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IMPROVED METHOD FOR THE ANALYSIS OF ESTROGENIC STEROIDS IN PREGNANCY URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The use of microparticulate packing materials and large injection volumes gives significant improvements in the analysis of complex samples, such as urine extracts by high-performance liquid chromatography. Lower detection limits and improved accuracy can now be attained. In addition, the combined use of adsorption and reversed-phase chromatography leads to reduced uncertainty in peak identification and gives more reliable quantification.

INTRODUCTION

Large quantities of estrogens, particularly estriol, are produced in the female body during the later months of pregnancy, and are eventually excreted in the urine mainly in the form of sulphate and glucuronide metabolites. We have previously described [1] a method for the analysis of these compounds in urine using high-performance liquid chromatography (HPLC). While demonstrating the potential of the technique, the separation was inadequate to allow accurate quantification. The availability of small-diameter porous packing materials and efficient slurry packing techniques [2] has since led to significant improvements in resolution. In addition, it has been suggested [3, 4] that use of large injection volumes can give improved sensitivity with little degradation of column performance. These factors should have important consequences, particularly in the field of body fluid analysis, where extracts are both dilute and subject to complex interferences.

Recent publications [5—7] have described the application of HPLC to the analysis of a variety of constituents in urine, and Trefz et al. [8] have analysed

human plasma for cortisol and related compounds. Synthetic mixtures of estrogens have been separated on chemically bonded stationary phases in an isocratic system by Butterfield et al. [9] and using gradient elution by Majors and Hopper [10]. However, the determination of estrogens in urine extracts has normally been performed using other techniques, such as thin-layer chromatography with spectrodensitometry [11], column chromatography followed by combined gas chromatography—mass spectrometry [12], and gel chromatography [13, 14].

This communication describes the application of improved column and sampling technology to the analysis of estrogens in pregnancy urine. Samples were analysed by both liquid—solid adsorption chromatography (LSAC) and reversed-phase chromatography (RPC) and we will demonstrate that this combination provides confirmatory information.

EXPERIMENTAL

A 40-ml sample of urine was hydrolyzed with concentrated hydrochloric acid, and the estrogens were extracted into diethyl ether as described previously [1]. The extract was reduced to 1 ml and analysed by LSAC on porous irregular chips of silica gel (Partisil-5, Whatman, Maidstone, Great Britain) of 7-μm mean particle diameter, packed in a 150 mm × 4.9 mm I.D. stainless-steel tube using a balanced density packing technique [2]. The mobile phase used with this column was 5% (v/v) ethanol in *n*-heptane.

A second extract was evaporated to dryness in a stream of dry nitrogen, the residue was redissolved in 1 ml of a 55:45 (v/v) mixture of methanol and 0.1% ammonium carbonate in water and analysed by RPC. This was performed on a column of Partisil-10 ODS (Whatman) which was purchased pre-packed in a 250 mm × 4.6 mm I.D. stainless-steel tube. This packing material consists of an octadecylsilane surface layer chemically bonded to 10-μm silica particles via Si—O—Si bonds. A 55:45 (v/v) mixture of methanol and 0.1% ammonium carbonate in water was used as mobile phase.

In both LSAC and RPC, mobile phase was delivered by a reciprocating piston pump and associated pulse damping equipment (Pye Unicam, Model 20LC chromatograph), and the components were detected using a UV detector (Cecil, Model CE212), operating at 280 nm. Samples were injected by means of a 75-μl loop valve (Valco, Model CV-6-HPA).

Synthetic mixtures were prepared using the pure estrogens purchased from BDH (Poole, Great Britain). The solvents were of 'AnalaR' grade and were variously supplied by BDH and Hopkin & Williams (Chadwell Heath, Great Britain). Deionised water was used in all cases.

RESULTS

Liquid—solid adsorption chromatography

The system was calibrated using standard solutions of the estrogens in diethyl ether and a typical chromatogram is shown in Fig. 1. The solvent peak was used for the calculation of capacity factors, which, together with the

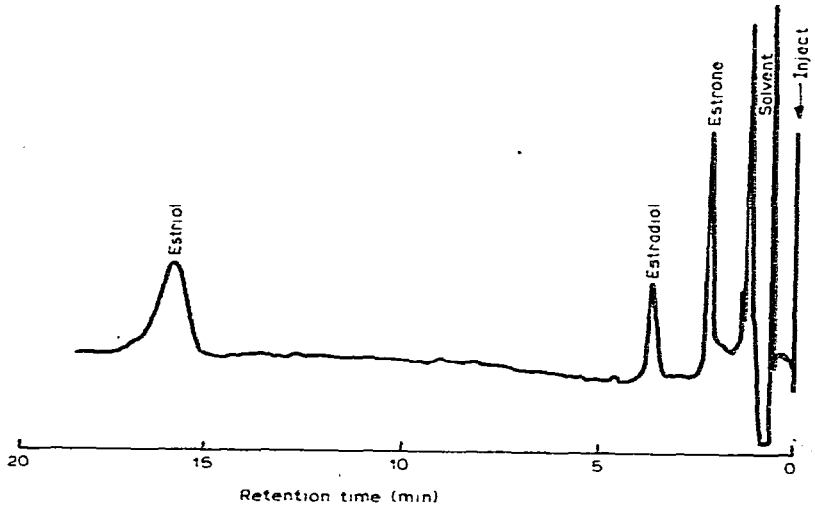

Fig. 1. Separation of estrogen mixture on Partisil-5. Mobile phase, 5% (v/v) ethanol in *n*-hexane; flow-rate, 5 ml/min; detector, 280 nm; range, 0.1 absorbance unit.

respective calibration factors, are listed in Table I. As mobile-phase flow-rate and retention times were constant, it was convenient to quantify peaks in terms of peak height, and calibration factors were defined as:

$$\text{Calibration factor} = \frac{\text{Peak height (fraction of f.s.d.) x attenuation (absorbance units)}}{\text{Amount of solute injected (mg)}}$$

A chromatogram of urine extract is shown in Fig. 2, with the peaks having capacity factors corresponding to estrone, estradiol and estriol indicated. While those peaks corresponding to estradiol and estriol are satisfactorily resolved, it is impossible to positively identify a peak for estrone. Confirmation by another method is clearly required.

In order to quantify the peaks for estradiol and estriol in the urine extract, it was necessary to determine the efficiency of extraction. The extraction was performed in the manner described, using 1.0 mg of estriol dissolved in 50 ml of male urine, and the chromatogram (Fig. 3) was compared with a blank

TABLE I

CALIBRATION AND CAPACITY FACTORS ON PARTISIL-5 SILICA GEL

Compound	Capacity factor (k')	Calibration factor (absorbance units/mg)
Estrone	2.7	17.5
Estradiol	5.3	15.4
Estriol	26.4	2.8

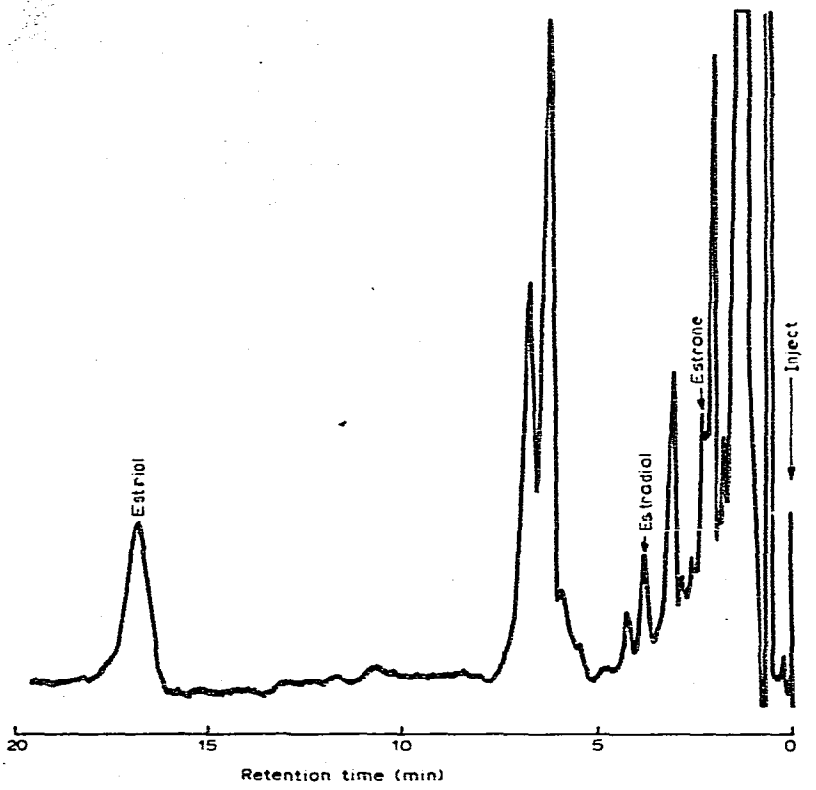

Fig. 2. Chromatogram of an extract of pregnancy urine on Partisil-5. Conditions as for Fig. 1.

obtained by extracting an 'unspiked' 50-ml sample of the same male urine. The height of the peak for estriol was measured and the extraction efficiency was determined as 27%.

The concentrations of each estrogen in the pregnancy urine were determined using the extraction efficiency, calibration factors and the volumes of extract (1 ml) and urine (40 ml). Six extractions were performed and the mean values and standard deviations are given in Table III.

Reversed-phase chromatography

The reversed-phase system was calibrated using standard solutions of estrogens in a methanol—0.1% ammonium carbonate in water (55:45, v/v) mixture. A typical chromatogram is shown in Fig. 4 and the corresponding capacity and calibration factors are given in Table II.

A chromatogram of urine extract on Partisil ODS is shown in Fig. 5, with the peaks having capacity factors corresponding to those of the estrogens indicated. In this case, all of the estrogens are adequately resolved for quantification. The extraction efficiency was not separately determined for the samples for RPC,

TABLE II

CALIBRATION AND CAPACITY FACTORS ON PARTISIL ODS

Compound	Capacity factor (k')	Calibration factor (absorbance units/mg)
Estrone	4.0	5.7
Estradiol	4.9	5.8
Estriol	1.9	20.0

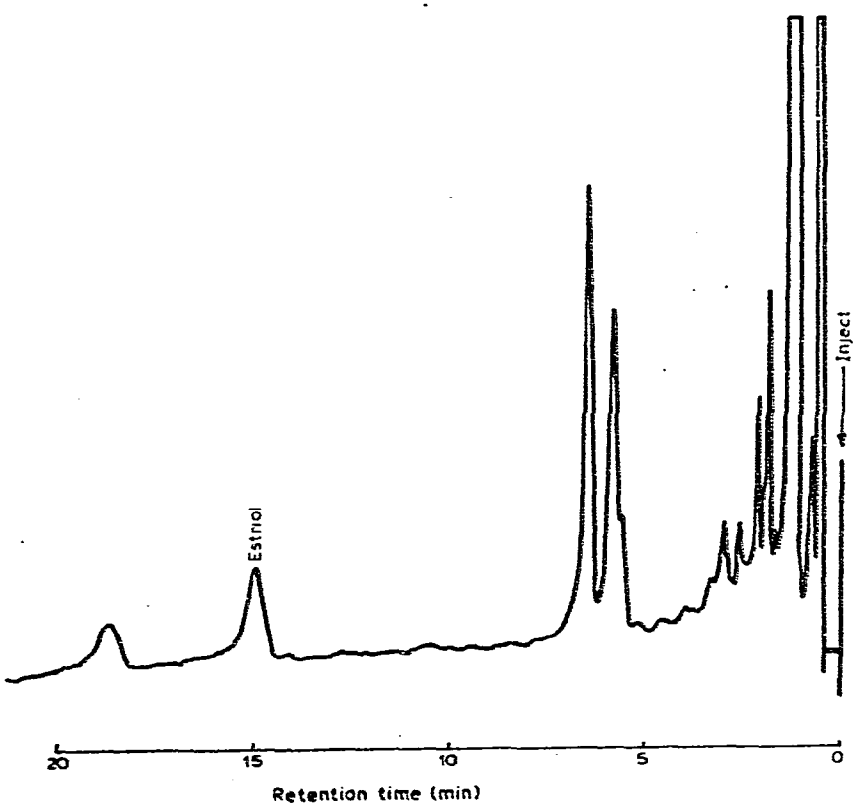

Fig. 3. Chromatogram of an extract of male urine, 'spiked' with estriol, on Partisil-5. Conditions as for Fig. 1.

but it was assumed that no loss of estrogens had been incurred in evaporating the extract to dryness and redissolving in methanol–ammonium carbonate solution. The previously measured extraction efficiency was then used to calculate the concentration of each estrogen in the pregnancy urine and the mean values obtained after analysing six extracts are given in Table III.

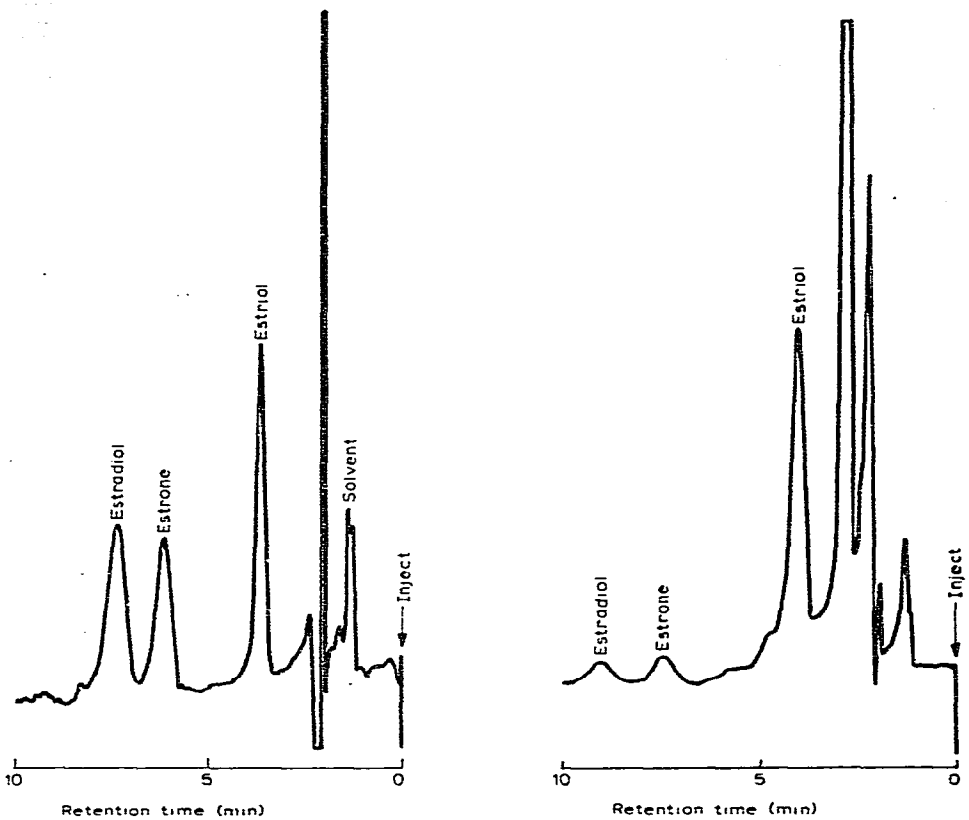

Fig. 4. Separation of estrogen mixture on Partisil-10 ODS. Mobile phase, methanol—0.1% aqueous ammonium carbonate (55:45, flow-rate 2 ml/min; detector range, 0.25 absorbance units.

Fig. 5. Chromatogram of an extract of pregnancy urine on Partisil-10 ODS. Conditions as for Fig. 4.

TABLE III

CALCULATED ESTROGEN LEVELS IN THE PREGNANCY URINE WITH RELATIVE STANDARD DEVIATIONS (RSD)

Compound	Concentration in the urine (g/l)	
	LSAC	RPC
Estrone	—	$7.3 \cdot 10^{-3}$ (RSD 18%)
Estradiol	$1.8 \cdot 10^{-3}$ (RSD 18%)	$2.5 \cdot 10^{-3}$ (RSD 18%)
Estriol	$33 \cdot 10^{-3}$ (RSD 15%)	$30 \cdot 10^{-3}$ (RSD 9%)

DISCUSSION

The results obtained from the two chromatographic systems show good agreement. Although quantification of the estrone peak on Partisil-5 was not possible, the well resolved peak obtained on Partisil ODS could readily be measured.

As would be expected, the concentration of estriol is considerably greater than that of either estrone or estradiol, and the figures are consistent with the levels normally obtained in the later months of pregnancy.

We have not attempted a rigorous determination of the extraction efficiency, but an indication of the precision is included with the results in Table III (standard deviations less than 20%).

The extraction efficiency of 27% is considerably lower than would have been anticipated from previous work [15] and merits further comment. A shorter extraction procedure was investigated, wherein a sample of 'spiked' urine was acid-hydrolysed and extracted into three 50 ml aliquots of diethyl ether which were combined and reduced in volume to 1 ml. The efficiency of this extraction was greater than 90%, indicating that considerable amounts of estriol must be removed in the usual neutralisation stages, but without neutralisation acidic co-extractants degraded the resolution and interfered in the determination of estrone and estradiol.

Huber et al. [15] emphasised the importance of reducing the pH of the neutralising sodium hydroxide solution to 10 as otherwise losses of up to 60% (w/w) of the extrogens were observed. Although care was taken over this point, our extraction efficiency was still low, and the extraction procedure could merit further investigation.

A comparison of the chromatograms in the present paper with those included in our previous publication [1] indicates the greatly enhanced resolution that can be achieved with microparticulate packing materials. This leads to important advantages in the ease and accuracy of quantification. The resolution is not significantly degraded by the use of large injection volumes (75 $\mu\ell$), which in turn lead to useful improvements in signal-to-noise ratios of the detected peaks. Such improvements would allow the quantification of much less concentrated estrogen solutions, as would be obtained when monitoring the urine in the earlier months of pregnancy.

HPLC can therefore offer the two important advantages of reduced analysis time and greater sensitivity compared with the more conventional column chromatography using Sephadex LH-20 [13, 14].

CONCLUSIONS

Advances in column packing material and techniques over the last three years lead to significant improvements in the analysis of complex samples such as urine extracts. Microparticulate adsorbents also allow the use of large sample volumes, without significant loss in efficiency, offering lower detection limits and improved accuracy.

The combined use of LSAC and RPC has led to reduced uncertainty in peak

identification and gives more reliable quantification. If only estriol is to be determined, LSAC alone would prove adequate. However, when it is necessary to quantify all three estrogens, RPC yields the more useful information. A disadvantage of RPC arises from the higher viscosity of the mobile phase, which results in a three-fold increase in pressure for a given flow-rate. This could mean that reduced flow-rates must be used in RPC because of the pressure limitations of the equipment. However, even at a lower flow-rate, the retention times are often lower with RPC than with LSAC.

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