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Note

High-performance liquid chromatographic determination of oestrogens in human urine

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Much research has been done to improve methods for oestrogen determination in body fluids [1—3]. The results of this analysis are very important in clinical chemistry for the information that can be obtained during pregnancy. In the last months of pregnancy the oestriol concentration should rise rapidly, while low levels, or a decrease in its excretion, are indicative of malfunctions in the regular progress of the pregnancy.

It would consequently be very advantageous to have quantitative results in a fast and simple way. For routine analysis the urine can be frequently collected and the oestrogen concentration monitored daily.

Among the various methods used for oestrogen determination, colorimetry and spectrometry are not very specific, radioimmunoassay is very sensitive but difficulties are encountered in the use of radioactive compounds (e.g. only authorised laboratories can handle these compounds). Gas chromatography has been successfully used, but normally only after derivative formation, and consequently the sample treatment is more complicated and time-consuming. High-performance liquid chromatography (HPLC) has been advantageously introduced for oestrogen analysis in urine by adsorption or in the reversed-phase mode [4—6] and with selective detection method [7].

In this paper we describe the HPLC determination of oestrogen in urine using a simplified procedure for sample treatment. Various mobile phases have been investigated for an optimum HPLC separation and the results obtained for urine samples before and after enzymatic hydrolysis are reported.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Series 2 liquid chromatograph was used with a variable-wavelength detector, LC 55, and a scanning system to record the spectra

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of the eluted peaks. Two columns were used (25 × 4.6 mm) packed in our laboratory with derivatized silica LiChrosorb RP-18 or DIOL, 10 μm (Merck, Darmstadt, F.R.G.). The first column is normally used for routine analysis, the second one in only a few cases for confirmation of oestrogen peaks. By running the same sample in both the columns of different polarity or also by comparing the ultraviolet spectra of the chromatographic peak with the reference compounds, identification of the oestrogen peak and purity are established. All the solvents used (acetonitrile, methanol, isopropanol, hexane, and tetrahydrofuran) were for HPLC (Carlo Erba, Milan, Italy). The flow-rate was 1.5 ml/min. The steroids were detected at 280 nm. Sample solutions and standards were injected (50—100 μl) with a Rheodyne valve using a 175-μl loop.

Procedure

The oestrogens in urine are partly present in the free form but mainly as glucuronide and sulphate conjugates. To determine the total concentration the sample is hydrolyzed; 5 ml of urine are mixed with 100 μl of β-glucuronidase—arylsulphatase (Merck) and 2 ml of 0.1 *M* acetate buffer at pH 5.5 and incubated for 24 h at 4°C.

To determine the free oestrogens, 20 ml of urine are used as such. In both cases the sample is centrifuged and passed through a Sep-Pak C_{18} cartridge (Waters Assoc.) previously washed with methanol and subsequently with water. Only after this treatment do new cartridges show reproducible retention. After urine passage the Sep-Pak is washed with 10 ml of water and the oestrogens are recovered by elution with 2 ml of methanol. The solvent is

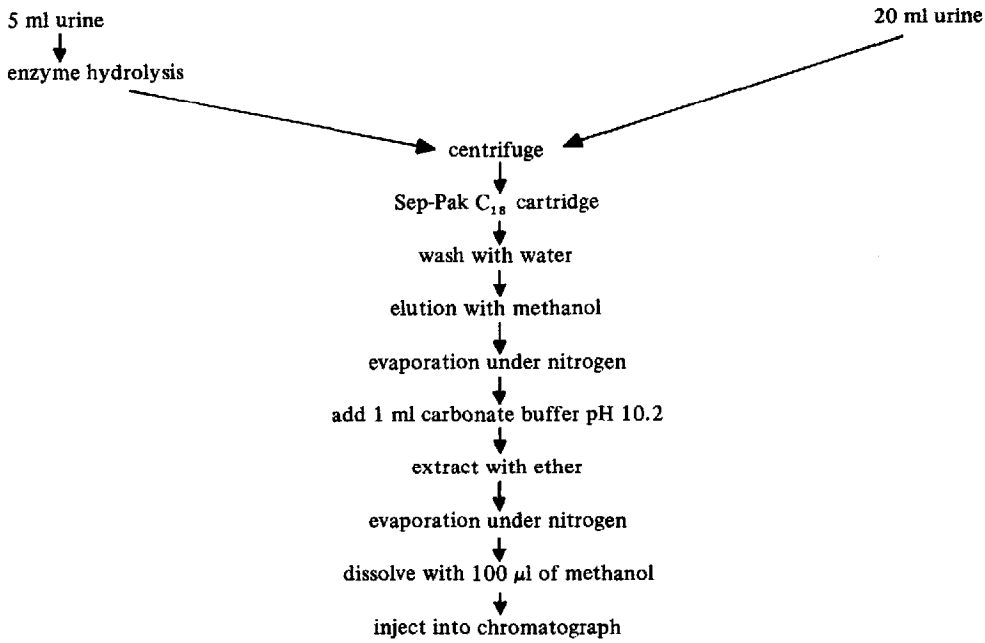

Fig. 1. Flow diagram of the oestrogen extraction from urine, and sample treatment.

evaporated under nitrogen and the residue is dissolved in 1 ml of 0.1 *M* carbonate buffer at pH 10.2 and about 0.1 g of sodium sulphate is added. The steroids are extracted with ether (3 × 1 ml) and the ether extracts are washed with 1 ml of water, dried over sodium sulphate and evaporated under nitrogen. The residue is dissolved in 100 μl of methanol and injected into the chromatograph. This extraction procedure is outlined in Fig. 1.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of a standard mixture of oestriol, oestradiol and oestrone, with the RP C_{18} column. The first part of the chromatogram is run in isocratic conditions (acetonitrile—water, 23:77) and, after the elution of oestriol, with a gradient up to 73% of acetonitrile in water at 3% per min.

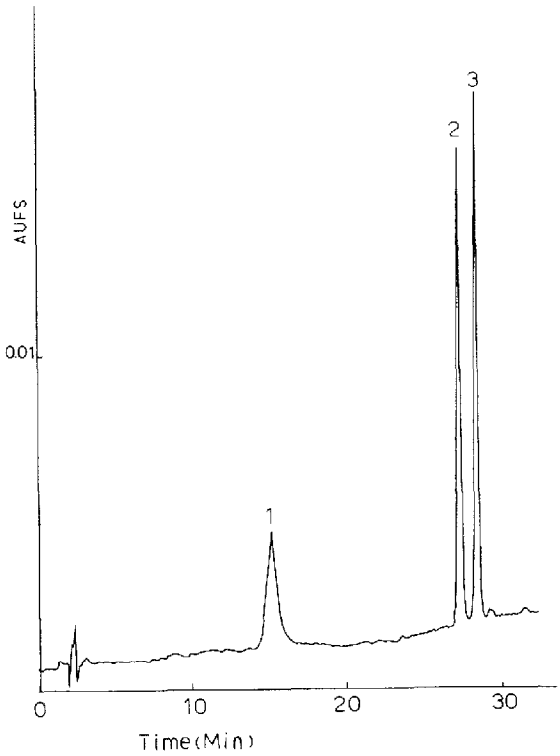

Fig. 2. Chromatogram of the oestrogen mixture on RP-18 column (10 μm, 25 × 4.2 mm). Flow-rate = 1.5 ml/min. Mobile phase: 23% acetonitrile in water for 15 min and then programmed to 77% acetonitrile in water at 3% per min. Peaks: 1 = oestriol, 2 = oestradiol, 3 = oestrone.

Because of the large difference in polarity between oestriol and the other oestrogens it is advisable to use the gradient. Table I shows the values of capacity ratios and the separation factors with various solvents. The percentage of water is regulated in order to have comparable k' values for the last peak (oestrone). The best separation of oestrone and oestradiol is achieved

TABLE I

SEPARATION FACTORS (α) AND CAPACITY FACTORS (k') OF OESTROGENS WITH DIFFERENT MOBILE PHASES (RP-18 COLUMN)

Mobile phase		k'			α
		Oestriol	Oestradiol	Oestrone	Oestrone/ oestradiol
Methanol—water	45:55	2.2	13.5	15.5	1.1
Acetonitrile—water	35:65	1.0	9.9	15.8	1.6
Tetrahydrofuran—water	35:65	2.3	10.3	13.5	1.3
Methyl acetate—2-propanol—water	25:13:62	1.7	9.6	13.6	1.4

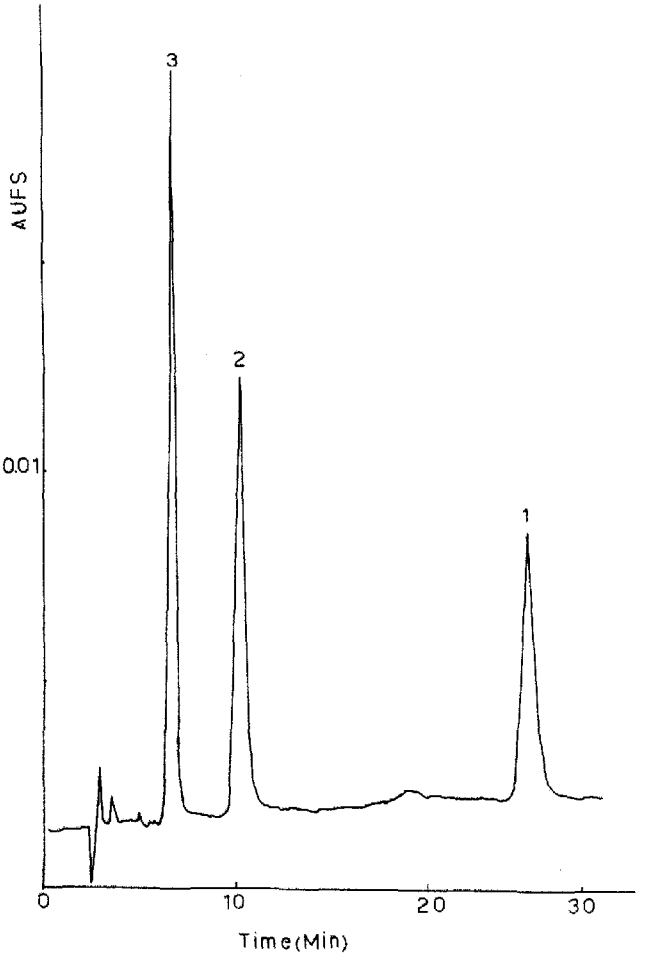

Fig. 3. Chromatogram of the oestrogen mixture on DIOL column (10 μm, 25 × 4.2 mm). Flow-rate = 1 ml/min. Mobile phase: *n*-hexane—isopropanol (9:1) for 10 min and then programmed to 30% of isopropanol in *n*-hexane at 1% per min. Peaks as in Fig. 2.

with acetonitrile—water. With the DIOL column, as shown from the chromatogram of Fig. 3, the elution order is completely changed and this fact can be usefully employed as a confirmatory test.

By using a Sep-Pak C_{18} cartridge a very fast and simple extraction of free and conjugated oestrogens is obtained, without the formation of emulsion which usually occurs with solvent extraction. The sample clean-up is performed by partition of the oestrogens between ether and a buffer solution at pH 10.2. Under these conditions oestrogens can be extracted from ether while many impurities (acidic and phenolic compounds) remain in the water phase. Recoveries of 85—90% were obtained from urine spiked with the oestrogens and extracted according to the full procedure reported in Fig. 1 for conjugated steroids.

Figs. 4 and 5 show the chromatograms obtained from a urine sample before and after enzymatic hydrolysis.

Under the working conditions described, many polar constituents are eluted before oestriol and do not interfere with the oestrogen determination. Table II shows the oestrogen concentration of different samples. Sample 1 is the

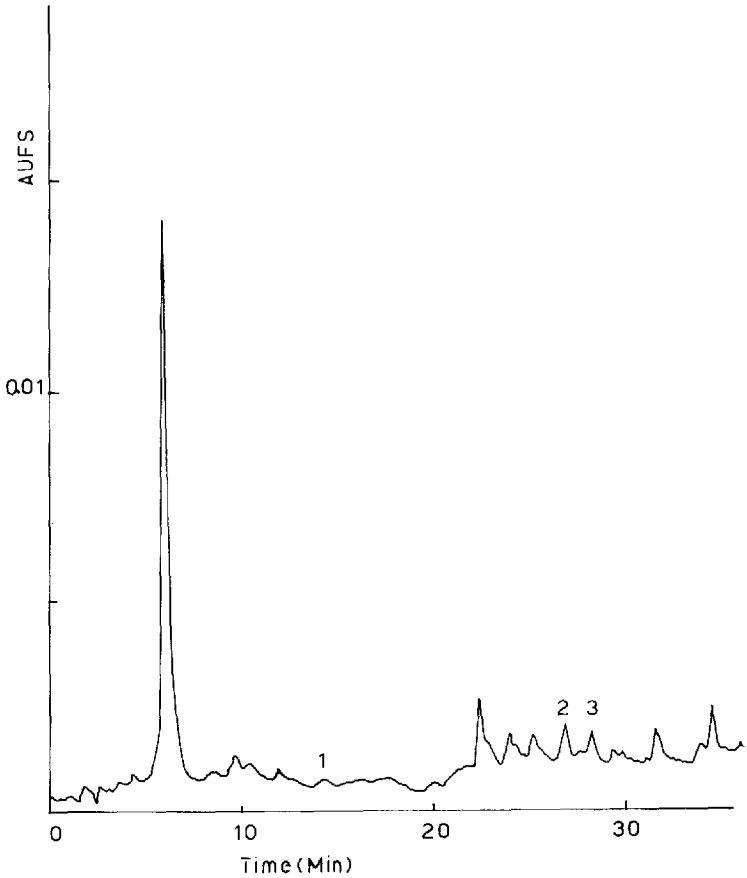

Fig. 4. Chromatogram of an extract of a non-pregnancy urine. Conditions and peaks as in Fig. 2.

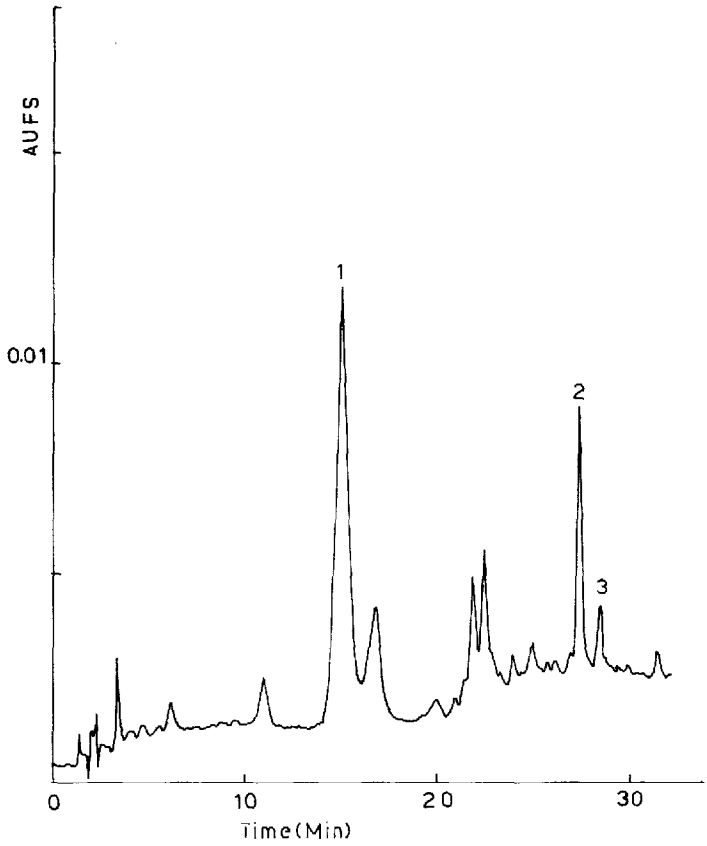

Fig. 5. Chromatogram of an extract from a pregnancy urine after hydrolysis. Conditions and peaks as in Fig. 2.

TABLE II

HPLC DETERMINATION OF OESTROGEN CONCENTRATION IN URINE

Sample No.	Months of pregnancy	Oestriol (mg/l)	Oestradiol (mg/l)	Oestrone (mg/l)
1	0	0.2	0.2	0.4
2	9	25.0	3.8	0.8
3	9	11.4	0.2	0.2
3	9	11.4	0.3	0.3
4	9	22.0	3.0	0.9
4	9	21.8	3.0	0.7
4	9	21.5	3.5	0.7
4	9	21.6	3.5	0.8
4	9*	0.06	0.05	0.04
5	4	2.5	0.3	0.3
6	8	7.2	1.1	1.3

*Not hydrolyzed.

urine of a non-pregnant woman and, as expected, low levels of oestrogens are observed. To show the reproducibility of the method the values obtained from duplicate analysis of sample 3, and from four analyses of sample 4 are reported. These analyses were repeated on different portions of the same urine sample. Sample 4 was also analyzed without hydrolysis and, as observed by other authors [5], the concentration of oestrogens in the free form is very small compared with the concentration of the conjugated compounds. The oestriol concentration rises sharply in the last period of pregnancy and is a very useful indicator of the patient's situation. Samples 2, 3 and 4 are from different subjects at the end of pregnancy, and consequently there are natural differences in the oestriol concentrations, but all of them show high values of oestriol, while the oestradiol and oestrone concentrations are at lower levels.

By HPLC many routine determinations of oestrogens can be carried out for clinical analysis with a simple and efficient extraction procedure and sample treatment.

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