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Studies on the Mass Fragmentographic Determination of Plasma Estriol

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Abstract—Three mass fragmentatographic methods for the determination of unconjugated estriol in pregnancy plasma and unconjugated and conjugated estriol in plasma of nonpregnant women after estriol administration were developed and tested as to their reliability and practicability. The methods were found to fulfil appropriate reliability criteria especially with regard to specificity. Unconjugated estriol could be assayed without prior chromatography in late pregnancy plasma, but a chromatographic step was needed for plasmas with a low estriol titre, and both a methylation step and chromatography is needed to achieve the required specificity if plasma conjugated estriol is determined or if assays are carried out following estriol administration. Unconjugated estriol in normal late pregnancy plasma was found in concentrations from 4.3 to 9.3 μg/l. The highest value recorded, 16.3 μg/l, was found in a prediabetic subject with mild hypertension, who delivered a child weighing 4150 g by Cesarean section in the 39th week. Low values were found in severe hypertension and in toxemia, and in general the results from the pathological material investigated seemed to correlate well with the clinical findings. However, only a few samples (five to ten) can be processed so rapidly that the results can be obtained the same day, which in addition to the expensive and complicated instrumentation limits the usefulness of the methods in routine clinical chemistry.

Introduction

ATTEMPTS to quantitate plasma steroids by mass fragmentography (m.f.) has only been carried out to a limited extent[1–12] and few publications present actual clinical data obtained using the techniques developed.[7–9,11,12] This is probably due both to the expensive instrumentation required and to the fact that there still are many problems encountered in using this approach. In order to further evaluate the usefulness of mass fragmentography in clinical chemistry we have studied the possibilities of carrying out rapid determinations of unconjugated estriol in pregnancy plasma for the purpose of monitoring fetal well-being and of plasma unconjugated and conjugated estriol for research purposes.

Experimental

Material

The blood samples were drawn into heparinized tubes and following centrifugation the plasma was stored at −20 °C until processed. Most of the plasma samples investigated were from pregnant women with various complicating diseases and no restrictions as regard to medication were made.

Reference steroid

Estriol(1,3,5(10)-estratriene-3,16α,17β-triol)(Mann Research Laboratories, New York, U.S.A.).

Solvents and reagents

Hexamethyl-d_{18}-disilazane and trimethyl-d_9-chlorosilane were obtained from Merck Sharp & Dohme of Canada Ltd, Montreal, Canada. All other reagents and solvents used in the present work have been described previously.[13]

Mass fragmentography

Two instruments were used in the present study, an LKB 9000 and a Varian MAT CH-7 gas chromatograph-mass spectrometer. The Varian instrument was equipped with a peak matching system, which allowed monitoring and separate amplification of two ions recorded on a two-pen recorder. The conditions used for mass fragmentography with this instrument have been described previously.[11] The LKB instrument was equipped with an accelerating voltage alternator (a.v.a.) system allowing simultaneous measurement of three ions. Some of the experiments were performed with this instrument after modification, as described previously.[8,11] However, it was found to be impossible to work on routine analyses using a system where the signals are recorded on a single-channel recorder (either the u.v. recorder or a potentiometric recorder), because the amount of steroid to be analyzed is unknown and it is not always possible to add the correct amount of internal standard. If too much or too little is added, one of the signals goes off scale. In addition, it was found that a high signal from one of the ions interfered with the height of the smaller signal, if the

internal standard had the same or almost the same retention time, as is the case when deuterated compounds are used as internal standards. Two of the three signals obtainable with the a.v.a. system were therefore fed to separate Hitachi recorders to make it possible to cope with different degrees of background suppression (zeroing) and also different amplification factors resulting from signals differing greatly in intensity. This was performed in a simple way using a Reed relay, which, synchronously with the change in accelerating voltage caused by the a.v.a., switched the multiplier signal alternately to one of two active low pass filters (cut-off frequency about 0.3 Hz). Each of the filters was connected to the input of a multirange recorder with adjustable zero. The coil of the Reed relay (12 V) was connected in parallel with the coil (L 1) of one of the relays switching the high voltage divider in the a.v.a. unit. A d.c. coupled operational amplifier with an amplification factor of 25 was connected between the Reed relay and the multiplier-preamplifier for the purpose of reducing the effects of contact potentials and resistances of the relay upon the measurement.

For further details on the conditions employed for m.f. with the LKB instrument see Methods below.

Calculation of results

This was first carried out as described previously.[11] Later, only the peak heights and not the peak areas were measured, because this was more simple and did not alter the precision of the method. Correction for losses during purification was made using the results of simultaneously performed recovery experiments in duplicate or triplicate.

Methods

Three different methods were investigated as to their suitability for determination of plasma estriol.

Method A for unconjugated estriol in pregnancy plasma. 100 to 200 µl of plasma was diluted with 200 to 400 µl of 1.5 mol/l sodium acetate buffer, pH 4.1 and extracted with 2 × 3 to 6 ml of diethyl ether. The ether extract was evaporated to dryness completely in a stream of nitrogen on a sand bath heated to a maximum of 50 °C. Formation of the trimethylsilyl ether derivative of estriol was carried out with 200 µl of a mixture of redistilled pyridine + hexamethyldisilazane + trimethylchlorosilane (9:3:1 v/v).[14] The solvents were evaporated to dryness and the silylated steroid extracted with *n*-hexane. A known amount of standard estriol was silylated in the same way with hexamethyl-d_{18}-disilazane and trimethyl-d_9-chlorosilane, and the *n*-hexane extract of the silylated standard estriol was combined with the *n*-hexane extract of the sample in a graduated microtube. The solvent was evaporated to dryness and 20 to 30 µl *n*-hexane added, of which 5 µl was needed for each assay. M.f. was carried out on a 1% QF-1 or a 3% OV-210 column (length 2.7 m, internal diameter 2 mm), at temperatures of approximately 225, 240, 250 and 290 °C for flash heater, column, separator and ion source, respectively. Other conditions (LKB 9000): full accelerating voltage, 3.5 kV, multiplier voltage 3.7 kV, entrance slit 0.1 mm, collector slit 0.6 mm, ionization energy 70 or 55 eV. The ions *m/e* 504 and 531 (molecular ion of estriol trimethylsilyl ether and of the deuteriated internal standard) were monitored. Instrument calibration was carried out at 2 h intervals. The instrument was allowed to warm up for at least 2 h with a mass marker setting at the highest mass used for analysis, otherwise considerable drift occurred due to changes in the temperature of the magnet.

Method B for unconjugated estriol in pregnancy plasma. Following extraction as in method A the ether was evaporated almost to dryness and the extract dissolved in 5 ml of benzene and the remaining ether blown off. The benzene extract was transferred to a 2 g alumina column (acid alumina, activity grade I, Merck, Darmstadt, Germany: prewashed with distilled ethyl acetate, reactivated at 110 °C overnight[15] and then deactivated with 7% (v/w) of distilled water). When the extract had percolated through the column, 10 ml of 1% ethanol in benzene (v/v) was added and the fraction obtained discarded. The estriol was eluted with 30 ml of 20% ethanol in benzene (v/v). This fraction was evaporated to dryness in a stream of nitrogen with the tube in a sand bath (maximum temperature 50 °C). Following silylation the procedure was exactly as described for method A.

Method C for unconjugated and conjugated estriol in plasma. 0.5 to 1.0 ml of plasma was diluted with two volumes of 1.5 mol/l sodium acetate buffer, pH 4.1, and extracted twice with 5 to 8 ml of diethyl ether. The water phase containing the conjugated estriol was diluted with water to give a final buffer concentration of 0.5 mol/l. The tube was incubated at 39 °C for 10 min and the enzyme solution (Suc d'Helix Pomatia, Industrie Biologique Francais, Gennevilliers, France) added to a final enzyme concentration of 1000 Fishman units of β-glucuronidase/ml of incubation mixture. Hydrolysis was carried out in a water bath for 16 to 40 h at 39 °C; thereafter the hydrolysate was chilled with tap water for 10 min and extracted 3 times with 10 ml of ethyl ether. The ether was evaporated to a smaller volume (4 to 5 ml) and then this (the conjugated estriol fraction) and the ether phase containing the unconjugated estriol fraction were processed in the same way, as follows.

The ether phases were extracted twice with 2 ml of 1 mol/l NaOH. After the first extraction the water phase was removed with a Pasteur pipette to another tube and after the second extraction the ether phase was removed by suction and the NaOH phase combined with the first extract. 2 ml of 1 mol/l NaOH and 9 ml of distilled water were added to the tube and the fraction was methylated according to the method of Brown[16] but using one third of all reagents at all stages of the methylation procedure. Following methylation 3.3 ml of 5 mol/l NaOH was added, the tube was

allowed to stand for 30 min and then its contents were extracted with 8 ml of benzene. The benzene extract was washed once with 1 ml of distilled water and chromatographed on alumina[16] (neutral alumina, Merck, activity grade I, deactivated with 9.5% of distilled water). When the extract had percolated through the column, 12 ml of 1.4% ethanol in benzene (v/v) was added and the fraction obtained was discarded. The methylated estriol was eluted with 16 ml of 3% ethanol in benzene (v/v), the solvents were evaporated and the dry residue processed as described above. The deuteriated trimethylsilyl ether derivative of standard estriol 3-methyl ether was added as internal standard for m.f., which was carried out in the same way as described for method A, but monitoring the ions *m/e* 446 and 464 (molecular ions of methylated and silylated estriol and the corresponding deuteriated derivative of the reference standard).

Results

Methodological results

Method A. When this method was used in the analysis of pregnancy plasma samples it was found that despite the speed with which a single determination could be made the impure nature of the extract submitted to mass fragmentography enforced a long waiting time before the next analysis could be made. A great number of other compounds in the samples had ions of the same mass as used for monitoring estriol and the internal standard. This problem could not be overcome if ions of lower mass were used. The method was found to be specific only when the estriol concentration was rather high; in early pregnancy other compounds interfered. In addition a nonpolar phase (1% SE-30) was tried but this did not result in any improvement. The recovery of added estriol was more than 95% and the precision depended principally on the precision of m.f., which is dependent on the instrument used. Because of the high background the method was found impractical and was only used for high titre plasmas when some few samples needed to be investigated.

Methods B and C. Because the only difference between these methods is the methylation step included in method C and as the results were similar, they will be presented together. The most apparent difference when compared to method A is that samples can be injected about every 8th to 10th min because no interfering peaks occur after the estriol peak during the mass fragmentographic analysis.

The *specificity* of both methods for unconjugated estriol is good. However, only method C, involving methylation, can be used for measuring conjugated estriol, due to the effects of interfering compounds when method B is used. Because the molecular ion is monitored most estrogens cannot interfere and only estrogen triols or tetrols need be considered. The silylated derivatives of 2-hydroxyestradiol-17β, 6α- and 6β-hydroxyestradiol-17β, 7α-, 11β-, 15α-hydroxyestradiol-17β and 16,17-epiestriol all have considerably shorter retention times on the columns used (for QF-1 data see Ref. 17) than the trimethylsilyl ether (TMS) of estriol and none of them have yet been detected in plasma. The retention time relative to cholestane of the TMS derivative of 16-epiestriol is 14% longer than that of the corresponding estriol derivative,[17] but the TMS derivative of 17-epiestriol has almost the same retention time as estriol.[17] The amount of 16-epiestriol both conjugated and unconjugated, e.g. in pregnancy plasma is less than a tenth of the concentration of estriol and the concentration of 17-epiestriol is less than one hundredth of that of estriol.[8,11] In addition, these two estrogens are partly eliminated during the alumina chromatography. Previously it was demonstrated that methylated 16-epiestriol is to a large extent eliminated during chromatography.[18] The tetrols have much longer retention times[17] and therefore cannot interfere with the assay. It may therefore be concluded that none of the estrogens known to be present in plasma and also none of the other possible occurring ones can interfere with the assay.

When the *accuracy* of the methods was tested by adding known amounts of estriol to eight plasma samples it was found that the recovery was the same for both procedures: 86 $\pm$ 10 (S.D.)%. Serial measurements (8 and 9 determinations) carried out with methods B and C on two different plasma pools revealed almost identical mean values 5.8 and 6.0 μg/l and 9.1 and 8.9 μg/l, respectively.

The ultimate *sensitivity* of the determination of the TMS derivative of estriol by mass fragmentography (Varian MAT CH-7) has been found to be 2.1 pg giving a peak 2.4 cm high with the noise level six to seven times smaller.[19] However, it is not always practicable to keep the instrument at its optimal performance and any impurities occurring in the final extract also decrease the sensitivity of the assay. Method C is the most sensitive of the three techniques because of the more pure final extract. One should avoid working with estriol amounts below 50 pg/injection with method B and below 150 to 200 pg/injection with method A. This means that method A can be used for late pregnancy plasma samples and method B for samples with concentrations down to 1 to 2 μg/l of plasma. For method C the smallest amount that may be injected varies from 10 to 30 pg depending on the condition of the instrument. The sensitivity of method C may be increased by taking larger samples of plasma. It should be mentioned that we have not tried to measure plasma estriol in non-pregnant individuals only in estriol-treated subjects. In experiments with pregnancy plasma, standard samples containing 50 to 300 pg, depending on the estriol level expected, are injected.

When studying the *precision* of the methods it was soon realized that it is difficult to obtain satisfactory precision using our old LKB 9000 instrument and the

TABLE 1. Results of the evaluation of the precision of methods B and C as calculated from serial determination in plasma pools

Pooled samples	No. of assays	Mean value μg/l	Coefficient of variation c.v. (%)	Instrument
Method B	11	11.8	7.7	Varian MAT CH-7
Method B	9	9.1	16.7	LKB 9000
Method B	8	5.8	19.0	LKB 9000
Method C	9	8.9	14.4	LKB 9000
Method C	8	6.0	9.7	LKB 9000

a.v.a. system. Some improvement has been achieved with the modifications made on the instrument. The coefficient of variation was calculated from serial analyses carried out with the two methods on plasma samples pooled from several individuals and the results are shown in Table 1. As can be seen the coefficient of variation was the smallest (7.7%) in one experimental series with a newer Varian MAT CH-7 instrument (including transportation of the purified samples from Helsinki to Bremen) and varied in the other experiments from 9.7 to 19.0% (all experiments were carried out by the same person). Method C seems to be slightly more precise probably because of the higher degree of purity of the samples.

To throw some light on the above results, the precision of the mass fragmentographic assay was calculated from serial determinations of reference standards, and the coefficient of variation was found to be 1.2% in one experimental series using the Varian MAT CH-7 instrument and varied from 4.1 to 17.6% with a median value of 8.9% using the LKB 9000 instrument (eight series with at least 5 determinations in each). These experiments were carried out with the same amount of standard (about 100 to 200 pg) as injected when plasma samples are analyzed. The injections were made between other routine sample injections over an entire working day in order to get a view of the stability of the instrument. It may be concluded that the precision of the assays depends, to a great extent, on the precision of the final mass fragmentographic measurement, which is largely dependent upon the stability of the instrument.

If the determinations of plasma unconjugated estriol are being undertaken routinely for monitoring fetal well-being the *practicability* of the method has to be very good. Method A is very rapid if one has only a few (about five) samples. But because every mass fragmentographic run takes 20 to 30 min (including 10 to 20 min waiting time for a horizontal base line after the estriol-TMS peak) the procedure starts to be very impractical when longer series have to be analyzed. The chromatographic step adds about 90 min to methods B and C and the methylation step about the same time to method C. In practice only about 5 to 10 analyses could therefore be carried out during a working day with these methods. During two working days about 20 analyses of unconjugated estriol can be carried out with method C.

Clinical results

The aim of this investigation was not only to develop suitable methods but also to evaluate if it is feasible to use m.f. for the determination of plasma unconjugated estriol in a routine clinical situation. Therefore a series of 41 determinations of plasma unconjugated estriol involving thirty women with normal or pathological pregnancy was carried out and the results including some clinical data are shown in Tables 2 to 4. Method B was used for all analyses and they were with few exceptions carried out in duplicate. The normal values in the small series fell within the range 4.3 to 9.3 μg/l from the 31st week of pregnancy to term. In the pathological series it can be seen that two patients with hypertension, one with twin pregnancy and hypertension and in addition two subjects with pruritus gravidarum, showed higher than normal values for unconjugated estriol. Lower values than seen in the

TABLE 2. Results of determinations of plasma unconjugated estriol in some normal pregnant women

Patient No.	Diagnosis	Pregnancy week	Estriol (μg/l)	Birth weight (g)	Weight of placenta (g)	Remarks
1	Normal	40	9.3	3360	520	Day of delivery
2	Normal	40	4.9	3520	650	Day of delivery
3	Normal	40	7.7	3110	480	Day of delivery
4	Normal	34	5.0	3810	—	
5	Normal	39	5.7	4850	—	
6	Normal	31	5.1	3310	600	
7	Normal	33	7.5	4250	820	
κ	Normal	28	4.3			
		36	6.7			
		38	6.2	3200	625	
9	Normal	36	4.3			
		37	5.2	3640	740	
	Pool of 10 normal	36–40	7.1[a]			

[a] Value obtained using a more elaborate mass fragmentographic technique for the determination of 11 estrogens in various biological fluids.

TABLE 3. Results of determinations of plasma unconjugated estriol in pregnant women with toxemia or hypertension

Patient No.	Diagnosis	Pregnancy week	Estriol (μg/l)	Birth weight (g)	Weight of placenta (g)	Remarks
10	Hypertension	24	4.7	—	—	Patient delivered elsewhere
	Glomerulonephritis	26	4.5	—	—	
11	Hypertension	38	6.3	1710	400	Cesarean section, 39th week
12	Hypertension (severe)	36	7.3	2610	660	
13	Hypertension	38	10.8			
	Diabetes mellitus latens	39	16.3	4150	750	Cesarean section, 39th week
14	Hypertension (severe)	36	9.9	2780	1150	
	Twin pregnancy			3400		
15	Hypertension (severe)	38	11.8	3760	780	
16	Hypertension (severe)	35	3.3			
		37	3.7	2230	400	Cesarean section, 38th week
17	Toxemia, pre-eclampsia	38	6.3	3270	800	Cesarean section, 39th week
18	Toxemia	37	5.5	3170	600	Cesarean section, 39th week
19	Toxemia (severe)	28	4.7			
		29	3.4			
		31	2.7	1740	400	Cesarean section, 31st week Child died 3 days later
20	Toxemia	41	2.7	3650	720	Cesarean section, 41st week

TABLE 4. Results of determinations of plasma unconjugated estriol in pregnant women with pruritus gravidarum, diabetes mellitus or other diseases

Patient No.	Diagnosis	Pregnancy week	Estriol (μg/l)	Birth weight (g)	Weight of placenta (g)	Remarks
21	Pruritus gravidarum	38	11.1	3420	580	
22	Pruritus gravidarum	39	12.5			
		40	6.6	4220	840	
23	Pruritus gravidarum	33	6.3	2870	500	
24	Recurrent jaundice of pregnancy	40	5.1	3300	650	Cesarean section, 40th week
25	Pruritus gravidarum	36	6.0	3150	475	
26	Pruritus gravidarum	37	8.0			
		38	6.5	3000	620	
27	Diabetes mellitus	33	7.3	3740	530	
28	Diabetes mellitus	40	8.3			
		40	7.7	4680	750	
29	Proteinuria	40	5.6	3310	500	
30	Anaemia	40	7.3	3890	770	

normal group were found for one patient with severe hypertension and two patients with toxemia, all three pregnancies ending in emergency Cesarean section. One of the children died three days after delivery and it can be seen that the series of three determinations made well reflected the poor situation of the fetus (patient No. 19). In the two cases with diabetes mellitus the values were normal and the patients delivered normally.

Method C has been used in a study of the effect of estriol administration on the menstrual cycle (Vähäpassi and Adlercreutz, to be published). 6, 20 or 40 mg of estriol was administered daily to normal women and the plasma concentration of unconjugated and conjugated estriol was determined every second or third day. Method C proved very useful for this study. To give a view of the concentrations found the results obtained in three subjects are shown in Table 5. Surprisingly the level of unconjugated estriol did not

TABLE 5. Plasma levels of unconjugated and conjugated estriol following daily oral administration of 6, 20 or 40 mg of estriol to normally menstruating women (according to Vähäpassi and Adlercreutz, to be published)

Subject No.	No. of assays	Treatment estriol/day	Unconjugated estriol μg/l (±SD)	Conjugated estriol μg/l (±SD)
1	12	6 mg	1.75 ± 0.55	11.6 ± 3.5
2	14	20 mg	1.58 ± 0.27	29.7 ± 18.1
3	3	40 mg	2.14 ± 0.66	68.8 ± 31.4

increase with increasing estriol load, but the amount of conjugated estriol increased clearly.

Discussion

From the methodological point of view it can be concluded that the sensitivity and accuracy of the methods developed are comparable to those found for radioimmunological procedures. However, with our old mass spectrometer it was very difficult to achieve satisfactory precision despite internal standardization which was readily obtained with a newer instrument, in this case a Varian MAT CH-7. For method B a coefficient of variation of 7.7% was then obtained, which compares well with those of about 7 to 16%[20–27] reported for competitive protein binding methods for estriol assay.

The practicability of methods B and C is comparable to that of competitive protein binding methods which include a chromatographic step,[20,22,23] and method A may in that respect well be compared to radioimmunological methods in which only extraction of the free estriol is carried out before the competitive protein binding assay. It seems to us that only these latter methods can serve the need of the clinician in an adequate way, as results must be made available the day the samples are taken. If only a few samples need to be analyzed, the methods which include a chromatographic step may well be used, but the need is rarely so limited.

A disadvantage of the mass fragmentographic technique is the complicated instrumentation required, which can be subject to breakdown requiring spare parts and/or expert service which may not be readily and speedily available. Thus, in the Helsinki laboratory we have had interruptions of work due to instrument failure frequently for several days, occasionally for up to two weeks and on two occasions, for two and four months. We conclude, therefore, that it is not practical to consider m.f. for routine clinical work until one is in the position to have and keep the mass spectrometer running reliably on a daily basis. This may require not only considerable expertise within the laboratory but also the acquisition of a simple and reliable mass spectrometer. While smaller instruments can be used confidently for the measurement of respiratory gases, the extension of this reliability to instruments used for the determination of higher molecular weight compounds is as yet only in its first stages.

The greatest advantage of mass fragmentographic techniques is their high specificity, which is probably much superior to the competitive protein binding procedures. The use of the molecular ion for monitoring purposes excludes interference from all estrogens with less than three hydroxyl groups and those with three or more hydroxyl groups are usually well separated from estriol on the gas chromatographic column.[17] Previously, when gas chromatographic methods were used in this laboratory, numerous mass spectra of the estriol fraction from various biological fluids have been taken and the experience gained indicates that the most specific measurements are obtained with QF-1 or OV-210 columns. The problem of reagent peaks, which sometimes disturb in g.c., never occurs in the higher mass region in m.f. If deuteriated internal standards that could be added directly to plasma were commercially available the methods could serve as reference methods for the determination of estriol in pregnancy plasma.

Thus, we have not been able to find any indication of interference of other compounds with the assay, not even in the patients treated with considerable numbers of drugs. It is therefore of interest to compare the present results with those obtained recently with some of the more specific competitive protein binding methods. The highest mean values at term (about 16 μg/l) are obtained with methods without chromatographic steps and utilizing antibodies obtained with 6-oxoestriol carboxymethyloxime-BSA as antigen.[21,25] The same high values (16.1 μg/l, 32nd to 40th week of gestation)[27] are obtained with antibodies to estriol-16-17-dihemisuccinate despite the inclusion of Sephadex LH-20 chromatography. According to Goebel and Kuss[25] the introduction of a chromatographic step decreases their values by about 30%. This decrease is due partly to the elimination of estradiol despite the fact that this steroid does not react with the antibody when the cross-reactivity is assessed according to Abraham,[28] but because the presence of estriol increases the cross-reactivity of estradiol considerably. This phenomenon which leads to overestimations was first observed by de Lauzon *et al.*[29] and is a serious complication in the competitive protein assay methods. This is because its adequate correction necessitates extensive purification of the steroid being measured if the antibody is subject to increased cross-reactivity to some component of the extract stimulated by the presence of other steroids.[29] Similar mean values in late pregnancy (14 to 16 μg/l) to the abovementioned are obtained with methods which include Sephadex LH-20 chromatography and employ antibodies to estradiol-17-hemisuccinate-BSA.[22,26] Two other methods based on competitive protein binding with rabbit uterine cytosol[20,24] give mean values at term of 11 to 13 μg/l, all being higher than the normal values obtained in the present investigation. Loriaux *et al.*[23] used antiserum obtained with estrone-17-oxime-BSA as antigen and included Sephadex LH-20 chromatography and obtained a mean value of 6 μg/l in the 35th week of pregnancy. This corresponds well with the values (4.3 to 9.3 μg/l) obtained in the present and previous mass fragmentographic or gas chromatographic investigations carried out in this laboratory.[8,30,31] Several of the published methods have not been adequately investigated with regard to interference by cross-reactivity to 'nonclassical' estrogens and none of them according to the method of de Lauzon *et al.*,[29] and it can be seen from Table 6 that estrogens with three or four oxygen

TABLE 6. List of estrogens which may interfere with estriol assay in competitive protein binding methods and their approximate concentration in late pregnancy plasma

Steroid	Concentration μg/l	Method used for assay	Reference
2-hydroxyestrone	2.0	Radioimmunoassay	Yoshizawa and Fishman[32]
15α-hydroxyestrone	0.8	Mass fragmentography	Adlercreutz[8]
16α-hydroxyestrone	1.0	Mass fragmentography	Adlercreutz[8]
16β-hydroxyestrone	0.9	Mass fragmentography	Adlercreutz[8]
16-oxoestradiol-17β	0.6	Mass fragmentography	Adlercreutz[8]
16-epiestriol	0.6	Mass fragmentography	Adlercreutz[8]
17-epiestriol	0.1	Mass fragmentography	Adlercreutz[8]
15α-hydroxyestriol	2.0	Radioimmunoassay	Fishman and Guzik[33] Giebenhain *et al.*[34]
Total	8.0		

functions occur in significant amounts in unconjugated form in plasma at term,[30–34] the total amount of known estrogens with three or four oxygen functions not including estriol being about 8 μg/l. Because other unknown estrogens are also most likely present, the higher results obtained with competitive protein binding methods as compared to gas chromatographic and mass spectrometric methods may well be explained by the presence of other estrogens in the extracts used for final analysis. Whether this overestimation is of any consequence in the proper judgement of the fetal well-being is unknown and needs to be investigated.

It has been suggested that plasma unconjugated estriol levels may give a better indication of fetal well-being than total plasma estriol or urinary estriol levels. This is because the unconjugated estriol is primarily produced by the fetoplacental unit and the conjugates are maternal metabolites. It is also easier to take a blood sample than to collect urine. There is some evidence suggesting that plasma estriol measurements are superior to urinary estriol assays for monitoring fetal well-being.[25,35] As judged from our small clinical material the fetal status was well reflected by the plasma estriol values obtained with method B.

It may be concluded that mass fragmentographic estriol determination for monitoring fetal well-being in pregnancy has not as yet reached a stage where it can be included as a routine method. However, the methods are ideal for research purposes because of their specificity. Further development of method A in order to reduce the extensive background during m.f., perhaps employing a derivative which would generate an ion of higher mass suitable for monitoring, or by some other means without including any chromatographic step, would be advantageous. Computer controlled ion measurement as already developed in some laboratories[3,8,36–39] would also increase the practicability of the method and make it possible to report the results the same day even when larger series have to be analyzed.

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