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THE ANALYSIS OF ESTROGENIC STEROIDS IN URINE BY HIGH-SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

The extraction and subsequent rapid analysis of urine samples for specific estrogens using a simple liquid–solid chromatographic system is described. Samples were analysed at pressures between 100 and 3000 lbs./in.^{2} and flow-rates between 0.5 and 3.0 ml/min using a high-pressure sampling valve and a pulseless pump. The estrogens were separated within 6 min on a 1-m Corasil I column with an ethanol–*n*-hexane eluent. Detection limits were approximately 20 ng.

Two different extraction procedures were compared. Using a simple ether extraction, the total analysis time was less than 1 h.

INTRODUCTION

It is universally accepted by pathologists and clinicians that much valuable information on the condition of a patient can be obtained by monitoring the level of certain chemicals in the body fluids. Possibly the most convenient source of such information is the urine, which can readily be analysed on a day-to-day basis. The concentrations of estrogenic steroids in female urine form a particularly useful source of knowledge in two distinct areas.

Urine analysis is now an accepted part of monitoring a pregnancy, and under normal circumstances large amounts of estrogens, particularly estriol, are produced in the body and excreted during the later months. A deficiency in the excretion level of estrogens is indicative of possible malfunction of the placenta and rapid counteractive measures by the clinician can reduce the possibility of any permanent damage to the foetus. The concentration of estriol in the urine during the later months of pregnancy is normally of the order 3 mg/l.

A second requirement for estrogen analysis, and one that has only become important in recent years, concerns females who are taking the so-called "fertility drugs". In non-pregnant females, the total estrogen concentration in the urine is normally of the order of 10 μg/l, but as the drugs themselves contain estrogenic steroids, this level can rise significantly. It has been suggested that the incidence of multiple births among females taking these drugs is associated with a high concentration of estriol in the body at the time of conception. It would therefore be advantageous

if the level of estrogens in the urine could be monitored on a day-to-day basis, in order that the amount of the drug administered can be carefully controlled. This non-pregnancy urine presents the more difficult analysis problem in that the total concentration of each estrogen is of the order 3 μg/l, and a 10-fold increase could be significant.

The most common method of analysis, which has been in use in hospital laboratories for a number of years, involves a long and complex extraction procedure, ending with the Kober[1] colour reaction. The intensity of the colour, which is measured by colorimetry or fluorimetry, indicates the amount of estrogens present. Unfortunately, this colour reaction does not differentiate between individual estrogens. Specificity can be introduced by the inclusion of an additional chemical extraction stage (*e.g.*, Givner *et al.*[2]), but this greatly increases the time involved and is completely unsuitable for a day-to-day monitoring method. Although it is widely believed that the concentration of estriol is most significant, the time required for a full analysis by traditional methods means that many hospital laboratories settle for the compromise of simply determining the total estrogen content of the urine.

Liquid chromatography offers an alternative separation method for the estrogens in urine. Conventional column chromatography is, however, a notoriously slow process and as such offers no time advantage over the traditional extraction method. Recent developments in liquid chromatographic theory and instrumentation, however, have resulted in a highly efficient separation method that is ideally suited to the rapid analysis of complex mixtures. Huber *et al.*[3] have described a method for the rapid separation and determination of estrone, estradiol and estriol in pregnancy urine using column liquid–liquid chromatography and on-line detection. These workers used a ternary two-phase system of water–ethanol–2,2,4-trimethylpentane mixtures.

This paper describes the analysis of both pregnancy and non-pregnancy urine by means of liquid–solid chromatography. This is a potentially simpler technique than liquid–liquid chromatography with no problems introduced by the loss of stationary phase.

URINE EXTRACTION PROCEDURES

As reported by Huber *et al.*[3], it can be calculated from liquid–liquid distribution data published by Engel *et al.*[4] that at least 99.9% (w/w) of estrone, estradiol and estriol can be removed from a given volume of an aqueous solution by three equal volumes of diethyl ether. This can be used either as a complete extraction in itself or as part of a comprehensive extraction procedure as outlined below.

Simple ether extraction

A 50-ml sample of the urine was shaken with three successive 50-ml volumes of diethyl ether. The three ethereal extracts were combined and reduced in volume to 0.5 ml by evaporation at room temperature in a continuous stream of nitrogen.

Full extraction

The full extraction procedure was essentially the same as that used by Huber *et al.*[3]. A 50-ml sample of the urine was heated under reflux with 7.5 ml of concentrated hydrochloric acid for 30 min. This acid hydrolysis was designed to break down

the estrogen conjugates yielding free estrone, estradiol and estriol. The hydrolyzed sample was extracted three times with 50 ml of diethyl ether and the ethereal extracts were combined. Unwanted acidic components were removed from the diethyl ether by shaking with 20 ml of a sodium carbonate solution at pH 10.5, and then with 4 ml of 0.1 *M* sodium hydroxide, adjusting the pH to 10.0 with 0.05 *M* sodium hydrogen carbonate. Finally, the ethereal phase was washed with 4 ml of an 8% (w/w) solution of sodium hydrogen carbonate, followed by 3 ml of deionized water. The ethereal extract was reduced in volume to 0.5 ml as described previously.

The final extracts were transferred to 1 ml graduated Reacti-vials (Pierce Products) fitted with PTFE-lined septa and stored under refrigeration so as to avoid further evaporation of the solvent.

It was expected that in non-pregnancy urine, the estrogens would be present mainly as free compounds and as such should be removed, using the simple ether extraction. Pregnancy urine, on the other hand, was expected to contain principally the glucuronide and sulphate conjugates of the estrogens, in which case the full extraction would be more effective. In order to evaluate the efficiency of the two extraction procedures, pregnancy and non-pregnancy samples were extracted by both methods. The extracts were analysed by liquid–solid chromatography and the results were compared.

CHROMATOGRAPHIC INSTRUMENTATION

A schematic diagram of the liquid chromatograph is given in Fig. 1.

The pulseless pump unit, designed and constructed in these laboratories, consists of a heavy worm-driven jack, which pushes a piston into a smooth-walled stainless-steel cylinder. A 250-ml volume of liquid is delivered with each complete stroke of the piston and facilities are provided for fast flushing and refill operations. Using appropriate feed-back devices, the pump can be operated at either constant pressure or constant flow-rate, and for the purpose of the present work the constant-flow mode was used. Analyses were performed at flow-rates between 0.5 and 3.0 ml/min and pressures of 100–3000 lbs./in.^{2}, which were well within the working range of the pump.

The pressure transducer used in the feedback system is manufactured by Bell and Howell Ltd. (Type 4-366-0001, 0–5000 lbs./in.^{2}) and is used in conjunction with a Bell and Howell strain gauge amplifier (Type 1-176). A record of the pressure during an analysis is obtained by feeding the amplifier output signal direct to one channel of a two-pen recorder as well as back to the pump control unit. At any instant, the transducer can be checked against a 12-in. dial pressure gauge, calibrated from 0 to 6000 lbs./in.^{2} in divisions of 5 lbs./in.^{2}, which was supplied by the Heise Bourdon Tube Co.

The sampling valve, which was also designed and constructed in these laboratories, is capable of injecting extremely reproducible volumes of 1–15 μl of sample into a liquid stream at any pressure up to 5000 lbs./in.^{2}. By virtue of its design, the valve is most suitable for the injection of samples that are available in amounts of 0.5 ml or greater, and in this work was used only for the injection of synthetic mixtures of steroids. Urine extracts, reduced in volume to 0.5 ml in order to obtain measurable concentrations of the estrogens, were injected by syringe at a simple low dead volume injection head. It should be noted that a slight modification of the valve has now been made, which will allow the injection of samples that are available in

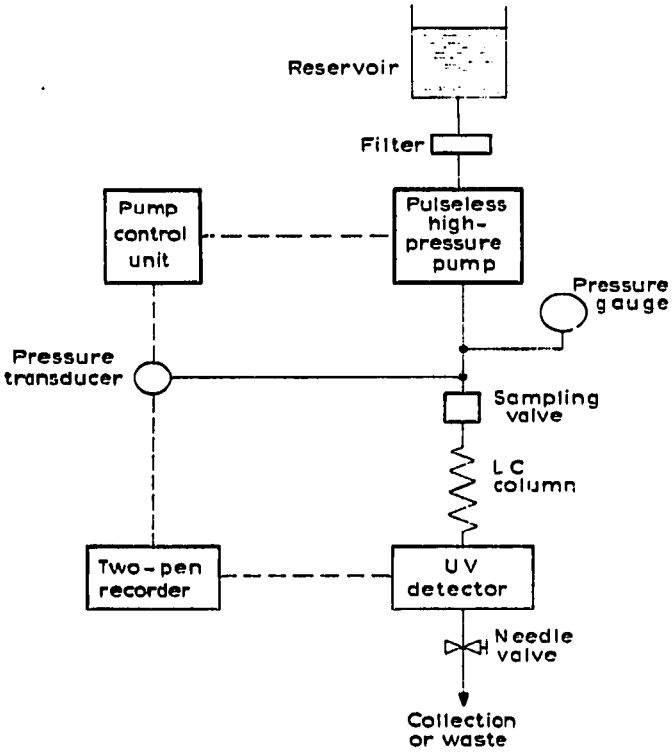


Fig. 1. Schematic diagram of the liquid chromatograph.

much smaller amounts than those indicated above. Using either the valve or the syringe, injection was directly on to the head of the column.

The column itself consisted of the superficially porous silica, Corasil I (37–50-μm particles from Waters Associates Ltd.), packed into a straight stainless-steel tube, 1 m long and 2.2 mm I.D. It was dry-packed using a "tap-fill" method similar to that recommended by Kirkland[5]. A drilled-out Kromlok coupling and 10 cm of 0.25 mm I.D. PTFE capillary tubing connected the column outlet to the flow-cell of a Cecil CE212 UV monitor. The variable wavelength facility allowed the determination and subsequent use of the optimum wavelength for estrogen detection, which was shown to be 280 nm. Output from the detector was recorded, with the pressure, on a Servoscribe two-pen potentiometric recorder (Smiths Industries).

Samples of pure estrone, estradiol and estriol, together with all the solvents and other chemicals used, were purchased from BDH Ltd.

Absolute ethanol (AnalaR) and *n*-hexane (designated free from aromatic hydrocarbons) were used without further purification as column eluents.

RESULTS

Calibration for estrone, estradiol and estriol

UV absorption spectra of standard solutions of each of the estrogens in ab-

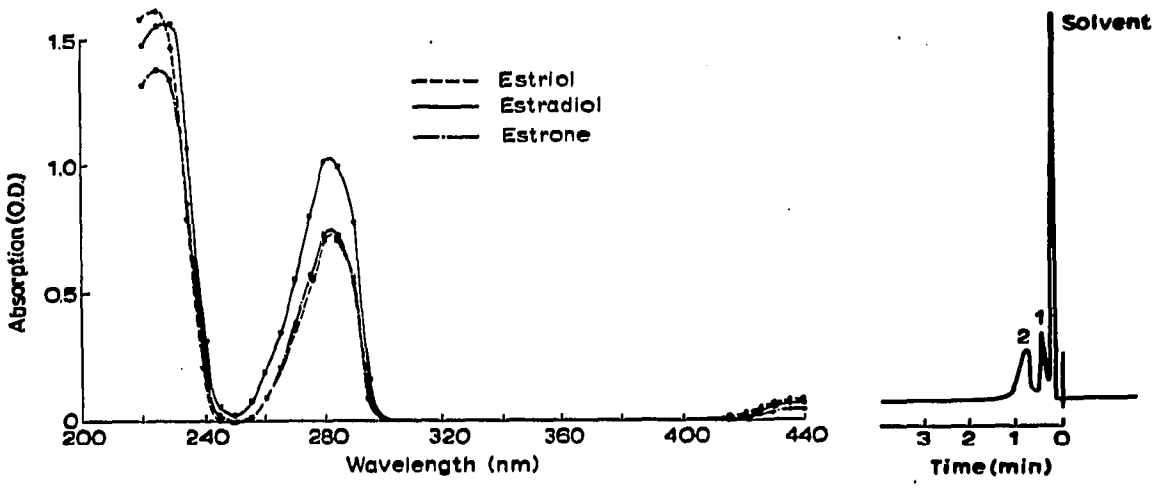


Fig. 2. UV absorption spectra of the estrogens.

Fig. 3. Separation of estrogens using tetrahydrofuran–*n*-hexane (1:9) as eluent. Column, Corasil I. Sample, 3 μl estrone, estradiol and estriol in tetrahydrofuran. Pressure, 1000 lbs./in.^{2}. Flow-rate, 3.0 ml/min. Detector range, 0.05 O.D. 1 = Estrone; 2 = estradiol.

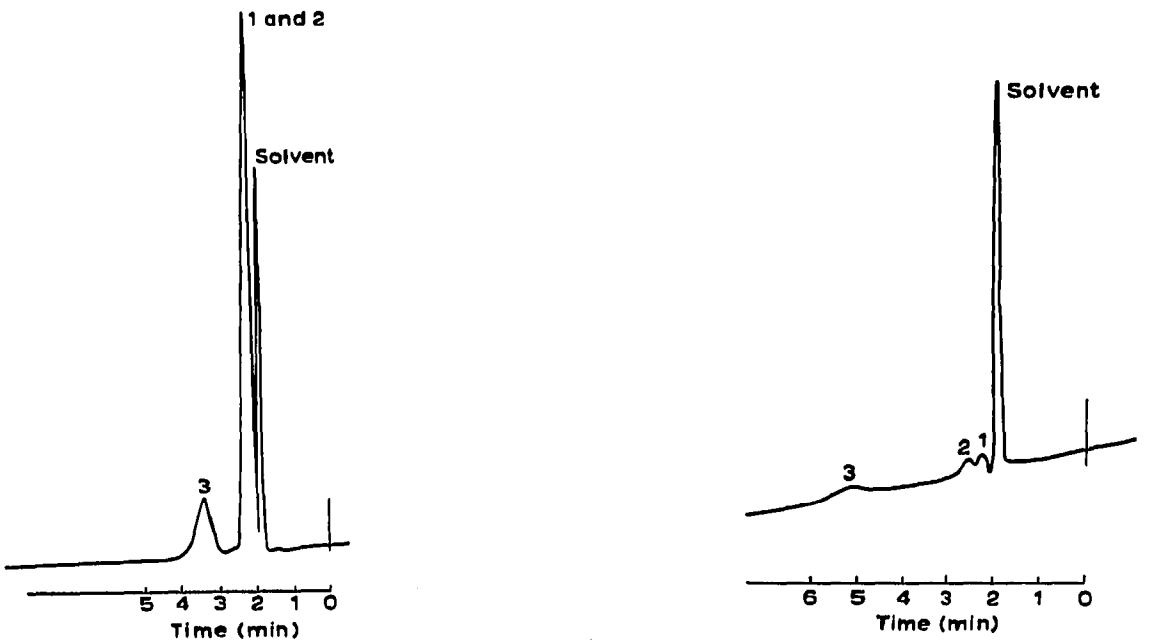


Fig. 4. Separation of estrogens using ethanol–*n*-hexane (1:9) as eluent. Column, Corasil I. Sample, 3 μl estrone, estradiol and estriol in ethanol. Pressure, 3000 lbs./in.^{2}. Flow-rate, 1.0 ml/min. Detector range, 0.05 O.D. 1 = Estrone; 2 = estradiol; 3 = estriol.

Fig. 5. Separation of nanogram amounts of estrogens using ethanol–*n*-hexane (4:96) as eluent. Column, Corasil I. Sample, 5 μl estrone, estradiol and estriol in ethanol. Pressure, 175 lbs./in.^{2}. Flow-rate, 0.85 ml/min. Detector range, 0.05 O.D. 1 = Estrone; 2 = estradiol; 3 = estriol.

solute ethanol were determined using the variable-wavelength Cecil UV detector. These spectra were measured using a 3-ml aliquot of a solution in a 10-mm path-length silica cell, and were compared with that obtained for 3 ml of absolute ethanol. Fig. 2 shows the differential spectra for each estrogen derived by allowing for the absorption due to the alcohol. In each case, a large absorbance peak centred around 280 nm and this wavelength was chosen as being the most suitable for monitoring the liquid chromatographic column eluant.

At this stage, it was necessary to identify a suitable solvent that could be used to separate a mixture of the three estrogens on a Corasil I column. Using tetrahydrofuran–*n*-hexane mixtures, good separations of estrone and estradiol were obtained, but estriol was not eluted from a 1-m column (Fig. 3). On the other hand, using an ethanol–*n*-hexane (1:9) mixture and the same column, estriol was eluted in 3 min but estrone and estradiol could not be separated (Fig. 4). An ethanol–*n*-hexane (4:96) mixture was found to provide the best compromise and by using this mobile phase the chromatogram shown in Fig. 5 was obtained for an ethanol solution containing 113 ng of estrone, 21 ng of estradiol and 56 ng of estriol. A flow-rate of 0.8 ml/min and column inlet pressure of 150 lbs./in.^{2} gave the best combination of speed of analysis and resolution. Capacity ratios of the three estrogens were determined under these conditions and are given in Table I. A synthetic estrogen solution was injected at intervals in order to monitor column performance and the capacity ratios remained constant to within $\pm$ 5% during the course of this investigation. The system was calibrated for each estrogen in turn, by measuring the peak heights obtained for 3 μl injections of successively diluted solutions. Calibration graphs are shown in Fig. 6.

The baseline noise of the UV detector was measured in order to determine the minimum detectable amount of each estrogen. The maximum peak-to-peak noise was 0.0002 optical density (O.D.) units and the total drift over 15 min was 0.0008 O.D. units. Taking the minimum detectable peak heights as twice the peak-to-peak noise level, the minimum detectable amounts listed in Table I were established.

TABLE I

CAPACITY RATIOS OF ESTROGENS

Compound	*Capacity ratio, k'*	*Minimum detectable amount (ng)*
Estrone	0.22	23
Estradiol	0.37	13
Estriol	1.77	19

Analysis of pregnancy urine

Aliquots of a sample of pregnancy urine were subjected to the two extraction procedures outlined above. Fig. 7 shows a chromatogram for a urine aliquot treated by the simple ether extraction method. Small peaks can be seen for estrone, estradiol and estriol. As a direct comparison, Fig. 8 shows a chromatogram for an extract of the same urine obtained by the full extraction procedure. In this case, a much larger peak was obtained for estriol, whereas those for estrone and estradiol were again small. A series of injections was performed for each extract and the estrogen peak heights were measured. The estrone peaks could not be quantified, but these were small in comparsion with those of the other two estrogens. In all of the chromatograms, the

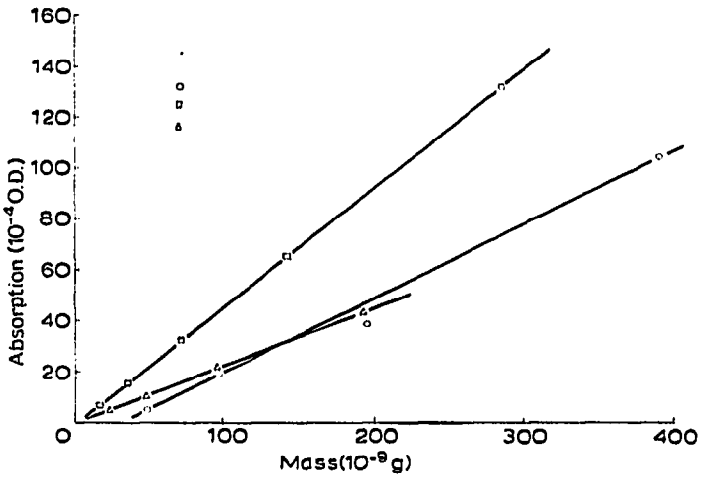


Fig. 6. Calibration of UV detector for estrogens. ○, Estrone; □, estradiol; △, estriol.

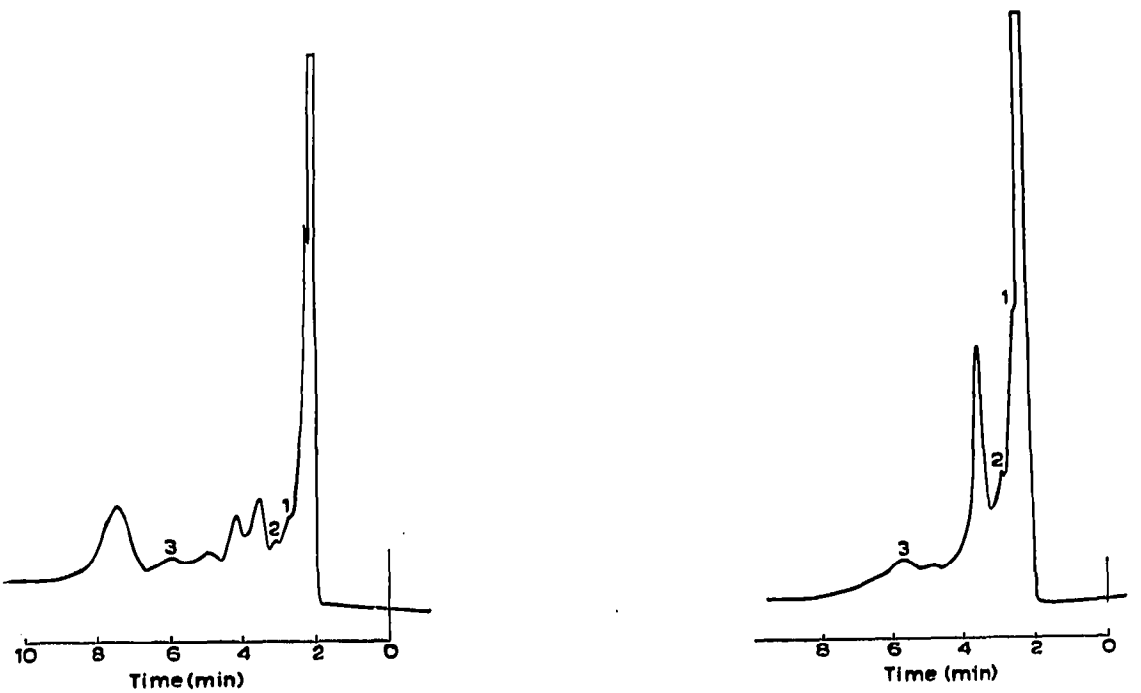


Fig. 7. Analysis of pregnancy urine using ether extraction. Column, Corasil I. Eluent, ethanol–*n*-hexane (4:96). Sample, 5 μl pregnancy urine extract. Pressure, 154 lbs./in.^{2}. Flow-rate, 0.80 ml/min. Detector range, 0.05 O.D. 1 = Estrone; 2 = estradiol; 3 = estriol.

Fig. 8. Analysis of pregnancy urine using the full extraction. Column, Corasil I. Eluent, ethanol–*n*-hexane (4:96). Sample, 2 μl pregnancy urine extract. Pressure, 157 lbs./in.^{2}. Flow-rate, 0.80 ml/min. Detector range, 0.2 O.D. 1 = Estrone; 2 = estradiol; 3 = estriol.

estrogen peaks appeared on the tail of a large impurity peak and quantitation was carried out by the tangent method.

TABLE II

CONCENTRATIONS OF ESTROGENS DETERMINED IN PREGNANCY URINE

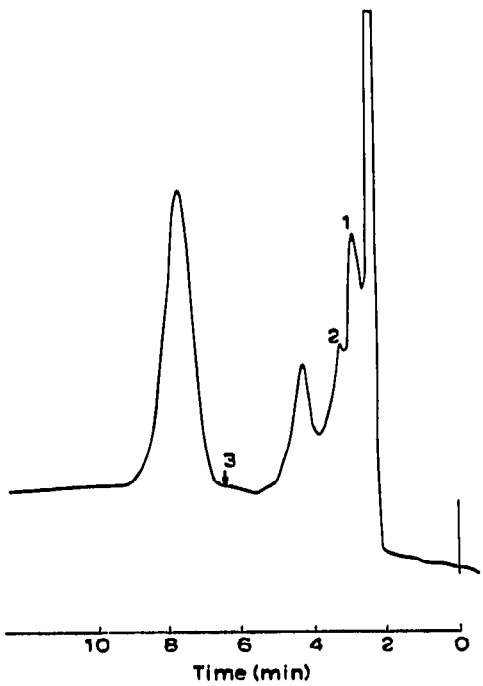
Compound	*Concentration in pregnancy urine (g/l)*	
	Ether extraction	*Full extraction*
Estrone	—	—
Estradiol	$0.03 \cdot 10^{-3}$	$0.60 \cdot 10^{-3}$
Estriol	$0.05 \cdot 10^{-3}$	$1.93 \cdot 10^{-3}$

The concentrations of estrogens in the pregnancy urine, as determined by the two extraction methods, are given in Table II.

It is apparent that a simple ether extraction of pregnancy urine removes only a fraction of the estrogens, probably due to their existence in a conjugated state.

Analysis of non-pregnancy urine

Aliquots of a non-pregnancy urine were also extracted by both of the methods previously described. Chromatograms for samples from the two extraction procedures are shown in Fig. 9 and 10. The chromatogram of the simple ethereal extract shows



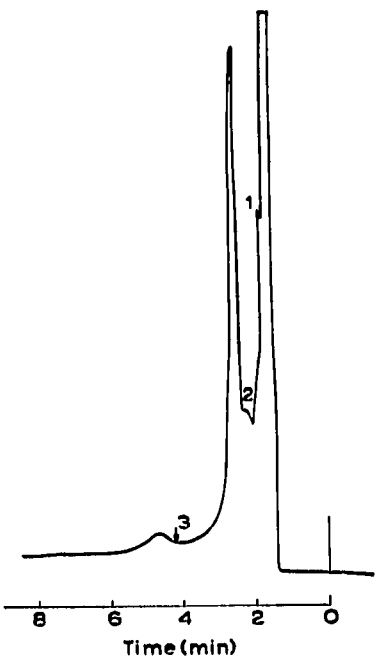


Fig. 9. Analysis of non-pregnancy urine using ether extraction. Column, Corasil I. Eluent, ethanol–*n*-hexane (4:96). Sample, 4 μl non-pregnancy urine extract. Pressure, 153 lbs./in.^{2}. Flow-rate, 0.80 ml/min. Detector range, 0.02 O.D. 1 = Estrone; 2 = estradiol; 3 = estriol.

Fig. 10. Analysis of non-pregnancy urine using the full extraction. Column, Corasil I. Eluent, ethanol–*n*-hexane (4:96). Sample, 2 μl non-pregnancy urine extract. Pressure, 214 lbs./in.^{2}. Flow-rate, 0.90 ml/min. Detector range, 0.1 O.D. 1 = Estrone; 2 = estradiol; 3 = estriol.

TABLE III

CONCENTRATIONS OF ESTROGENS DETERMINED IN NON-PREGNANCY URINE

Compound	*Concentration in non-pregnancy urine (g/l)*	
	Ether extraction	*Full extraction*
Estrone	$0.24 \cdot 10^{-3}$	—
Estradiol	$0.03 \cdot 10^{-3}$	—
Estriol	—	—

distinct peaks for estrone and estradiol, but no indication of the presence of estriol. Analysis of the sample obtained from the full extraction procedure gave similar results, although the peaks for estrone and estradiol were less distinct. Concentrations of estrogens in non-pregnancy urine, as indicated by the two extraction methods, are given in Table III.

It would seem that the simple ether extraction is more suitable for non-pregnancy urine, as the estrogens are efficiently extracted while some of the masking impurities are left behind in the aqueous layer.

CONCLUSIONS

The liquid–solid chromatographic system described in this paper has proved most useful in the analysis of both pregnancy and non-pregnancy urine. It offers considerable advantages over the more traditional methods of analysis in terms of speed and specificity. Different extraction procedures have been investigated in conjunction with the liquid chromatographic system in order to optimize the total analysis. In the case of pregnancy urine, it has been established that acid hydrolysis followed by a series of chemical extractions provides the most efficient method. The total time between receiving the sample and completing the analysis is 2 h.

A simple ether extraction of non-pregnancy urine is acceptable and the total analysis time is reduced to 1 h. An interesting point arose with the analysis of non-pregnancy urine. The sample analyzed contained significant amounts of estrone and estradiol but no detectable estriol. This was contrary to expectations in that estriol was thought to be present in the greatest concentration. As previously mentioned, estriol is thought to be the most important of the estrogens in terms of its role in the onset and progress of a pregnancy. The traditional method of analysis simply determines the total estrogen content and, as in the present case, this could possibly lead to erroneous conclusions about the condition of a patient. Analysis by liquid chromatography removes the possibility of any ambiguity in this context.

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