, followed by 3 ml of chloroform—methanol (15:85, v/v). The oestriol was eluted with chloroform—methanol (60:40, v/v) and 3 ml of the eluate were collected, starting from the moment the eluting solution was applied to the column. The solution was evaporated under a stream of nitrogen at 60°C. The residue was reconstituted in 60 μl of the solution used as mobile phase and 20 μl were injected into the chromatograph. The total oestriol concentration of standard and patient samples was calculated by comparing the height of the peak produced by oestriol in the sample with that of an authentic oestriol standard. The latter is prepared for chromatography by evaporating 10 μl of one of the unconjugated oestriol working standard solutions and reconstituting with 60 μl of the mobile phase. The response of the fluorimetric detector is linearly related to amount of injected oestriol within the range 0.08—30 ng.

RESULTS AND DISCUSSION

Enzyme purification

The hydrolysis of the oestriol conjugates is the critical point in this assay. The crude solution from *Helix pomatia* contains a lot of organic impurities which are not completely eliminated by the isolation procedure of the oestriol freed by hydrolysis. These compounds produce a number of chromatographic peaks, some obscuring that of the oestriol. Neither the use of purified *Helix pomatia* extracts commercially available nor a purification procedure involving the use of XAD-2 [6] gave satisfactory results.

The procedure elaborated by us, which exploits removal of organic impurities from the extract by adsorption onto the Carbopack B surface, completely eliminates any positive bias in the analysis of total oestriol. Under the experimental conditions selected, repeated (n = 6 in each case) activity tests showed no loss of the β-glucuronidase activity, while only a 6% loss of arylsulphatase activity occurred owing to the purification procedure. Fig. 1 shows typical chromatograms of serum samples hydrolysed with the purified enzyme preparation and carried through the procedure.

The amount of Carbopack B and the preliminary dilution ratio of the *Helix pomatia* juice with acetate buffer are both critical parameters in the purification procedure. We observed a significant loss of enzymic activity by increasing the amount of the adsorbing material or the dilution ratio. This loss can be explained by assuming that the adsorption rate of complex large molecules such as proteins is much lower than that of simpler molecules. Therefore, the enzyme passes unretained through the column provided that the percolation time is made short enough. On the other hand, insufficient enzyme purification was noted when the enzyme preparation was passed undiluted through the Carbopack column, probably because impurities bound to enzymes via non-specific interactions are not retained by the Carbopack column. Dilution of the enzyme preparation has the effect of weakening these interactions.

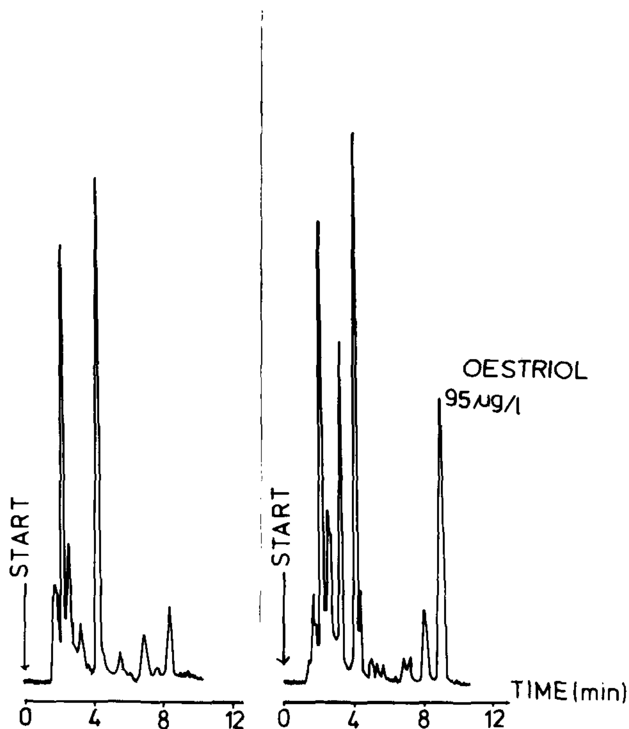

Fig. 1. Chromatograms of purified plasma from a non-pregnant woman (left) and from a pregnant woman at 34 weeks of gestation (right).

Recovery and precision

We assessed the analytical recovery of oestriol and the within-run precision of the method by adding increasing amounts of authentic $E_3$16-G and $E_3$3-S to oestriol-free pooled serum (Table I). The average recovery in the concentration we considered was 92.9%. Each serum sample was assayed eight times during a month. The day-to-day C.V. ranged from 4.0% at 20 µg/l to 2.3% at 400 µg/l.

Sensitivity

The limit of sensitivity (signal-to-noise ratio = 3) was set at 5 µg/l of serum. At this concentration the C.V. was 7.9%.

Specificity

The washing of the Carbopack B column with 10 ml of acidified methanol followed by 3 ml of chloroform—methanol (15:85, v/v) removed residual impurities in the enzyme preparation. The analytical procedure adopted has the same effectiveness in eliminating interfering compounds which may be present in a serum sample as that reported for unconjugated oestriol [5]. Moreover, we observed that either plasma or serum can be used for this assay. EDTA was used as anticoagulant.

TABLE I

RESULTS OF NINE REPLICATE ANALYSES OF OESTRIOL CONJUGATES ADDED TO POOLED SERUM

Added* (μg/l)	Found (μg/l, mean ± S.D.)	C.V. (%)	Recovery (%)	
			Mean	Range
20	18.6 ± 0.73	3.9	93.0	89.1—96.6
100	92.1 ± 2.7	2.9	92.1	89.6—95.6
200	185 ± 5	2.7	92.5	90.5—95.7
400	375 ± 9	2.3	93.8	91.3—96.1

*Data are expressed as oestriol.

Method comparison

Serum samples from 40 patients in their last trimester of pregnancy were analysed by our method and by a commercially available total oestriol RIA kit [Amerlex oestriol (total) RIA kit, Amersham International, U.K.]. Linear regression analysis of data obtained by our HPLC procedure (y) and by the RIA method (x) gave the following results: slope = 0.942, intercept = —0.318 μg/l, r = 0.966. The mean of the HPLC values was 102 (S.D. 74.2) μg/l; the mean of the RIA values was 108 (S.D. 76.1) μg/l.

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