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A GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF OESTRIOL IN HUMAN PLASMA DURING THE SECOND HALF OF PREGNANCY

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SUMMARY

A method for the determination of oestriol in plasma during the second half of pregnancy is described. After acid hydrolysis the plasma extract is chromatographed on thinlayer plates. Quantitation is achieved by gas–liquid chromatography of the oestriol as its trimethylsilyl ether derivative and correction of the result is made on the basis of the recovery of tritium-labelled free oestriol. Data on specificity, accuracy, precision, sensitivity, and reproducibility are given.

INTRODUCTION

URINARY oestriol determination has been found to be of considerable value in obstetric practice as an indication of the foetal state[1]. The determination of oestriol in blood or plasma is of interest too. Since gas–liquid chromatography (GLC) is a versatile analytical tool, the procedure was investigated to see if it could be used for this purpose.

During recent years some methods for measuring oestrogens in plasma by colorimetry and fluorimetry have been described. On the basis of Brown's method [2] for the determination of oestrogens in urine, Roy and Brown[3] have developed a micro method for measuring oestrone, oestradiol-17-β and oestriol in pregnant women's blood. Ittrich[4] has also described a very sensitive method. Nachtigall *et al.*[5] deserve special mention for describing a quick method for determination of plasma oestriol during pregnancy.

On the basis of a double isotope method[6], Svendsen and Sørensen have determined un-conjugated oestrone and oestradiol-17-β both during pregnancy[7] and during the normal menstrual cycle[8]. A similar method was used by Baird[9].

Since the early work on separation of oestrogens by GLC[10], there have been relatively few publications of methods concerning the determination of these substances in blood by GLC, presumably because a thorough purification of the extract is necessary before it is applied to the gas chromatograph.

Adlercreutz[11] describes a method for determination of conjugated oestrogens in pregnancy blood, and Touchstone and Murawec[12] have measured free and conjugated oestrogens during pregnancy. Kroman *et al.*[13] state that they have measured oestrogens in normal plasma. By means of heptafluorobutyrate derivatives and electron capture detection, Wotiz *et al.*[14] have determined oestrone, oestradiol-17-β and oestriol in plasma from non-pregnant subjects. Eik-Nes *et al.*[15] described a method for oestradiol-17-β, and Attal *et al.*[16] for free oestrone in plasma. The aim of the method of Ratanasopa *et al.*[17] is to estimate oestriol in plasma during pregnancy.

EXPERIMENTAL

Materials

Reagents. All organic solvents were twice fractionally distilled through a Vigreux column. Pyridine was refluxed over sodium hydroxide pellets, distilled and stored over barium oxide [18].

Acetone p.a. Merck.
Benzene p.a. Merck.
Carbon tetrachloride p.a. Merck.
Chloroform p.a. Riedel-De Haën (Special quality "Seruminstitut").
Cyclohexane for chromatography Riedel-De Haën.
Ethanol 99 per cent w/w.
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.
Sodium sulphate anhydrous p.a. Merck.
Silica gel H, acc. to Stahl, Merck.
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. Oestriol (Leo Pharmaceutical Products, Ikapharm) Oestriol-6,7-^{3}H, specific activity 9·15 mc/mg (The Radiochemical Centre, Amersham).

Thin-layer chromatography (TLC). Glass plates of 20 × 20 cm are coated with silica gel in 0·5 mm layer thickness. The plates are air dried and activated at 110°C for 90 min. Then they are developed about two hours in the system benzene–ethanol 80:20 in order to have impurities washed out. After re-activation the plates can be used.

GLC. Pye 104/4 and 104/24 gaschromatographs are used with flame ionization detectors. The glass columns are 7 ft. packed with 1·2 per cent SE-30 on Gas-Chrom Q and cured at 250°C for 48 hr before operating.

Method

Hydrolysis. 5 ml plasma from a heparinised blood sample and 0·1 ml solution of tritium-labelled oestriol (about 16,000 cpm) are diluted with 15 ml distilled water in a 100 ml centrifuge tube with a ground glass joint. A few granules of carborundum are added, and the tube is placed in an oil bath at 40–50°C and connected with an Allihn condenser. The temperature is raised to 95°C and 3·5 ml concentrated hydrochloric acid are then added. The mixture is quickly brought to gentle boiling, and the hydrolysis is carried out for a total of one hour.

Extraction. The acidic hydrolysate is extracted three times, each time with 40 ml chloroform during vigorous stirring. The emulsion formed is separated by centrifugation for ten minutes. The combined extract is dried with anhydrous sodium sulphate. The volume of the chloroform extract is reduced at 50°C

under vacuum and nitrogen to about 1 ml. This residue is transferred into a small test tube with a total of 5 ml chloroform–methanol (1 : 1) and evaporated to dryness.

Solvent partition. According to Svendsen[6] this chloroform extract is dissolved in 1 ml ethanol–water (1 : 1) and washed three times, each time with 0·5 ml pentane. The ethanol–water mixture is then evaporated. The residue is re-dissolved in 1 ml carbon tetrachloride–chloroform (5 : 1). The oestriol is extracted three times with water (0·5 ml), and it is re-extracted three times with chloroform (0·5–1 ml). After evaporation a coloured extract is formed, and this extract is thin-layer chromatographed.

TLC. Two analyses are streaked on each plate with a microsyringe. Authentic oestriol standard is applied on each side of the plate. After development in benzene–ethanol 80 : 20 the plate is air-dried, the analyses are covered, and the standards are sprayed with concentrated sulphuric acid and heated for marking of oestriol. After the colour reaction, the corresponding areas of the analyses are scraped off into test tubes and eluted three times with 3 ml acetone; the eluates are transferred into small tubes with very pointed ends and with ground glass joints and stoppers. A one-tenth aliquot of the eluate is removed for liquid scintillation counting to determine the percentage recovery.

Derivative formation. For GLC it has been found most expedient to use trimethylsilyl ethers (TMSi)[19] as derivatives. From a fresh solution of pyridine, hexamethyldisilazane, and trimethylchlorosilane (9 : 3 : 1), 0·2 ml is added to the evaporated eluate from TLC. The tube is carefully closed and shaken and the mixture is then left overnight in a desiccator. The surplus reagents are evaporated immediately before GLC, and the precipitate is dissolved in an adequate quantity of chloroform: 25, 50, or 100 μl, depending on the anticipated concentration.

From carefully measured standard solutions of oestriol in ethanol, there are also prepared oestriol–TMSi solutions in known concentrations for the quantitative determination by GLC.

GLC. The analyses are carried out at 230°C with nitrogen as carrier gas. Liquid injection is performed with a 10 μl Hamilton syringe. For the calibration e.g. 10, 30, and 50 ng oestriol–TMSi are injected on several suitable occasions. Quantitation is achieved by measuring the peak height. When dealing with low plasma concentrations of oestriol, as shown in Fig. 1, it may be more accurate to measure the peak area.

With the present equipment in this laboratory one technician can process 10–12 blood samples in three days, viz. 5–6 duplicate analyses. Four samples can be carried through in two days.

RESULTS

Specificity. The investigations are based on the maintenance of a constant retention time (R_t) for oestriol–TMSi by GLC. By using male plasma in the procedure no peak appeared in the chromatogram at the spot for oestriol (Fig. 2). When oestriol was added in known quantities to male plasma, the recovery (Table 1) corresponded to the added material. When the same specimen is tested on different phases which separate more or less polarly and the results of the measurements are compared, it is possible to determine whether it is the same material measured on the two columns. From two plasma pools from pregnant

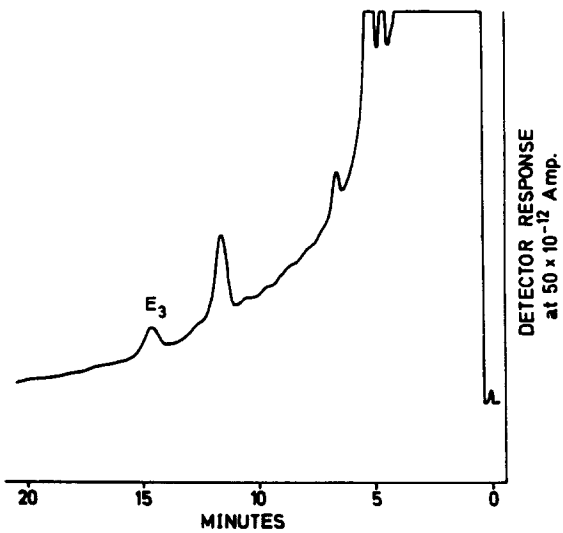

Fig. 1. Gas chromatogram of pregnancy plasma with a low concentration of oestriol: 1·6 μg per 100 ml plasma.

women near the term, each sample was injected on QF-1 and XE-60, as well as on QF-1 and SE-30, respectively (Table 2, Figs. 3 and 4). The first injection was made by turns between the instruments. By comparison between the strongly polar QF-1 and the less polar XE-60 and the non-polar SE-30, respectively, was found an average overestimation of 12–15 per cent by quantitation on QF-1.

Furthermore, samples from the same plasma pool from pregnant women were tested in two thin-layer systems with different R_f-values for oestriol: System A, ethylacetate–cyclohexane–ethanol (45 : 45 : 10), and system H, benzene–ethanol (80 : 20)[20, 21] with R_f-value about 0·35 and 0·51–0·55, respectively. In these systems, a clear separation of oestriol from oestrone and oestradiol is obtained. Furthermore, oestriol is separated from such epimers as 16-epi- and 17-epi-oestriol. Gas chromatographically there are no difficulties in separating the TMSi-derivatives of oestrone, oestradiol, and oestriol on SE-30, but there is no great difference in R_t between oestriol-, 16-epi-, and 17-epi-oestriol TMSi[22]. On QF-1, oestrone- and oestriol-TMSi are not completely separated, whereas the ethers of all the three classic oestrogens are very well separated on XE-60[23].

In Table 3 are stated the results of such a test with TLC in the two systems and GLC of all samples on two phases. Both hydrolysis and extraction as well as the chromatographies took place on different days of tests. There is no significant difference between the average values in the four sections.

Accuracy. In the pregnancy plasma, oestriol occurs mainly in conjugated form as 3-sulphate, 16-glucosiduronate, and 3-sulphate-16-glucosiduronate, while only about 10 per cent is free[11, 12, 24]. In the individual sample nothing is known about the ratio between these fractions. As stated by other authors in the discussion of this problem, the existing conditions cannot be imitated, and consequently the accuracy of the method, viz. its approximation to the real value[1] cannot be tested, even though this has been attempted[5].

An elucidation of the accuracy is obtained from the results of experiments with

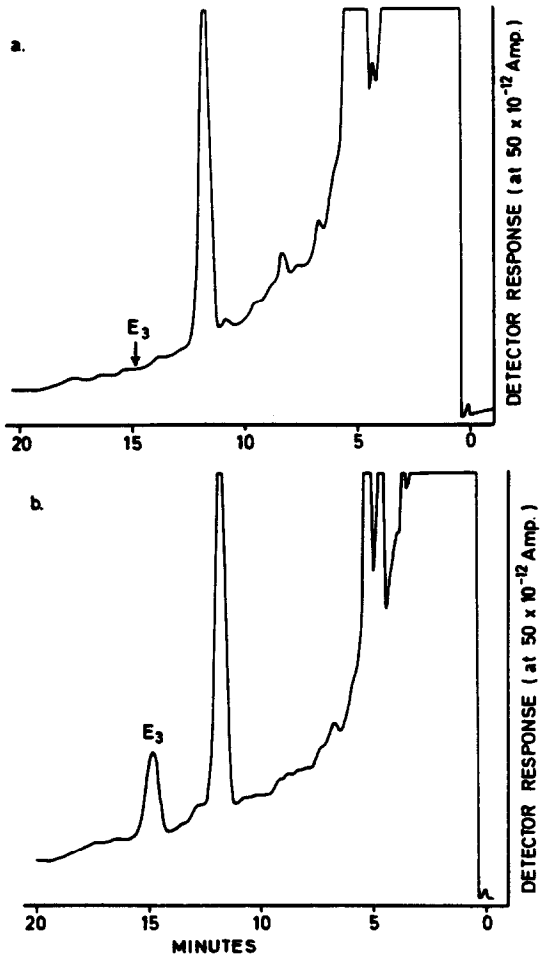

Fig. 2. (a) Gas chromatogram representing male plasma from which was injected ¼th of an extract from 5 ml. No peak corresponding to oestriol is seen. (b) Gas chromatogram of pregnancy plasma from 24th week serial tested with the above male plasma. Concentration: 4·0 μg oestriol per 100 ml plasma.

addition of oestriol to male plasma (Table 1). From this it appears that the average total recovery with the radioactive correction will be very close to the value of the added, free steroid. The tritium-labelled oestriol is added before hydrolysis and is thus correcting for the loss which will result from this. It gives an indication of the recovery of hydrolysed, free oestriol, but not about the material which might not be hydrolysed. The reproducibility is acceptable as shown by the results in Table 3. When ten samples from the same plasma pool were assayed by three different persons, a mean value ($\pm s$) of $18{\cdot}2 \pm 0{\cdot}8$ μg oestriol per 100 ml plasma was obtained. Hydrolysis was performed on different days, but in spite of this, the results show it to be consistent.

The recovery of radioactive oestriol from male plasma (the last column in Table 1) was 60 ± 5 per cent. In some later individual duplicate analyses the percentage radioactive recovery was 69 ± 3 per cent. Since the first investigations a certain improvement in recovery has taken place, but it cannot be dispensed with.

Table 1. Recovery of oestriol added to male plasma

Column	3% QF-1*	3%QF-1*	1% SE-30†
Number of samples, N	10	12	8
Imitated concentration of oestriol per 100 ml	20·0 μg	10·0 μg	10·0 μg
Measured concentration of oestriol per 100 ml mean value $\bar{x}$	21·2 μg	10·2 μg	9·9 μg
Standard deviation $s = \sqrt{\frac{\Sigma(\bar{x}-x)^2}{N-1}}$	±1·5 μg	±0·9 μg	±0·6 μg

*5 ft, temp. 225°C, R_t 13·9 min.
†5 ft, temp. 220°C, R_t 13·6 min.

Table 2. Oestriol concentration in samples from two plasma pools of pregnant women at term measured on QF-1 and XE-60 and on QF-1 and SE-30. Mean values ± standard deviations in μg per 100 ml

	Number of samples	Columns 3% QF-1*	2% XE-60†
Pool 1	5	27·0 ± 1·1	23·4 ± 1·1
		3% QF-1*	1% SE-30‡
Pool 2	8	33·9 ± 3·0	30·1 ± 2·3

*5 ft. temp. 225°C, R_t 10·4 min.
†5 ft, temp. 207°C, R_t 11·6 min.
‡5 ft, temp. 221°C, R_t 12·5 min.

The methodological loss is not unreasonable, since many operative steps are involved, including hot acid hydrolysis and TLC.

Precision is stated in Table 4 for different concentrations of oestriol. The standard deviation s is estimated from duplicate analyses according to 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 is the number of such analyses performed [25].

Sensitivity can be calculated from the standard deviation for the lowest measured concentration range, listed in Table 4. When $P = 0{\cdot}05$ the smallest amount of oestriol which is distinguishable from zero is 0·6 μg per 100 ml plasma, i.e. twice the standard deviation.

DISCUSSION

Since repeated oestriol estimations during pregnancy may be necessary, it is important that the procedure used can be carried out on a suitably small quantity

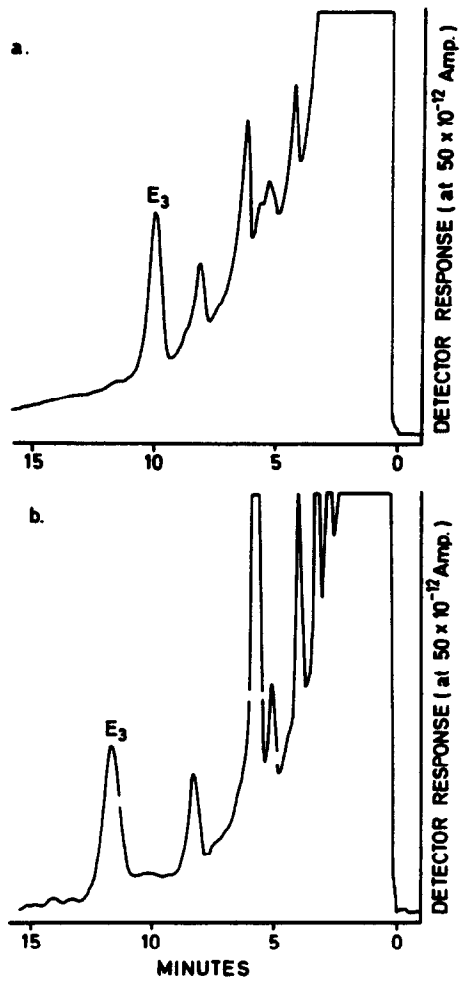

Fig. 3. Same sample of pregnancy plasma injected on 3% QF-1 (a) as well as on 2% XE-60 (b). Example from Table 2, pool 1.

of blood. From the statements in the literature on the concentration of oestriol in plasma, about 0·5–3 μg per 100 ml can be expected between the 20th and 24th week of the pregnancy, and 10–40 μg per 100 ml at term. In pathological conditions the values may be lower. If an allowance is made for the loss during analysis and for the fact that gas chromatography with flame ionization detection rarely measures less than 5 ng in a biological extract, a suitable volume for this technique can hardly be much less than about 5 ml plasma. It should further be considered that only an aliquot of the extracted oestriol can be injected into the gas chromatograph with liquid injection technique.

Of the methods previously mentioned Roy and Brown [3] use a similar volume, while the fluorimetric methods of Ittrich [4] and Nachtigall *et al.*[5] require 2–4 ml plasma and 1 ml plasma, respectively. The other authors are using bigger volumes. Ratanasopa *et al.*[17] can, however, perform their analysis on 5 ml plasma at term.

When there is only a small quantity of steroid present, it would be better to

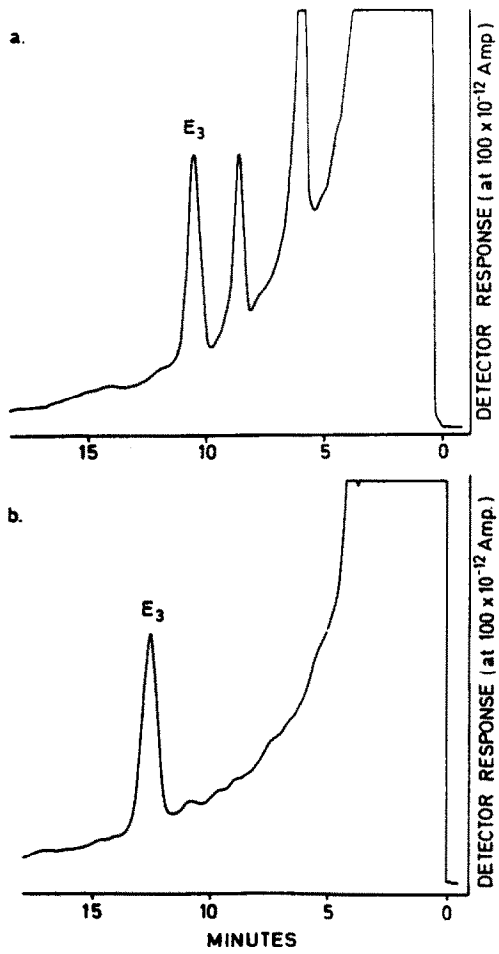

Fig. 4. Same sample of pregnancy plasma injected on 3% QF-1 (a) as well as on 1% SE-30 (b). Example from Table 2, pool 2.

have the total quantity injected by a solid sample injection technique instead of the aliquot limited by liquid injection. A number of methods—often rather ingenious—of solid sample technique for gas chromatography have been published[26–32]. However, the method described by Menini[33] and Menini and Norymberski[34] is quite simple. Unfortunately, the present author has had difficulty in obtaining a satisfactory, quantitative application of the extract on to the gauze cylinder.

In the actual method acidic hydrolysis with hydrochloric acid is used. The measured oestriol concentrations are thus 'total oestriol'. As mentioned under the question of accuracy, the hydrolysis seems to give reproducible results. Enzymatic hydrolysis has been considered, but is rather time-consuming. It is possible that a rapid method for enzymatic hydrolysis of oestrogens in the urine [35] could be transferred to plasma. Enzymatic hydrolysis in connection with gel filtration seems to be superior to the acidic hydrolysis[36].

Chloroform was chosen as extraction solvent as Svendsen[6] obtained a purer extract using this solvent than either with ether or acetone. He evaporated

Table 3. Oestriol concentration in samples from same plasma pool of women at term. Thin-layer chromatography was done in two different systems. Each sample was measured by GLC on SE-30 as well as on XE-60. Mean values ± standard deviations in μg per 100 ml

TLC system	Number of samples	Columns 1% SE-30*	2% XE-60†
A: Cyclohexane–ethyl acetate–ethanol, 45:45:10, $R_f = 0{\cdot}35$	7	22·3 ± 1·0	22·9 ± 1·3
H: Benzene–ethanol, 80:20, $R_f = 0{\cdot}51$–$0{\cdot}55$	7	22·5 ± 0·6	23·5 ± 1·4

*5 ft, temp. 221°C, R_t 12·9 min.
†5 ft. temp. 206°C, R_t 9·8 min.

Table 4. The precision with which oestriol is estimated in duplicates for different concentration ranges

Concentration range μg per 100 ml	Number of duplicates	Standard deviation μg $\sqrt{\frac{\Sigma(x_1 - x_2)^2}{2N}}$
1·6–5·0	56	±0·3
5·1–10·0	62	±0·4
10·1–15·0	21	±0·7

equal amounts of the three solvents and chromatographed and esterified residues on paper. The chloroform was purified by simple distillation. When using ether the peroxides must be removed as they are destructive to the oestrogens [1].

A fractionation and analysis of free, sulphate-, glucosiduronate-, and doubly conjugated oestriol might have a clinical importance, but at present it will be a most complicated analysis to use extensively [11, 12, 24].

The specificity of the method has been tested with different systems in TLC and on different phases in GLC, and the results suggest the probable identity of the compound with the reference standard. The specimens could also have been tested as different derivatives, e.g. as acetates [37] as well as trimethylsilylethers. The considerably smaller response of the acetate compared with the trimethylsilylether is very inconvenient when dealing with the small amounts of steroid as in this study. Furthermore, the irreversible adsorption with necessary priming of the column interferes impedingly with the use of acetates.

A very simple supplement to the specificity tests is to add a known quantity of the steroid in question to the sample analyzed [14]. It is then seen whether an increase corresponding to the quantity added has taken place and whether any broadening or skewing of the peak measured has occurred.

There has been no opportunity to carry out a more direct study of specificity such as gas chromatography combined with mass spectrometry.

The pools of pregnancy plasma for the studies of the reliability of the method (Tables 2 and 3) have oestriol values just at the upper limit of the normal range

presented in a later paper[38]. The plasmas for the pools were chosen from women with high urinary oestriol excretion.

It would be desirable to obtain a more ideal chromatogram where the peak of the steroid would be free of the solvent front and the impurities. One possibility is the above mentioned solid sample injection by which the solvents are evaporated before their introduction into the gas chromatograph. There will, however, still be disturbing impurities, and the impurities increase proportionally to the greater amount of steroid injected. By adequate extensive and careful cleaning of glasswares, solvents, and TLC-technique, most impurities in the chromatogram seem to appear during the TMSi-derivation after TLC. It is necessary to add a certain surplus of the hexamethyldisilazane and trimethylchlorosilane, and these reagents contain an amount of impurities which cannot easily be removed. Therefore it would seem more appropriate to carry out the derivative formation before TLC, as could be done with acetates. The lability of the oestrogen trimethylsilyl ethers by an acid-catalysed hydrolysis does not make these derivatives suitable for TLC. After elution from the thin-layer plate the derivative formation must be carried out again. Several reports are available about TLC of TMSi-derivatives of other groups of substances: e.g. steroles and terpenoles[39], glycoles[40], carbohydrates[41], and of hydroxysteroids[42].

The preparation of halogenoalcylsilylethers of steroids as chloro- or bromomethyldimethylsilyl ether[43, 44] should among other things give more stable derivatives for TLC. Their area of application has so far mainly been for 11-deoxy-17-oxysteroids[45].

Column chromatography (silicic acid) of oestriol-TMSi can be performed on extracts of pregnancy urine[46]. During the elution from the column there was, however, added a small quantity of hexamethyldisilazane to protect the phenolic TMSi-ethers from partial hydrolysis.

In a later paper are the results obtained when the method is applied to a study of plasma oestriol levels in late pregnancy.

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