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Gas chromatography of methylated estrogens and application of the method to the analysis of human late pregnancy bile

Satisfactory identification and quantitation of the structurally closely related estrogens of a biological sample is only possible with an analytic technique of the highest quality. The suitability of gas chromatography as a method for the separation and estimation of these steroids was realized after the pioneer work of VANDENHEUVEL, SWEELEY AND HORNING[1]. However, the sensitive and blind detection systems of gas chromatography are a disadvantage if the purity of the samples is not equal to the capacity of detection. After adequate purification, the compounds to be estimated should be the main components on the recording chart. Further, the best identification of the compounds can be achieved if all the available potentialities of gas chromatography are used (see ref. 2 for review). The retention times are then short and the peaks are sharp and well separated on the baseline, with a number of different liquid phases at moderate temperatures.

The "classical estrogens" may be readily separated as trifluoroacetates[3] or as acetates[4-7]. The trimethylsilyl ethers seem to offer a possibility for the identification of all the known estrogens; the relative retention times of eleven estrogens with each of four types of liquid phases have been determined earlier[8,9].

The present investigation is concerned with a combination of a previously published method for the purification of estrogens[10] and gas chromatographic analysis of the final extracts in the case of one of the most complex biological fluids, *i.e.* bile. The gas chromatographic properties of methylated estrogens are presented, too, and their usefulness as starting material for double derivatives pointed out.

A Chromalab model 110 (Glowall Corporation, Pa. (U.S.A.)) gas chromatography apparatus was used. Bile samples were obtained by duodenal intubation during late pregnancy or by T-tube drainage of the main bile duct and processed as described by ADLERCREUTZ[10]. Steroids were converted to trimethylsilyl ethers as reported previously[8,9] and the preparation and use of the steroid columns were based on the methods employed in Dr. HORNING's laboratory[11]. The following stationary phases

TABLE I

RELATIVE RETENTION TIMES (TO ANDROSTERONE)

*Liquid phase** *Temperature* *Pressure*	*1% XE-60* *212°* *2 kg/cm²*	*1% QF-1* *180°* *2 kg/cm²*	*1% SE-30* *190°* *2 kg/cm²*
Estrone 3-methyl ether	0.92	0.89	1.06
17β-Estradiol 3-methyl ether	0.86	0.58	1.13
Estriol 3-methyl ether	2.82	1.53	2.06
Estrone acetate	1.93	2.58	1.59
Estrone TMSi**	0.80	—	1.33
Estrone	—	1.33	1.19
17β-Estradiol	—	0.82	1.26
Estriol	—	2.06	2.32
Androsterone time	4.3 min	6.4 min	6.3 min

* All phases on 100–140 mesh Gas-Chrom P, 6 ft × 4 mm coiled-glass column.
** TMSi, trimethylsilyl.

were used: SE-30 (dimethylsilicone polymer), QF-1 (fluorinated alkyl silicone polymer), XE-60 (cyanoethylmethylsilicone polymer) and NGS (neopentylglycol succinate polyester).

Table I gives the relative retention times (to androsterone) of methylated estrone, 17β-estradiol and estriol. For comparison, the relative retention times of their parent compounds and of representatives of other estrogen derivatives have been determined. Gas-chromatographic analysis of the methylated estrone in the "sulphate"

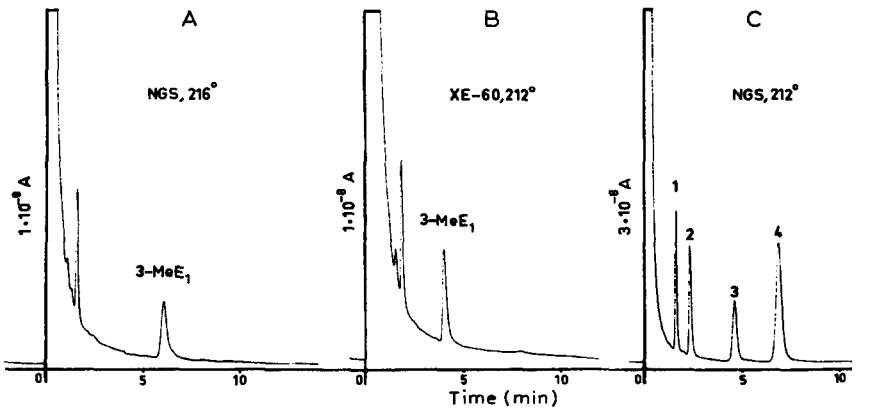

Fig. 1. Gas chromatography of estrone 3-methyl ether (3-MeE_1) obtained from late pregnancy bile, on two different stationary phases (A and B), and (C) of 3,17β-ditrimethylsilyl ether of estradiol (1), 17β-trimethylsilyl ether of estradiol 3-methyl ether (2), and 3-trimethylsilyl ether of estrone (3) and estrone 3-methyl ether (4). Pressure, 2 kg/cm².

fraction (0.1 μg) of a late pregnancy bile sample on two liquid phases (Figs. 1 A and B) demonstrates that purification was satisfactory. The possibility of estimation and identification is almost ideal. The results represent additional important evidence for the presence of estrone in human late pregnancy bile[10,12].

From the practical standpoint, the methylated derivatives of estrogens are an obvious choice, because they are more stable than the free compounds and one of the existing purification methods is based on a phase-change purification step including a methylation of the estrogens[13]. However, the gas-chromatographic properties are better when estrogens with more than one hydroxy group are converted to double derivatives with hexamethyldisilazane after methylation. The use of these double derivatives, in addition to other derivatives, makes the identification of an unknown compound still more conclusive. It appears from Figs. 1 C, 2 and 3 that methylated estriol and its ditrimethylsilyl ether obtained from the "glucosiduronate" fraction of human bile, as well as standard preparations of methylated estrone, trimethylsilyl ethers of estrone and 17β-estradiol and a double derivative of 17β-estradiol, can be separated and estimated with high accuracy and specificity. From Figs. 2 and 3 it can be well seen that estriol is the main compound on the chart, which confirms previous findings regarding the specificity of the purification procedure used[10].

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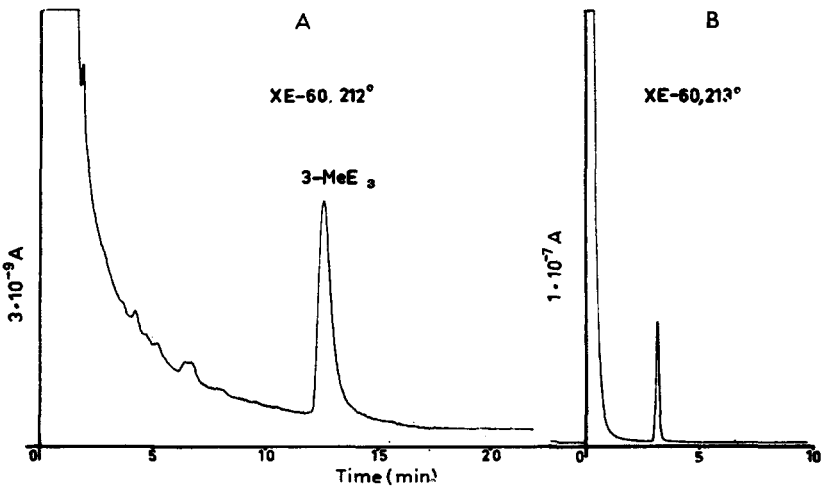

Fig. 2. Gas chromatography of estriol 3-methyl ether (A) obtained from human late pregnancy bile and of 16,17-ditrimethylsilyl ether of estriol 3-methyl ether (B) obtained from a bile sample of a patient with T-tube drainage of the main bile duct after intravenous administration of 12.5 mg estriol. Pressure, 2 kg/cm².

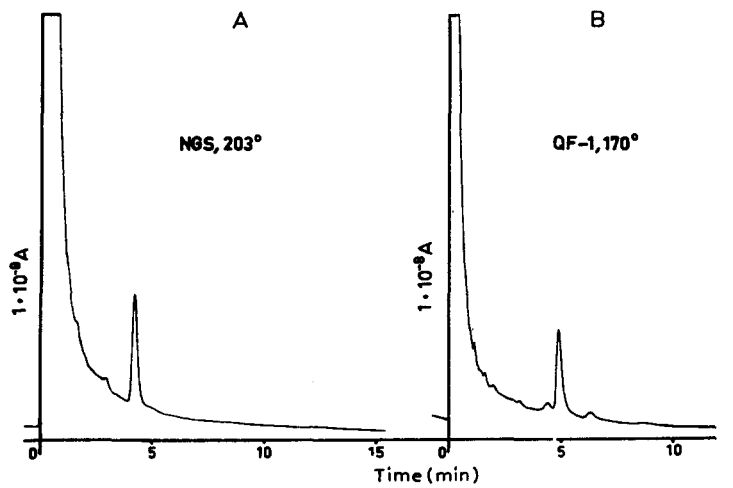

Fig. 3. Gas chromatography of 16,17-ditrimethylsilyl ether of estriol 3-methyl ether obtained from human late pregnancy bile, on two different stationary phases. Pressure, 2 kg/cm².

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Use of trypsin to eliminate residual snake-venom phospholipase A activity in lysolecithin prepared from ovolecithin

MARPLES AND THOMPSON[1] have reported that lysolecithin prepared from ovolecithin by the action of cottonmouth mocassin venom contained residual phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) in an active state, confirming similar observations of SAUNDERS AND THOMAS[2]. These workers further reported that crystallization of lysolecithin from hot ethanol resulted in complete removal of the phospholipase activity as tested both by CO_2 evolution and by acid titration.

Under certain conditions, however, we have found that the residual phospholipase activity is still demonstrable after 5–6 recrystallizations from hot ethanol of lysolecithin prepared from ovolecithin treated with naja naja snake venom. When equal molar concentrations of the above prepared lysolecithin and ovolecithin were incubated at 37° in the presence or absence of buffer at pH 6.0 or 9.0, significant phospholipase A activity was observed qualitatively on thin-layer chromatograms by large increases in fatty acids and lysolecithin with a concomitant decrease in lecithin within 2 h. If the incubations were carried out under the conditions described by MARPLES AND THOMPSON[1], or by the preparative method of HANAHAN, RODBELL AND TURNER[3], or when lecithin is in excess of lysolecithin (3 to 1 mole ratio), very little residual activity in the recrystallized lysolecithin could be observed either by thin-layer chromatography or by fatty acid determinations, because the methods were not sufficiently sensitive to demonstrate the small amount of phospholipase A remaining. When incubated for 24 h as above in equal molar concentrations, no hydrolysis of lecithin occurred with lysolecithin prepared by the sodium methoxide method of MARINETTI[4].

When the above lysolecithin preparations were treated with trypsin and subsequently isolated, they did not exhibit any residual phospholipase activity after 24 h incubation with equal molar concentration of lecithin. Recovery of these preparations