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QUANTITATIVE ANALYSIS OF TRACE AMOUNTS OF ESTROGENIC STEROIDS IN PREGNANCY URINE BY COLUMN LIQUID–LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

J. F. K. HUBER AND J. A. R. J. HULSMAN*

Laboratory of Analytical Chemistry, University of Amsterdam, Amsterdam (The Netherlands)

AND

C. A. M. MEIJERS

Laboratory for Clinical Chemistry, St. Anna Hospital, Geldrop (The Netherlands)

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SUMMARY

The influence of the sample volume on peak height and resolution in elution column chromatography was studied. Graphs which enable the choice of the optimum sample volume are given. The quantitative analysis of estrogens by column liquid–liquid chromatography with ultraviolet detection was investigated.

The method has been applied to the rapid quantitative determination of estriol in urine. After preparing the urine sample for chromatography, the quantitative information can be obtained in 20 min with a precision of 5 % for a concentration of 3 mg l. The noise of the sample-free detector corresponds to 60 ng of estriol.

INTRODUCTION

The analysis of steroid hormones in body fluids is characterized by the large number and variety of compounds and the wide range and low values of concentrations. In urine, several hundred steroids are known to occur and the number of steroids in blood is even higher. The amounts of the individual steroids are generally very low and correspond to a daily excretion level in the nanogram to milligram range. A number of methods have been developed for the determination of steroids in body fluids. It would be ideal if a physical quantity could be measured which depended only on the compound to be determined. In general, however, a physical measurement alone is not specific enough. In order to increase the specificity of the analysis method, chemical reactions and separations from interfering compounds have to be carried out prior to the physical measurement. Separation and measurement can be combined off-line and on-line. The second procedure is less laborious and more precise than the first.

The oldest method for the chemical analysis of estrogens is based on a highly specific colour reaction[1] followed by colorimetric or fluorimetric measurement. The

* Present address: Laboratory for Clinical Chemistry, St. Anna Hospital, Geldrop, The Netherlands.

identification and determination of the individual estrogens, however, requires an efficient separation which can be performed only by extraction and chromatography. More than 100 papers deal with the application of chromatography to the chemical analysis of estrogens in body fluids. This literature is discussed in a number of reviews[2-26] with exception of the most recent papers[27-33]. The first step in the analysis of estrogens in body fluids is the hydrolysis of the conjugates, followed by the extraction of the free estrogens and other phenolic compounds, chromatographic separation of the phenolic fraction and the determination of the components by a selective measuring method. In many cases derivatives have to be prepared to increase the specificity of the separation or detection. In gas chromatography, the preparation of derivatives seems to be a necessity for high performance. In classical liquid chromatography, the separation is slow and the detection is generally performed off-line. As a result of these factors, both methods are not yet fully satisfactory with respect to speed, simplicity and reliability. Automation can not be achieved easily. The use of modern liquid column chromatography should ensure a great improvement. In this paper it is shown that a rapid and precise determination of low levels of estriol in the phenolic fraction of human urine can be achieved by column liquid–liquid chromatography with on-line UV detection.

THEORY

The description of the chromatographic transport of a compound is based on the mass balance in a section of the column which is described by a partial differential equation. With some assumptions and simplifications, it is possible to find a single solution for given boundary and initial conditions. This solution is called the transport function, $<c_i^m> = f(z,t)$, and it describes the concentration[34], $<c_i^m>$, of the sample in the moving fluid as a function of the coordinate, z, in the direction of flow and the time, t.

A partial form of the transport function is the temporal distribution function, $<c_i^m>_z = f(t)_z$, which describes the concentration as a function of time at a given location. The temporal distribution functions at the beginning ($z = 0$) and the end ($z = L$) of the column are called the input and output functions, respectively.

The output function, $<c_i^m>_L = f(t)_L$, approximates asymptotically to a Gaussian function[35] if: (i) the variance of the input function, $\sigma_{t_0}^2$, is much smaller than that of the output function, $\sigma_{t_L}^2$; and (ii) the standard deviation, σ_{tL}, of the output function is much smaller than the retention time, t_R. For these conditions, the output function can be written as:

$$<c_i^m>_L = <c_i^m>_L^{max.} \exp\left(-\frac{1}{2}\frac{(t_{Ri} - t)^2}{A(\sigma_t^2)_L}\right)$$

with

$$t_{Ri} = \frac{L}{<v>}(1 + K_i q)$$

$$A(\sigma_t^2)_L = \frac{LH(1 + K_i q)^2}{<v>^2}$$

$$<c_i^m>_L^{max.} = \frac{Q_i}{v\sqrt{2\pi A(\sigma_t^2)_L}}$$

where

$\langle c_i^m \rangle_L$ = concentration of a component i in the moving phase m averaged over the flow cross-section at the end of the column

$\langle c_i^m \rangle_L^{max.}$ = maximum value of $\langle c_i^m \rangle_L$

t = residence time

t_{Ri} = retention time = average residence time

$\Delta(\sigma_t^2)_L$ = variance of the output function arisen in the column

L = length of the column

$\langle v \rangle$ = fluid velocity averaged over the cross-section of flow

$K_i = c_i^s / c_i^m$ = distribution coefficient

$q = V_s / V_m$ = volume ratio of the stationary and moving phase

$H = L\Delta(\sigma_t^2)_L / t_{Ri}^2$ = theoretical plate height

Q_i = amount of the component injected.

The equation for the maximum concentration can be modified if the expressions $Q_i = c_{i0} V_0$ and $\Delta(\sigma_t^2)_L = \langle v \rangle^2 \Delta(\sigma_t^2)_L$ are substituted:

$$\langle c_i^m \rangle_L^{max.} = \frac{V_0 c_{i0}}{\sqrt{2\pi \Delta(\sigma_V^2)_L}} \tag{2}$$

where V_0 and c_{i0} are the injection volume and the concentration of the component in the original sample, respectively, and $\langle v \rangle$ is the flow rate.

For accurate quantitative analysis, a sufficiently high concentration in the detector is required to ensure a high signal-to-noise ratio. According to eqn. 2, the maximum concentration of a compound in the detector can be increased by increasing the injection volume as well as by concentrating the component in the sample before the injection.

The concentration which may be injected is limited by (i) the saturation concentration in the mobile liquid, which may be very low, and (ii) the non-linearity of the distribution isotherm which causes additional non-symmetric peak broadening.

The input variance contributes to the output variance according to the equation[36]

$$\sigma_{V,L}^2 = \sigma_{V0}^2 + \Delta(\sigma_V^2)_L \tag{3}$$

where

$$\sigma_{V,L}^2 = \langle v \rangle^2 \sigma_{t,L}^2 \text{ and } \sigma_{V0}^2 = \langle v \rangle^2 \sigma_{t0}^2.$$

An expression for the dilution of the sample as a function of the injection volume can be derived[37] if it is assumed that the output function remains Gaussian so that after replacement of $\Delta(\sigma_t^2)_L$ by $\sigma_{t,L}^2$, eqn. 2 can be applied. Together with eqn. 3, the following expression is obtained:

$$\frac{\langle c_i^m \rangle_L^{max.}}{c_{i0}} = \left(\frac{2\pi\sigma_{V0}^2}{V_0^2} + \frac{2\pi\Delta(\sigma_V^2)_L}{V_0^2} \right)^{-1/2} \tag{4}$$

The first term describes the dilution at the injection, the second term the dilution in the column. If the output function is not Gaussian, eqn. 4 may not be applied.

Nevertheless, the influence of the injection volume on the dilution will follow the same trend.

In order to optimize the sample loading, it is necessary to take the resolution into account. The resolution of two compounds A and B is defined as:

$$R_{BA} = \frac{t_{RB} - t_{RA}}{\sigma_{tL}} \tag{5}$$

The standard deviation of the component A or B as well as their average value may be chosen for σ_{tL}. Owing to the influence of the variance of the input function on the variance of the output function, the resolution will decrease with increasing sample loading. Substitution of eqn. 3 in eqn. 5 gives the relation between the resolution and the width of the input curve:

$$\frac{R_{BA}}{R_{BA}^{\max.}} = \left(\frac{\sigma_{V0}^2}{\sigma_{VL}^2} + 1 \right)^{-1/2} \tag{6}$$

where

$$R_{BA}^{\max.} = (t_{RB} - t_{RA}) / \sqrt{\sigma_t^2}_L$$

On the basis of eqns. 4 and 6, it is possible to determine the practical optimum between the gain in peak height and loss in resolution.

APPARATUS

The chromatographic apparatus (Fig. 1) is similar to that described earlier[38].

Separation unit

The eluent flow was provided by a pulsating pump (Orlita DMP 1515) fitted with a Bourbon tube manometer and a capillary restriction as a damping device.

The sample was injected by a precision syringe (Hamilton) into the eluent stream in an injection port which effected only negligible mixing. Two coexistent phases of the ternary system water–ethanol–2,2,4-trimethylpentane[39] (molar ratios 0.229:0.680:0.091 and 0.019:0.177:0.804) were used as the stationary and moving phases. This phase system has proved to be useful for thes eparation of estrogens[40,41]. Diatomaceous earth with particle diameter 28–32 μm was coated with the water-rich

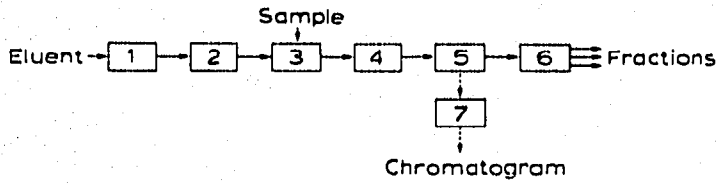

Fig. 1. Block diagram of a liquid chromatograph. 1 = thermostatted eluent reservoir; 2 = pump with damping device; 3 = sampling system; 4 = separation column with thermostat; 5 = detector; 6 = fraction collector; 7 = recorder.

phase and packed tightly into a thick-walled borosilicate glass tube of length 50 cm and I.D. 0.27 cm. The water-poor phase was pumped through the fixed bed. The eluent stock and columns were kept at constant temperature (22 ± 0.2°) by use of a thermostat. The column was connected to the detector with a capillary tube of length 3 cm and I.D. 0.5 mm, the contribution of which to the peak variance was negligible.

Detector unit

The detector was a UV spectrophotometer (Zeiss PMQ II) with a home-made[12] flow cell of capacity 7.5 μl. The flow cell was cylindrical with a diameter of 1 mm and a path length of 10 mm. The absorption measurements were carried out at a constant wavelength of 281 nm, corresponding to the absorption maximum of the estrogens. The spectrophotometer amplifier had a range of 10 mV. The relation between the concentration in the detector cell and the output potential is

$$1 - \log U \sim E \sim \varepsilon dc \qquad (7)$$

where U is the potential (V), E is the absorbance, c is the concentration, ε is the molar absorbance and d is the optical path length.

The detector signal was fed to a logarithmic recorder (Servogor RE 514.9) in order to obtain a deflection which is proportional to the concentration. The voltage may be considered to decrease proportionally to the concentration, however, in the range $10 \geqslant U \geqslant 8$ mV, corresponding to the absorbance range of $0 \leqslant E \leqslant 0.1$.

For the measurement of low concentrations, scale expansion was therefore applied and the signal in the range $8 \leqslant U \leqslant 10$ mV was recorded with a 2-mV recorder (Servogor type RE 511) which was provided with a ball and disc integrator in order to record the line integral of the recorder deflection. For the determination of the noise level, the voltage was amplified 100-fold by a DC-Millivoltmeter (Philips PM 2436).

QUANTITATIVE ANALYSIS OF TEST SOLUTIONS

Sample feed

The influence of the increase in the sample volume was investigated. In order to find a correlation with eqn. 3, the relation between the volume variance of the output function and the square of the injection volume was considered. An example of the plotting of the results is given in Fig. 2. It can be seen that a linear relationship exists, which corresponds to eqn. 3 if it is assumed that σ_{f0} increases in proportion to the injection volume. $\Delta(\sigma_f^2)_L$ can be found by extrapolation from graphs according to Fig. 2.

The influence of the injection volume on the concentration in the detector can be demonstrated by plotting the concentration ratio, c_f^{max}/c_{f0}, as function of $V_0/[\Delta(\sigma_f^2)_L]^{1/2}$. In order to obtain a general plot, dimensionless numbers are used. Fig. 3 shows an example of such a graph. The results agree satisfactorily with eqn. 4 if the ratio σ_{f0}^2/V_0^2 is assumed to be constant.

An increase in the maximum concentration in the detector due to the increase of the injection volume is coupled with an increase of the peak width and therefore with a loss of resolution. The influence of the injection volume on the resolution can be shown in general by plotting the relation in dimensionless numbers. Fig. 4 gives an

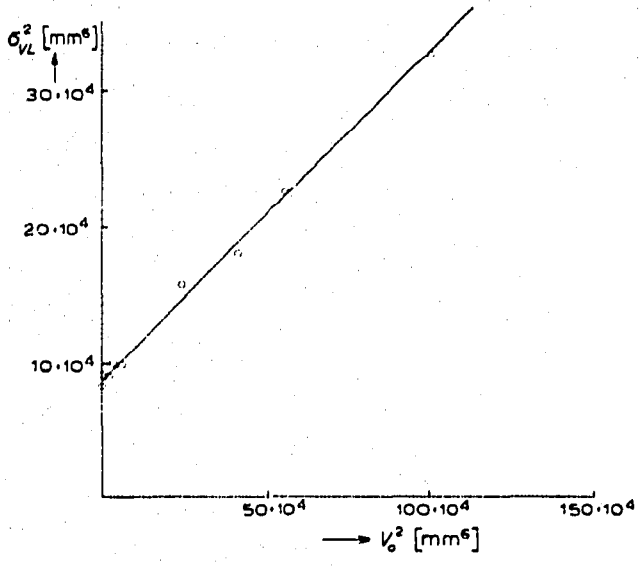

Fig. 2. Influence of the sample volume on the peak width. Sample compound: estrone.

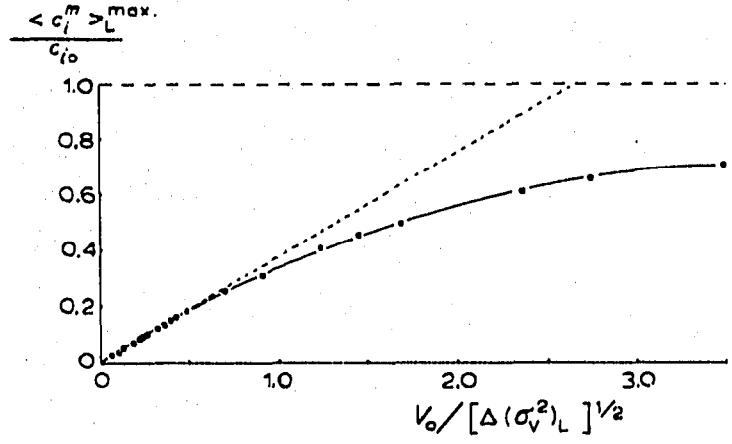

Fig. 3. Influence of the sample volume on the maximum value of the concentration peak at the column outlet. Sample compound: estriol.

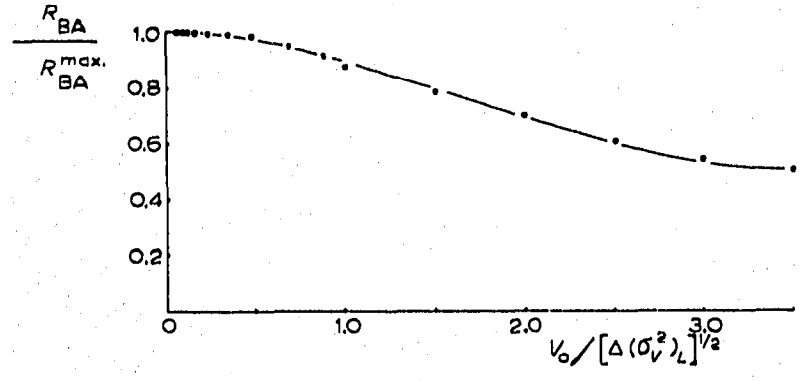

Fig. 4. Influence of the sample volume on the resolution. Sample compounds: estrone(A) and estradiol(B) ($R^{max.}_{estradiol/estrone} = 13.8$).

example of such a plot. The results agree fairly well with eqn. 6 if the ratio $\sigma_{t,0}^2/V_0^2$ is assumed to be constant.

The injection volume has opposite effects on the maximum concentration in the detector and the resolution. In quantitative analysis, the systematic error increases if the resolution decreases and the statistical error increases if the peak height decreases. A different optimum value for the injection volume is found, therefore, in different cases. From Figs. 3 and 4, it can be concluded that the maximum concentration in the detector is half of the sample concentration, and a loss of 25 % in resolution occurs if the injection volume $V_0 = 1.7\ [A/(\sigma_{t,e}^2)_L]^{1/2}$.

Precision

The precision of the analytical results in chromatography is determined mainly by sampling, detection and data processing.

The detector responds also to other influences in addition to the sample concentration, so that the output signal fluctuates even if a constant concentration is fed to the detector. The random variation of the signal is called the noise and is characterized by the standard deviation of the signal amplitude. The noise-to-signal ratio determines the precision of the measurement. The noise of the absorbance has been determined for different values of the input signal (= concentration). Fig. 5 shows a typical example of the noise which is recorded when only eluent flows through the detector cell. In terms of absorbance, the standard deviation of the noise for zero input signal is found to be $\sigma_{E0} = 4 \cdot 10^{-4}$.

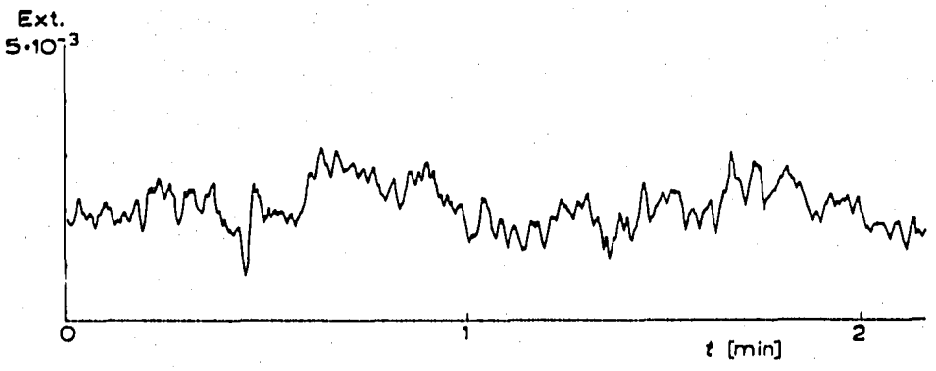

Fig. 5. Typical noise pattern of the base line of a low noise UV detector (Zeiss PMQ II).

In general a linear relation exists between the peak area and the amount of sample:

$$A_i = S_Q Q_i \tag{8}$$

where Q_i is the amount of sample compound i injected, S_Q the sensitivity of the detector with respect to Q_i, and A_i the peak area. The sensitivity was determined for estrone, estradiol and estriol with calibration mixtures of known composition, and was constant over the investigated range, $0.5 < Q < 10$ μg. The sensitivity values are included in Table I. If the sensitivity is known, it is possible to calculate the absolute amount of the compound present in a sample of unknown composition from the peak area.

TABLE I

SENSITIVITY DATA FOR THE UV DETECTOR FOR ESTROGENS

Compound	c_{10} (*mg/l*)	S_Q (*I.U./μg*)	σ_{S_Q}/S_Q	β_{is}	$\sigma_{\beta_{is}}/\beta_{is}$
Estrone	21.7	19.02	0.060	1.082	0.024
Estradiol	43.3	17.54	0.052	1.000	
Estriol	21.8	14.71	0.063	0.830	0.020

The standard deviation of the peak area is caused by random variations of the base line and of the signal. The relative standard deviation of the peak area was found to be 0.05 in the range 20–150 (arbitrary) integration units (I.U.) corresponding to e.g. 1.4–10 *μg* of estriol. Below the peak area of 20 I.U., the relative standard deviation increases, presumably because of the base line noise. The contribution of the random fluctuation of the base line can be estimated from the repeated integration of the base line with respect to an arbitrary zero line. The standard deviation of the base line integral over 3 min, which corresponds approximately to the integration time of the estriol peak, was determined to be 0.85 I.U.

From the experimental results, it can be concluded that the standard deviation of the peak area may be described by the equation

$$\sigma_A = [\sigma_{A0}^2 + (kA)^2]^{1/2} \tag{9}$$

where σ_A is the total standard deviation of the peak area, σ_{A0} is the standard deviation of the corresponding base line integral (0.85 I.U.), and k is a constant (0.05).

Fig. 6 shows the system characteristics for estriol. The average peak area, $\bar{A}$, is plotted as a function of the mass, Q, together with a confidence limit band of $\bar{A} \pm 3\,\sigma_A$.

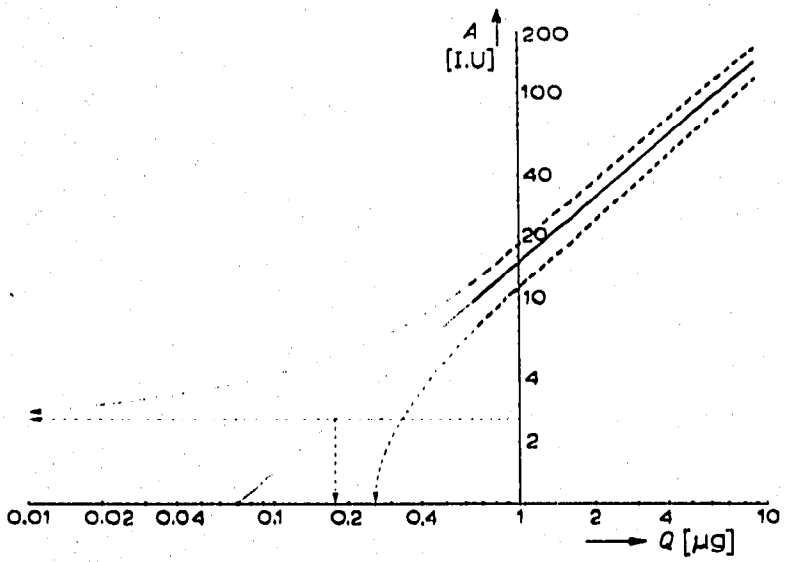

Fig. 6. Characteristics of the chromatographic system for estriol. The solid line represents the average value $\bar{A}$ and the broken line the confidence limit at $\pm 3\,\sigma_A$. The strong lines indicate the measured range.

The plot is obtained by the correlation of the experimental data according to the least squares method. The confidence lines in the range which was not measured were calculated according to eqn. 9.

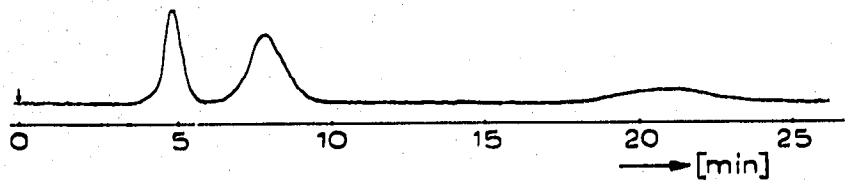

Fig. 7. Low-level chromatogram. Sample: 1.1 μg of estrone, 2.1 μg of estradiol and 1.1 μg of estriol in 50 μl of eluent.

A chromatogram of the three estrogens is shown in Fig. 7 in which the base line noise is noticeable. It illustrates that estrogens at a level of *ca.* 1 μg can still be determined without a significant decrease in the precision due to the base line noise. It can be shown from Fig. 6 that the standard deviation of the noise of the base line integral (0.85 I.U.) is equivalent to an amount of about 60 ng of estriol. Fig. 4 shows that an injection volume of $3.5\,[A(\sigma_I^2)_I]^{1/2}$, which is about 1 ml, is allowed in order to retain a resolution of 6 for estrone and estradiol, for which the maximum resolution is 13.8. The resolution of estradiol and estriol is not critical since this value is much larger. Therefore, an estriol peak with an area equivalent to the noise of the corresponding base line integral can be obtained with complete resolution of the three estrogens from a solution which contains 60 μg/l of estrol.

The error caused by the sampling is eliminated to a great extent by using the relative sensitivity, β_{is}, of a component i with respect to an arbitrary chosen standard component s. The relative sensitivity is defined by the expression

$$\frac{A_i Q_s}{Q_i A_s} = \frac{S_{qi}}{S_{qs}} = \beta_{is} \qquad (10)$$

The composition of an unknown mixture can be determined relative to a standard component from the peak areas if the relative sensitivities are known. The relative sensitivity has to be determined beforehand using a mixture of known composition. The sensitivity ratios of estrone and estriol relative to estradiol were measured in this way. Table I gives a compilation of the sensitivity data together with their standard deviations.

QUANTITATIVE ANALYSIS OF ESTRIOL IN PREGNANCY URINE

Extraction and chromatography

In connection with the complex character of a hydrolyzed urine sample, the group of estrogens is first separated by extraction from most of the other urine constituents. The extraction is based on the phenolic nature of the estrogens. Data on the liquid–liquid distribution of estrogens are available from literature[43], from which it can be calculated that a three-fold extraction of an aqueous solution of estriol with the same volume of ether yields 99.9 % (w/w) of the original amount of estriol in the ether phase. The collection of estrone and estradiol in the ether phase is even better. The separation of the phenolic species from the acidic compounds, which are also

extracted, can be achieved[43] by extraction of the ether phase with aqueous sodium bicarbonate solution or by similar procedures[44,45]. The further separation of the estrogens which remain in the ether fraction can be achieved by column liquid chromatography[44,46–49].

The column liquid chromatographic separations of estrogens described in the literature are slow. Detection is carried out discontinously in portions of the effluent which have been obtained by a fraction collector. Fluorimetry and colorimetry after a colour-developing chemical reaction have been used as the measuring methods. Column liquid chromatography, however, can be accelerated significantly[38,50]. The use of a detector with a flow cell increases the speed and precision of the analysis.

The precision and accuracy of the determination of free estriol in aqueous solution by extraction and chromatography were studied. Aqueous solutions of estriol (15.6 mg/l), adjusted to different pH values, containing 2 % (w/w) of ethanol in order to increase the solubility of estriol, were analysed repeatedly. 50 ml of the test solution were extracted three times with 50 ml of ether. The total ether phase was extracted first with 20 ml of concentrated aqueous sodium carbonate solution (pH 10.5) and then with 4 ml of an aqueous solution of 8 % (w/w) sodium hydroxide adjusted to pH 10 with sodium bicarbonate. The ether layer was first washed with 4 ml of an aqueous solution of 8 % (w/w) sodium bicarbonate followed by 3 ml of water. Finally, the ether fraction was evaporated to dryness and dissolved in a known volume of eluent. Aliquots of the solution were analysed by column liquid–liquid chromatography as described above. Each step of the extraction procedure was examined by analysing the ether fraction and the total recovery was found for each step. It proved to be important to reduce the pH of the sodium hydroxide solution to 10 as otherwise a loss of up to 60 % (w/w) was observed. The results are summarized in Table II which demonstrates the excellent accuracy and precision of the combined extraction and chromatography.

TABLE II

ACCURACY AND PRECISION OF EXTRACTION AND CHROMATOGRAPHY

Extraction conditions		*Concentration*		
pH	*Temperature* (°C)	*Added* (*mg/l*)	*Found* (*mg/l*)	*Relative standard deviation* (%)
1–7	20	15.6	15.6	4.1

Hydrolysis

Estrogens are excreted in the form of water-soluble compounds, mainly glucuronides and sulphates. Hydrolysis of the compounds is necessary prior to the extraction, separation and determination of the free estrogens. The two basic methods suitable for hydrolyzing estrogen compounds are acid hydrolysis[48,49,51–53] and enzymatic hydrolysis[51–57] with β-glucuronidase and sulphatase.

The milder conditions involved in enzymatic hydrolysis make it a suitable method for less stable steroids. However, certain difficulties are encountered when it

is used for routine estimation of steroids. The sample may contain inhibitors, as in the case of urine samples, so that the yield of free steroids is small and irreproducible. The time required for enzymatic hydrolysis is rather long as compared with the time for acid hydrolysis and the volume of the sample must be small to minimize the quantity of the expensive enzyme used. The optimization of enzymatic hydrolysis has been described[58]. A detailed comparison[51] of the results obtained by acid and enzymatic hydrolysis shows that, as far as estrogens are concerned, identical results ensue from both methods.

The main advantage of acid hydrolysis is the fact that a large volume of urine may be applied whereby a larger amount of estrogens becomes available for analysis. Furthermore, the hydrolysis time is usually shorter than the time required for enzymatic hydrolysis. The yield of free estrogens depends on a number of variables which include acid concentration, heating time and temperature. Optimum conditions have to be chosen since a loss of estrogens occurs during hydrolysis[51,59]. The disadvantage of acid hydrolysis is that, under the rigorous conditions required, damage can occur to less stable steroids.

Taking the characteristic properties of both methods into account, acid hydrolysis is preferred for the quantitative determination of estriol in urine.

The following hydrolysis procedure, which is widely used for conjugated steroids, was applied. 50 ml of the urine sample were heated to boiling and then 7.5 ml of concentrated hydrochloric acid were added. The solution was heated under reflux for 0.5 h.

Since loss of estriol due to oxidation has been reported[60], this effect was examined. First an aqueous solution of estriol was treated according to the hydrolysis conditions and analysed by extraction with ether and chromatography. No loss of estriol was observed. In order to study the behaviour of estriol in a urine matrix, a known amount of estriol was added to male urine. Total recovery of estriol was found by extraction and chromatography after treatment according to hydrolysis.

The yield of free estriol after hydrolyzing the conjugates was studied with pregnancy urine. The data in Table III demonstrate the influence of the hydrolysis time. It can be seen that the results obtained with hydrolysis time of 0.5 h and 1 h agree. With a hydrolysis time of 18 h, however, about half of the estriol was lost. If a known amount of estriol was added to a pregnancy urine sample, only 91 % of the calculated concentration increase was found. It can be seen from Table III that a concentration of about 3 mg/l of estriol in pregnancy urine can be determined with a precision of 5 %. Comparison of the chromatograms (Fig. 8) of a hydrolyzed pregnancy urine and an aqueous solution of three estrogens, both separated under the same conditions, shows

TABLE III

ACCURACY AND PRECISION OF HYDROLYSIS, EXTRACTION AND CHROMATOGRAPHY

Concentration	*Hydrolysis time (min)*		
	30	*60*	*1080*
Average value (mg/l)	3.15	3.14	1.65
Relative standard deviation (%)	4.0	4.7	10.4

the expected location of estrone and estradiol in the urine sample. It can be seen that estrone and estradiol are still overlapped by other components. This necessitates a further separation of the urine sample, which is being investigated further.

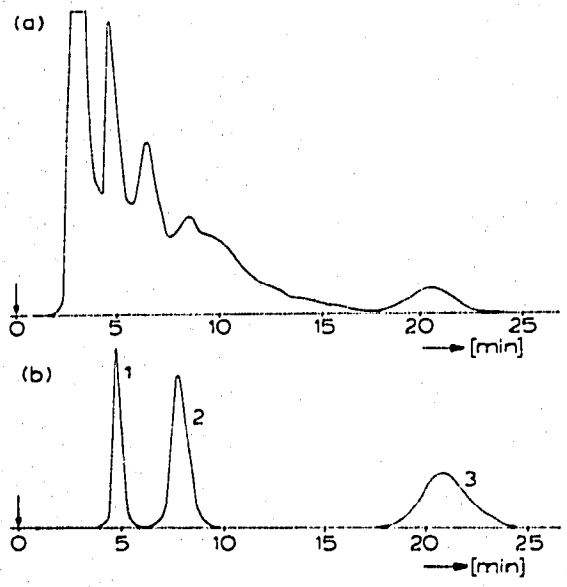

Fig. 8. Chromatograms (a) from 50 ml of pregnancy urine after hydrolysis and extraction, (b) from a test mixture of estrone (1), estradiol (2) and estriol (3). Column: 50 × 0.27 cm; liquid–liquid system water–ethanol–2,2,4-trimethylpentane, mole fractions stationary phase 0.220:0.080:0.001, mobile phase 0.010:0.177:0.804; volume ratio stationary phase to mobile phase 0.1; fluid velocity 0.32 cm s^{-1}; solid support diatomaceous earth, 28–32 μm; temperature 22°. Sample volume 50 μl.

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