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A GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF OESTRONE AND OESTRADIOL-17β IN HUMAN PLASMA DURING THE SECOND HALF OF PREGNANCY

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SUMMARY

A method for the determination of oestrone and oestradiol-17β in the plasma of pregnant women is reported. It is an extension of an earlier published method for the determination of plasma oestriol. Measurement of the three oestrogens can be made from a 5 ml plasma sample after acid hydrolysis, extraction, solvent partition and thin-layer chromatography. Quantitative measurement is made of the oestrogens' trimethylsilyl ether derivatives by gas chromatography. Results are presented after correction based on the recovery of tritium labelled free oestrogens. Data for specificity, accuracy, reproducibility, precision and sensitivity are presented.

INTRODUCTION

THE measurement of steroid metabolites in urine is of no little importance[1], and the determination of oestrogens in the blood, not only in the presence of gynaecological and endocrine disturbances, but also during pregnancy, is also of scientific and practical interest[2]. Oestriol perhaps best reflects the function of the foeto-placental unit, but oestrone and oestradiol-17β are also involved in pathological foetal conditions, as has been demonstrated in adrenal hypotrophy in anencephalics[3].

Apart from oestrogens and progesterone, the determination of the neutral steroid hormones as precursors of oestrogens during pregnancy may be valuable [4]. Considerable interest is currently centred on many hormone determinations – non-steroid as well as steroid, e.g. human placental lactogen and chorionic gonadotrophin – as possible prognostic pointers in abnormal pregnancies[5].

Methods of measuring oestrone, oestradiol-17β, and oestriol in the plasma of the pregnant woman by colorimetry[6] and fluorimetry[7] have been described. A double isotope method[8, 9] has also been reported for the determination of unconjugated oestrone and oestradiol-17β in plasma during pregnancy, and during the normal menstrual cycle.

There are relatively few reports of the measurement of plasma oestrone and oestradiol-17β by GLC. Adlercreutz[10] gives one method for the determination of conjugated oestrogens in plasma in pregnancy, where sulphate and glucosiduronate conjugated fractions in a 25 ml blood sample are measured, after enzyme hydrolysis. Touchstone and Murawec[11] have determined the free and conjugated oestrogens in plasma pools of 100 ml from pregnant women, and Kroman *et al.*[12] report the measurement of the three oestrogens in 90 ml blood samples from women not pregnant.

Some authors have determined oestrogens from smaller samples of pregnant

women's plasma by means of electron capture detection[13–16]. Adlercreutz *et al.*[17] have identified many oestrogens in both pregnancy plasma and cord plasma by a combination of gas chromatography and mass spectrometry.

Much effort has been expended in increasing the sensitivity of the methods of oestrogen determination in plasma, as appears from the reports of oestradiol-17β measurement by competitive protein-binding assay[18–21] and by radioimmunoassay[22–24].

For the study of plasma oestrogen concentrations not only in normal pregnancy, but also when there is placental dysfunction[25], and when pregnancy is prolonged, a method is here presented by which oestriol, oestrone and oestradiol-17β concentrations can be measured from a single plasma sample of 5 ml. The method is an extension of that earlier reported for the measurement of oestriol alone[26].

EXPERIMENTAL

Materials

Reagents. All organic solvents were twice distilled through a Vigreux Column. Pyridine was refluxed over sodium hydroxide pellets, distilled and stored over barium oxide[27].

Acetone p.a. Merck; benzene p.a. Merck; carbon tetrachloride p.a. Merck; chloroform p.a. Riedel–DeHaën (Special quality Seruminstitut); ethanol 99% w/w (Rigshospitalets Apotek); ethyl acetate p.a. Merck; hexamethyldisilazane purum Fluka; methanol p.a. Merck; n-pentane p.a. Merck; pyridine certified A.C.S. Fisher; trimethylchlorosilane puriss. Fluka; hydrochloric acid fuming p.a. Merck; sulphuric acid, concentrated p.a. Merck; sodium hydroxide (Rigshospitalets Apotek); sodium sulphate, anhydrous p.a. Merck; disodium hydrogen phosphate p.a. Merck; DC-Fertigplatten Kieselgel (without fluorescence indicator) Merck, (20 × 20 cm, 0·25 mm layer thickness); Gas-Chrom Q, 100–120 mesh, Applied Science Laboratories; SE-30 (methylsiloxane polymer), QF-1 (trifluoropropylmethylsiloxane polymer), XE-60 (cyanoethylmethylsiloxane polymer), F&M Scientific Corp.

Reference compounds. Oestrone, oestradiol-17β (Leo Pharmaceutical Products, Copenhagen, and Ikapharm, Ramat-Gan, Israel). [6,7-^{3}H]-oestrone, S.A. 136 mCi/mg, [6,7-^{3}H]-oestradiol-17β, S.A. 110 mCi/mg (The Radiochemical Centre, Amersham, England).

Method

Hydrolysis and extraction are carried out exactly as described for oestriol[26]. Five ml of plasma from heparinized whole blood is diluted with 15 ml distilled water after addition of 0·1 ml aliquots of oestriol, oestrone and oestradiol-17β solutions. The 3 oestrogens are tritium labelled – approx. 16,000, 17,000, and 24,000 c.p.m., respectively. Hydrolysis is effected with 3·5 ml concentrated hydrochloric acid at 100°C for one hour. The hydrolysate is extracted 3 times with 40 ml chloroform. The extract is dried with anhydrous sodium sulphate, and the extract volume is reduced under vacuum and nitrogen at 50°C to about 1 ml. The concentrated extract is transferred to a small test tube with 5 ml of chloroform–methanol (1 : 1, v/v), and evaporated dry.

Solvent partition. As described by Svendsen[28], the chloroform extract is dissolved in 1 ml ethanol–water (1 : 1, v/v), and washed 3 times with 0·5 ml

pentane. The ethanol–water is then evaporated off. The residue is again dissolved in 1 ml carbon tetrachloride–chloroform (5 : 1, v/v), and from this solution oestriol is extracted with water[26]. Oestrone and oestradiol-17β are extracted 3 times with 0·5 ml 1 N sodium hydroxide. This extract is acidified with 0·15 ml 10 N HCl, and oestrone and oestradiol-17β further extracted from this weak acid solution 3 times with 1 ml chloroform. Finally the chloroform extract is washed, first with 0·5 ml 0·1 M di-sodium phosphate, and thereafter with 0·5 ml water. The resultant is evaporated dry.

Thin-layer chromatography (TLC). The prepared plates are washed clean of impurities in a benzene–ethyl acetate system (50 : 50, v/v) for approx. 2 h. After re-activating at 115°C for 60 min they are deemed ready for use.

To further purify the extract after the solvent partition, and to separate oestrone and oestradiol-17β, TLC is undertaken in a system which gives reasonable differences in the R_F-values of the two hormones. Benzene–ethyl acetate (50 : 50, v/v) is used. The remnant, after the solvent partition and evaporation, is dissolved in sufficient acetone and introduced onto the thin-layer plates with a micro-syringe. Two analyses are made on each plate, and on each side of the plate reference quantums of oestrone and oestradiol-17β are also applied. When the oestrogen standards have developed in the system, they assume a reddish colouring, after spraying with sulphuric acid and warming[29, 30]. The analysis areas corresponding to oestrone and oestradiol-17β are separately scraped off, and the silica gel from each area is separately eluted with approx. 5 ml acetone. The eluates are transferred to small test tubes with finely pointed bottoms, and ground glass necks and stoppers. One tenth aliquots are pipetted for liquid scintillation counting to determine the radioactive recovery.

Derivative formation. The trimethylsilyl–ether (TMSi) derivatives of the oestrogens are prepared by adding 0·2 ml of a fresh preparation of pyridine, hexamethyldisilazane and trimethylchlorosilane, in proportions 9 : 3 : 1, to the eluates after evaporation. The test tubes are carefully closed, shaken, and stored at 56°C for 2 h, or left overnight in a dessicator at room temperature. The excess of reagents is evaporated off immediately before gas chromatography, and the precipitate dissolved in 25 or 50 μl chloroform, depending on the expected concentration. The preparation is now ready for injection into the gas chromatograph.

The TMSi derivatives of the carefully prepared standard solutions of oestrone and oestradiol-17β in ethanol were similarly obtained for comparative quantitation by GLC.

Gas–liquid chromatography (GLC). Pye 104/4 and 104/24 gas chromatographs with flame ionization detectors are used. The columns are of glass, 7 ft long.

The analysis is conducted at 224°C (approx.) for oestrone-TMSi, and at 229°C (approx.) for oestradiol-17β-TMSi. Five μl of the analysate and, for calibration, different known quantities of the authentic oestrogen derivative, are injected. As a routine, the peak height is used in quantitation. Possibly area measurement would give slightly more accurate results, but this is more difficult and time consuming[31].

For the final determination, columns packed with 1·2% SE-30 on Gas-Chrom Q were used. In the investigation of the specificity, columns with 2% XE-60 and 3·3% QF-1 were used. They were conditioned at 250°C for 48 h before use.

RESULTS

Specificity. These studies were based on a constant retention time for oestrone- and oestradiol-17β-TMSi by GLC. No oestrone or oestradiol-17β peaks were evident when male plasma was processed (Figs. 1, 2), but after addition of known quantities of the two steroids, the recovery corresponded well to the additions, after correction for loss in the analysis process, determined by the radioactive recovery (Table 1).

Further investigation of specificity was made by quantitative testing of plasma pools from pregnant women in the two different gas chromatographic phases. The samples were thin-layer chromatographed in the same system. Oestrone was studied on the non-polar SE-30 and the middle-polar XE-60 (Fig. 3), and oestradiol-17β on SE-30 and on the strongly polar QF-1 (Fig. 4). Table 2 gives the results

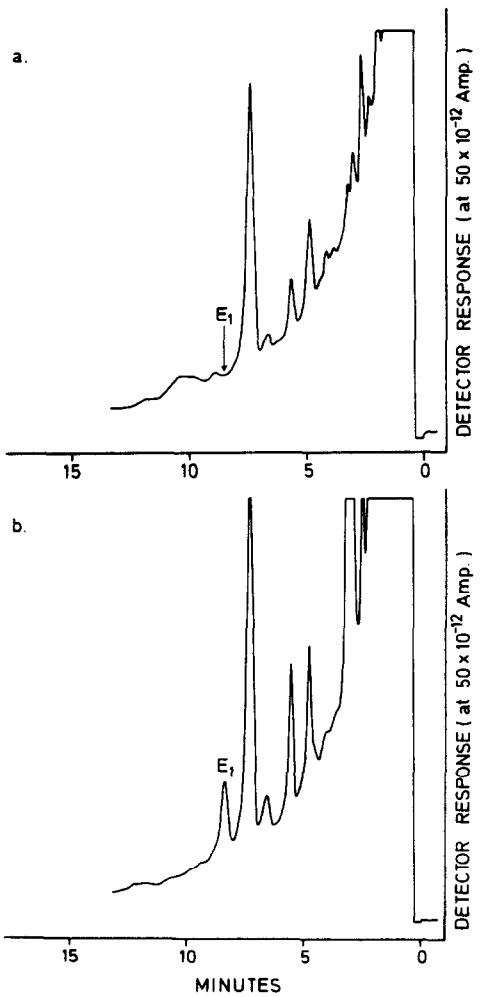

Fig. 1. (a). Gas chromatograph of male plasma. One fifth of the oestrone fraction extracted from 5 ml plasma was injected. No oestrone peak is evident. (b). Gas chromatograph of pregnancy plasma studied in series with the male plasma. An oestrone peak representative of a concentration of 633 μg/l plasma is seen.

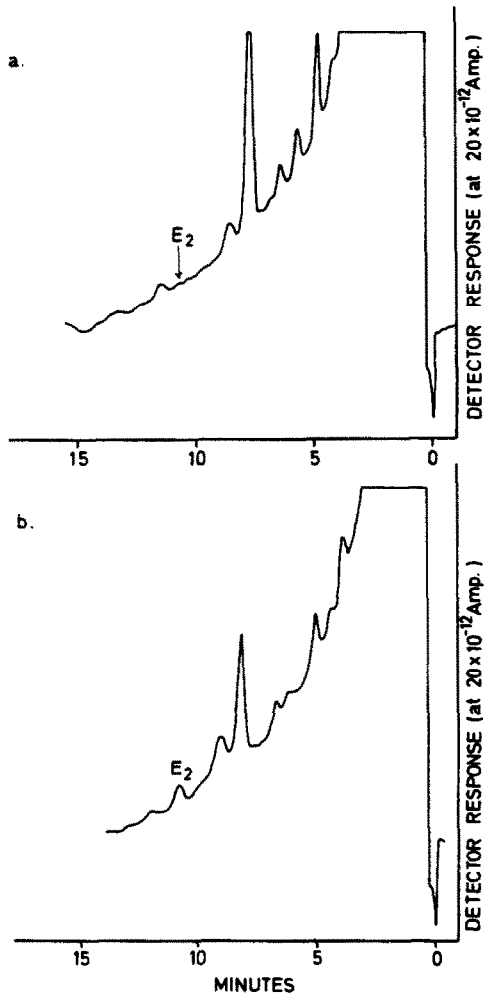

Fig. 2. (a). Gas chromatograph of male plasma. One fifth of the oestradiol-17β fraction extracted from 5 ml plasma was injected. No oestradiol-17β peak is evident. (b). Gas chromatograph of pregnancy plasma studied in series with the male plasma. An oestradiol-17β peak representative of a concentration of 13 μg/l plasma is seen.

Table 1. Recovery of oestrone (E_1) and oestradiol-17β (E_2) after addition of different quantities to male plasma. Mean value $\pm$ standard deviation μg/l.

Prepared concentration E_1	E_2	Measured concentration E_1*	E_2†	No. of tests
90	20	85 $\pm$ 5	18 $\pm$ 1	8
60	20	57 $\pm$ 3	18 $\pm$ 1	8
30	10	29 $\pm$ 4	9 $\pm$ 1	8

*1·2% SE-30, 7 ft, 226°C.
†1·2% SE-30, 7 ft, 229°C.

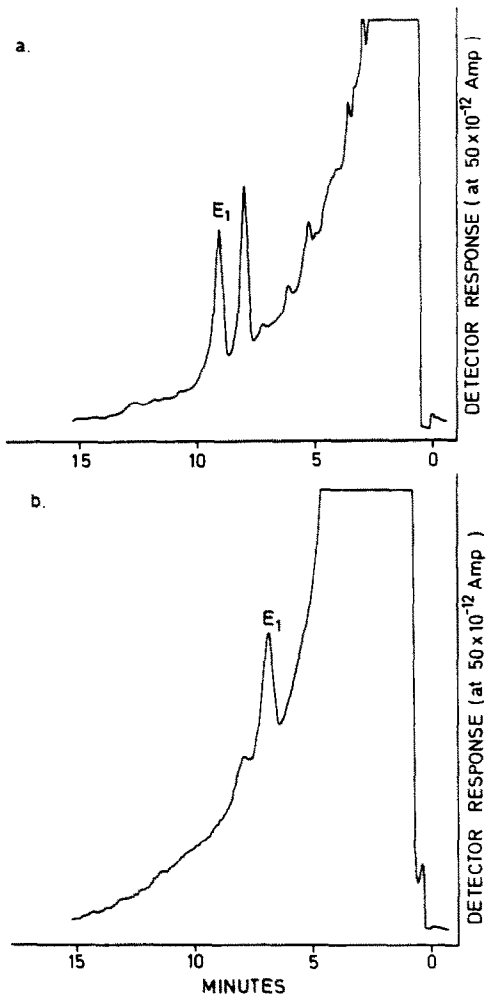

Fig. 3. Oestrone determinations from the same pregnancy plasma sample: (a). injection onto 1·2% SE-30; (b). injection onto 2% XE-60 (Table 2, Pool 1).

of tests from two pools. There was no significant difference between measurements in the non-polar and polar phases.

Table 3 gives the results of tests of samples from the same plasma pool in 2 different thin-layer systems. Four test samples from each of 5 plasma pools were put through each system. Gas chromatography was performed on the same phase. The average value of the 4 test results is given. The results agree well. The thin-layer systems used were 1) chloroform–acetone (90:10, v/v) (32), which gives R_F values for oestrone, oestradiol-17β and oestriol of, respectively, 0·78, 0·59 and 0·08, and 2) benzene-ethyl acetate (50:50, v/v) (33), where the comparative R_F values are 0·82, 0·71 and 0·15.

Accuracy. Oestrone and oestradiol-17β in pregnant women's plasma are partly free and partly conjugated with sulphate or glucosiduronate. The proportions in the individual sample are unknown, and, therefore, from a theoretical stand-point, actual evaluation of the method's accuracy is not possible [10, 34].

Table 2. The concentration of oestrone and oestradiol-17β in 2 pregnancy plasma pools measured on two different gas chromatography column phases. Mean value ± standard deviation μg/l.

	Plasma pool no.	Phases		No. of tests
		1·2% SE-30[a]	2% XE-60[b]	
	1	87 ± 4	81 ± 5	8
Oestrone	2	103 ± 9	106 ± 9	8
		1·2% SE-30[c]	3·3% QF-1[d]	
	1	19 ± 1	17 ± 1	8
Oestradiol-17β	2	16 ± 2	17 ± 2	6

[a]7 ft, 224°C.
[b]7 ft, 225°C.
[c]7 ft, 226°C.
[d]7 ft, 217°C.

Table 3. The concentration of oestrone (E_1) and oestradiol-17β (E_2) in pregnancy plasma pools. From each pool 8 samples were put through one or two different thin-layer chromatography systems—4 samples in each. Gas chromatographic measurement of each sample was on 1·2% SE-30. Mean values of 4 determinations, μg/l.

Plasma pool no.	Thin-layer chromatography: Benzene–ethyl acetate (50:50, v/v)		Chloroform–acetone (90:10, v/v)	
	E_1*	E_2†	E_1*	E_2†
3	51	17	51	15
4	54	23	53	23
5	72	28	75	25
6	95	32	109	35
7	137	27	137	29

*7 ft, 224°C.
†7 ft, 228°C.

Some idea, however, of the accuracy can be obtained from the recovery after addition of a known quantity of free oestrogens to male plasma. The average total recovery approximated closely the quantity of oestrogen added (Table 1). The unconjugated, tritium labelled oestrogens added before hydrolysis could be thought to give a basis for correction for the oestrogen which is lost during hydrolysis, but is no guide to any oestrogen fraction which is possibly exceptionally not hydrolysed.

The *reproducibility* of the method seems acceptable: 8 samples from the same plasma pool were studied on different days and the following mean values and standard deviations obtained: oestrone 76 ± 7 μg/l, and oestradiol-17β 26 ± 2 μg/l. Recovery of the radioactive oestrone and oestradiol-17β in male plasma (Table 1) was, respectively, 58 ± 4% and 54 ± 6%.

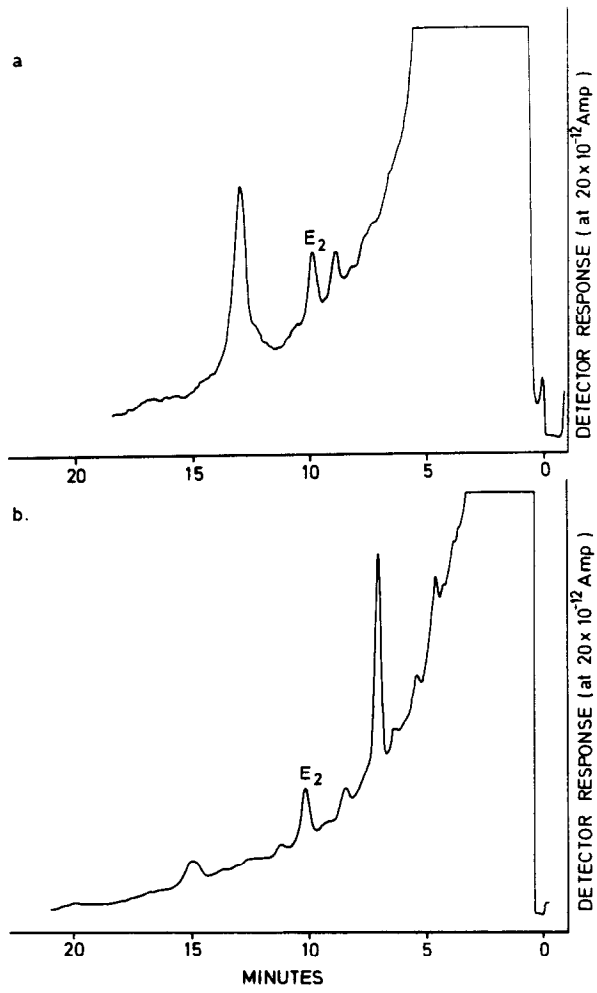

Fig. 4. Oestradiol-17β determinations from the same pregnancy plasma sample: (a). injection onto 3·3% QF-1; (b). injection onto 1·2% SE-30 (Table 2, Pool 1).

Precision for the different concentration ranges is shown in Table 4. The standard deviation is calculated from the formula:

$$s = \sqrt{\frac{\Sigma (x_1 - x_2)^2}{2N}}$$

where x_1 and x_2 are the results of the duplicate analysis, and N the total number of analyses performed.

Sensitivity can be calculated from the standard deviation in the lowest concentration range (Table 4). If $P = 0{\cdot}05$, then the smallest quantity which can be differentiated from zero is 5 μg/l for oestrone, and 3 μg/l for oestradiol-17β— that is, twice the standard deviation.

DISCUSSION

Usually plasma alone is studied, but some authors have felt that whole blood studies are equally reliable[6]. It is possible, however, that study of plasma alone

Table 4. The precision of oestrone and oestradiol-17β determinations on samples of pregnancy plasma in duplicates for different concentration ranges

	Concentration range (μg/l)	No. of duplicates	Standard deviation μg $\sqrt{\frac{\Sigma (x_1 - x_2)^2}{2N}}$
Oestrone	10–50	65	±2·5
	51–100	58	±4·3
	101–200	35	±7·8
	201–300	18	±15·7
Oestradiol-17β	9–30	117	±1·6
	31–55	49	±3·1

gives more accurate results[35]. In any event, many research workers have demonstrated that virtually 100% of the three oestrogens lie in the plasma fraction [6, 11, 36, 37].

As with the oestriol study, so with oestrone and oestradiol-17β, it is the 'total oestrogen' which is measured—that is oestrogen free in the first instance plus conjugated oestrogen freed after acid hydrolysis.

Many studies of the relative proportions of free and conjugated oestrogens in pregnancy plasma have been made[11, 38, 39, 40]. It is now generally accepted that, in the second half of pregnancy, most circulating oestriol is conjugated, most oestradiol-17β free, while oestrone circulates in both forms. Thus Adlercreutz *et al.*[17] found that more than 93% oestriol was conjugated, more than 84% oestradiol-17β was free, while conjugated oestrone varied from 86–93%.

The biological significance of the proportion between free and conjugated oestrogens in plasma is, as yet, not clear, and differentiation between the fractions is a time consuming process requiring extended analyses[10, 11, 39]. Conventional acid hydrolysis, as used in this study, involves a higher analysis loss than does enzyme hydrolysis[10]. Recently a more rapid enzyme hydrolysis of plasma oestrogens has been reported, which may be practical[41]. Oestrogen recovery with the present method was similar to that of Roy and Brown[6], in which there was 50–60% recovery after addition of oestrone and oestradiol-17β before hydrolysis. These same authors suggest that oestrogen loss during analysis of blood is greater than during analysis of urine, as a result of interference by hydrolyzed blood protein and lipid with the adsorption and emulsion formation process during extraction. It is of interest to note that Svendsen[28], whose extraction and preliminary purification procedure was to some extent used in this study, found an average recovery of oestrone and oestradiol-17β from non-hydrolyzed, non-pregnancy plasma, of 65 and 64%, respectively.

In regard to analysis loss, it should also be considered that thin-layer chromatography of the oestrogens is at the nanogram level, while a reducing recovery rate of oestrogens is already described at the microgram level[42]. A good elution technique seems, however, very significant in the attainment of a satisfactorily high recovery[43]. The results shown in Table 1, and the results of the reproducibility tests, seem to indicate that there is no great or variable loss or destruction of oestrogens during TLC, as has, for example, been described with cortisol[44].

The method's specificity was studied using thin-layer systems which ensured complete separation of the three classic oestrogens: oestrone, oestradiol-17β and oestriol. The preliminary solvent partition should separate the oestrogens from the neutral steroids, and from most of the lipids, as it also separates oestriol from oestrone and oestradiol-17β. Further separation is achieved during TLC and gas chromatography.

As earlier found in urine[45] and bile[46], other oestrogens in addition to the three classic oestrogens have recently been identified in plasma: 16-keto-oestradiol-17β, oestradiol-17α, 11-dehydro-oestradiol-17α, 2-methoxy-oestrone, 16α-hydroxy-oestrone, 16β-hydroxy-oestrone, 15α-hydroxy-oestrone, 16-epi- and 17-epi-oestriol[17]. 11-Dehydro-oestradiol-17α, quantitatively important in urine, is, however, destroyed by acid hydrolysis[47], and furthermore is of little quantitative importance in plasma, as is also true of the other oestrogens mentioned, apart from 16-keto-oestradiol-17β and 16α-hydroxy-oestrone which are present in concentrations similar to that of oestradiol-17β[17].

A benzene-ethylacetate system (50:50, v/v) will not adequately separate any 16-keto-oestradiol-17β which may be present from oestradiol-17β, but oestradiol-17α is well separated[38]. The second system used in this study – chloroform–acetone (90:10, v/v) – should resemble Lisboa's system L (chloroform–ethanol 95:5, v/v), except that ethanol is a little more polar than acetone. If so, it will not separate 16α-hydroxyoestrone from oestradiol-17β, but 16-epi- and 17-epi-oestriol and 2-methoxy-oestrone are well separated. Increasing knowledge of oestrogen metabolites is allowing the development of other solvent systems for use in TLC[48, 49].

Gas chromatography is effective in the separation of oestrone, oestradiol-17β and oestriol as trimethylsilyl–ethers on phases SE-30, QF-1 and XE-60[50, 51], even though oestriol and oestrone are less well separated on QF-1, and oestradiol-17β has only a short retention time on XE-60. The SE-30 phase, used routinely in this study, gives good separation of the TMSi ethers of oestradiol-17β, 16-keto-oestradiol-17β and 16α-hydroxy-oestrone[52]. Similarly the TMSi ethers of oestradiol-17β, oestrone and oestriol are also well separated on this phase.

However, interference peaks on the gas chromatogram need not be steroid, but can equally well be of low boiling point alcohol or lipid origin, for example. Notably, TMSi derivatives developing after TLC may give extra chromatogram peaks[53]. Using iodomethyldimethylsilyl–ether in place of TMSi, a derivative of oestradiol-17β has been obtained, which has all the good gas chromatographic characteristics (especially as regards electron capture detection) of the TMSi derivative, and is, furthermore, stable during TLC[54]. This additional characteristic allows separation of the excess silylizing reagents which otherwise cause significant extension of the gas chromatogram solvent front. The associated elevation of the base line complicates the measurement of the small quantities of steroid[55].

Further studies[56, 57], made concurrently with the present one, give the results of oestrone and oestradiol-17β determinations by the described method in a series of normal pregnant women. The results of the oestriol study in the same series of women has been previously published[58].

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