Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 845 852. Pergamon Press. Printed in Great Britain

# GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC ANALYSIS OF PERMETHYLATED ESTROGEN GLUCURONIDES

RICHARD M. THOMPSON
Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana 46202, U.S.A.

(*Received* 7 *May* 1976)

SUMMARY

Five different estrogen monoglucuronides have been separated at least partially by gas chromatography of the permethyl derivatives on OV-17 columns either isothermally or with temperature programming. The permethyl derivatives are formed quantitatively with a greater than tenfold excess of the methylsulfinylmethide carbanion and methyl iodide. On the other hand, quantitative derivatization (>98%) of the aglycones, estradiol and estriol, is effected only with a thirtyfold or greater excess of the carbanion. Mono-, di-, and trimethyl derivatives are found and all have characteristic mass spectra. The mass spectra of the permethylated glucuronides are also clearly different. All spectra contain molecular ions and fragment ions due to the permethylated glucuronic acid moiety, the aglycones, and M-$CH_3OH$. This data is being applied to the analysis of intact estrogen glucuronides in body fluids.

## INTRODUCTION

Estrogenic steroids in body fluids have been determined in the past by a variety of techniques including high-speed liquid chromatography [1], gas chromatography (GC) [2–4], single or multiple ion detection with a combined gas chromatograph–mass spectrometer (GC–MS) [5, 6], and fluorometry [7]. All of these methods utilize acid or enzyme hydrolysis to free conjugated estrogens, followed by solvent extraction and analysis either of the free compounds or of the trimethylsilyl derivatives in order to determine total estriol [2, 3, 7] or total estrogens [1, 4–6].

In 1972 Tikkanen and Adlercreutz reported an elegant study in which they quantitatively determined four conjugates of estriol in human urine [8]. Their procedure involved isolation of each conjugate by column chromatography on Sephadexes G-25 and LH-20 followed by enzymatic hydrolysis, ether extraction of the aglycones, methylation with dimethyl sulfate, chromatography on alumina, and determination of the trimethylsilyl derivatives by GC or of any radiolabelled compounds by liquid scintillation counting.

Other previous work has demonstrated that intact glucuronides of certain drugs, steroids, and other compounds could be determined by GC and GC–MS analysis of the permethyl derivatives [9, 10]. These derivatives were formed with the methylsulfinylmethide carbanion and methyl iodide. The GC separations were carried out with either SE-30 or OV-1 columns [9]. Steroid glucuronides which contained isolated $\alpha,\beta$-unsaturated ketone functions yielded multiple products due to "overmethylation" by the permethylation procedure [10].

More recently, two radioimmunoassay techniques have been developed for the quantitative determination of estriol-16-glucuronide, the major estriol conjugate in urine and plasma during pregnancy [11, 12]. No method is presently available, however, for obtaining total intact estrogen glucuronide profiles in body fluids. Therefore, the following studies were undertaken in order to understand the GC and GC–MS properties of these glucuronides so that systems could be developed for determining such profiles.

After this manuscript was submitted another paper concerning the GC–MS analysis of estrogen glucuronides as the trimethylsilyl-n-propyl ester derivatives appeared [16].

## EXPERIMENTAL

1,3,5(10)-estratrien-3,17$\beta$-diol (estradiol) ($E_2$), 1,3,5(10)-estratrien-3,16$\alpha$,17$\beta$-triol (estriol) ($E_3$), 1,3,5(10)-estratrien-3-ol-17-one (estrone), the 3- and 17$\beta$-glucuronides of $E_2$ ($E_2$-3G1 and $E_2$-17G1) (the latter as the sodium salt), and the 3-glucuronide of estrone were purchased from Steraloids, Inc., Wilton, N.H., 03086. The 3-, 16$\alpha$-, and 17$\beta$-glucuronides of $E_3$ ($E_3$-3G1, $E_3$-16G1, and $E_3$-17G1) were from Sigma Chemical Co., St. Louis, Mo., 63178, the 3-glucuronide as the sodium salt. Hydrocarbon standards were obtained from either Supelco, Inc., Bellefonte, Pa., 16823, or Applied Science Laboratories, State College, Pa., 16801.

All of the above steroids and steroid glucuronides were permethylated in dimethylsulfoxide in small screw-capped vials alone or in mixtures with the others (50–100 $\mu$g each) with the methylsulfinylmethide carbanion (1 M), prepared as described previously [13], and methyl iodide ($CH_3I$ or $CD_3I$). Varying substrate to carbanion ratios were used along with a large excess of methyl iodide in order to determine the best conditions for permethylation. The reactions were stopped by the addition of 1 ml of water. The products were extracted into chloroform and washed 3 × with water. The chloroform solutions were evaporated to dryness under a gentle stream of nitrogen

and the residues were redissolved in 100 μl of chloroform for GC and GC–MS analysis.

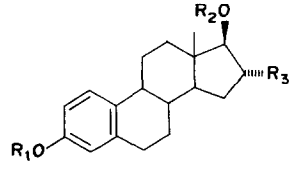
*Gas chromatography.* The analytical separations were carried out with a Tracor Model 550 dual column gas chromatograph equipped with 1.8 m × 4 mm or 3.6 m × 3 mm coiled glass columns packed with either 1% OV-17 on Gas-Chrom Q (80–100 mesh) (Applied Science Laboratories) or 1% SE-30 on Gas-Chrom Q (100–120 mesh). Both phases were prepared in this laboratory by solution coating. Operating parameters included: injection port temperature, 270°C; detector temperature, 300°C; nitrogen flow rate, 40 ml/min; and oven temperature, 285°C for the isothermal analysis. Temperature programming was carried out at either 2°C or 4°C/min from 100°C, 150°C, or 200°C. Quantification was done by measurement of peak areas with an Infotronics Model CRS-204 automatic digital integrator. Methylene unit values were determined by co-injection with an appropriate hydrocarbon mixture.

*Mass spectrometry.* The mass spectra were collected on an LKB-9000-S combination GC–MS instrument with a 1 m × 3 mm 1% SE-30 column (100/120 mesh Gas-Chrom Q) as the GC inlet. The ion source temperature was 270°C, the accelerating and ionizing potentials were 3.5 kV and 16 eV or 70 eV respectively, and the trap current was 60 μA. The GC oven temperature was programmed from 150°C or 250°C and the helium flow rate was 30 ml/min.

## RESULTS AND DISCUSSION

*Estrogens.* In a series of preliminary experiments prior to the analysis of the standard estrogen glucuronides, conditions for the permethylation of the free aglycones—$E_2$, $E_3$, and estrone—were determined. One hundred μg samples of each (0.37, 0.35, and 0.37 μmol respectively, representing 0.74, 1.04, and 0.37 μequiv of hydroxyl groups respectively) were methylated with amounts of the anion solution (1 μequiv/μl) varying from 1 to 50 μl.

Figures 1 and 2 show graphically the results obtained from the methylation of $E_2$ and $E_3$ respectively by using various substrate to carbanion ratios. Sample chromatograms showing the GC separations of the methylation products which are cleanly resolved on either OV-17 or SE-30 columns appear as insets in both figures. The methylene unit values




$E_2$: $R_1 = R_2 = R_3 = H$
$E_2$ – Me: $R_1 = CH_3$, $R_2 = R_3 = H$
$E_2$ – diMe: $R_1 = R_2 = CH_3$, $R_3 = H$
$E_3$: $R_1 = R_2 = H$, $R_3 = OH$
$E_3$ – Me: $R_1 = CH_3$, $R_2 = H$, $R_3 = OH$
$E_3$ – diMe: $R_1 = CH_3$, $R_2 = H$ or $CH_3$, $R_3 = OH$ or $OCH_3$
$E_3$ – triMe: $R_1 = R_2 = CH_3$, $R_3 = OCH_3$
$E_2$ – 3Gl: $R_1 = \beta$ – D – glucuronic acid, $R_2 = R_3 = H$
$E_2$ – 17Gl: $R_1 = R_3 = H$, $R_2 = \beta$ – D – glucuronic acid
$E_3$ – 3Gl: $R_1 = \beta$ – D – glucuronic acid, $R_2 = H$, $R_3 = OH$
$E_3$ – 16Gl: $R_1 = R_2 = H$, $R_3 = O$ – $\beta$ – D – glucuronic acid
$E_3$ – 17Gl: $R_1 = H$, $R_2 = \beta$ – D – glucuronic acid, $R_3 = OH$

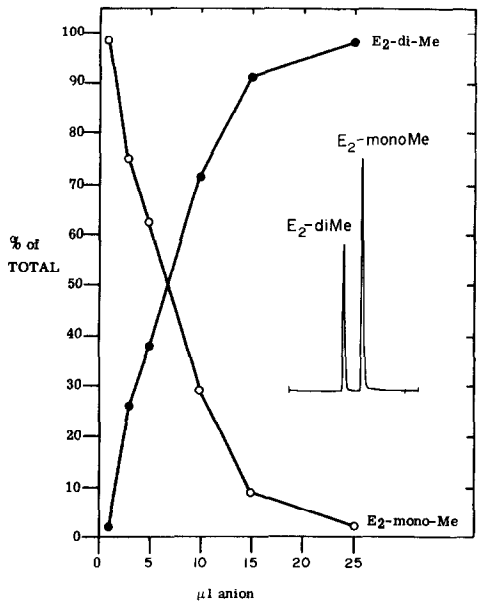



Fig. 1. Relative proportions of the products obtained from the permethylation of estradiol ($E_2$) with varying amounts of methylsulfinylmethide carbanion. The inset shows the GC separation of these products. Conditions: 1.8 m × 4 mm OV-17 on 80/100 mesh Gas-Chrom Q; injector temperature, 270°C; detector temperature, 300°C; $N_2$ flow rate, 40 ml/min; temperature programming @ 4°C/min from 180°C.

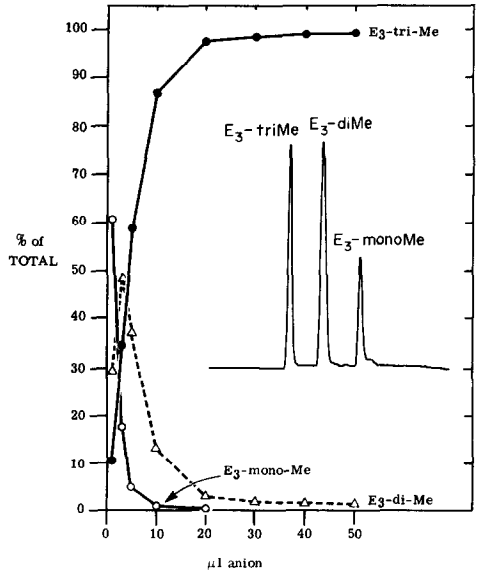


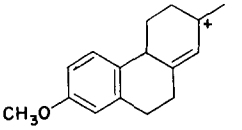

Fig. 2. Relative proportions of the products obtained from the permethylation of estriol ($E_3$) with varying amounts of methylsulfinylmethide carbanion. The inset shows the GC separation of these products. Conditions: 1.8 m × 4 mm OV-17 on 80/100 mesh Gas-Chrom Q; injector temperature, 270°C; detector temperature, 300°C; $N_2$ flow rate, 40 ml/min; temperature programming @ 4°C/min from 180°C.

Table 1. Methylene unit values for the various derivatives of estradiol ($E_2$), estriol ($E_3$), and the glucuronides (G1) on OV-17

| Product | MU value |
|---|---|
| $E_2$-monomethyl | 29.38 |
| $E_2$-dimethyl | 27.94 |
| $E_3$-monomethyl | 32.73 |
| $E_3$-dimethyl | 31.04 |
| $E_3$-trimethyl | 29.73 |
| $E_2$-3G1-pentamethyl | 42.40 |
| $E_2$-17G1-pentamethyl | 42.82 |
| $E_3$-3G1-hexamethyl | 43.87 |
| $E_3$-16G1-hexamethyl | 43.33 |
| $E_3$-17G1-hexamethyl | 43.72 |

are given in the Table. Figures 3 (a and b) and 4 (a, b, and c) contain the mass spectra of the mono- and dimethyl derivatives obtained from $E_2$ ($E_2$-Me and $E_2$-diMe) and the mono-, di-, and trimethyl derivatives from $E_3$ ($E_3$-Me, $E_3$-diMe, and $E_3$-triMe) respectively.

The graphic data presented in Figs. 1 and 2 clearly shows that the degree of methylation of $E_2$ and $E_3$ was dependent on the amount of anion used in the derivatization. $E_2$ yielded essentially only the monomethyl derivative (98.2%) with 1 $\mu$l anion, a slight excess, but the amount of the dimethyl derivative increased to 98% of the total product mixture with 25 $\mu$l anion, a 34-fold excess. These data along with the mass spectra of $E_2$-Me and $E_2$-diMe (Figs. 3a and 3b) indicate that the first methyl is added to the more acidic phenolic hydroxyl at the 3-position as expected and that the more highly hindered hydroxyl group at the 17-position is more difficult to methylate. The mass spectra contain appropriate molecular ions at *m/e* 286 ($E_2$-Me) and *m/e* 300 ($E_2$-diMe). The ion at *m/e* 227 in both spectra is due to the loss of carbons 15, 16, and 17, along with the C-17 substituent and a transferred hydrogen:




*m/e* 227

This ion contains one added methyl group as shown by pertrideuteriomethylation. Other ions at *m/e* 147, 160, 174, and 186 all containing one added methyl group are also present in both spectra. Therefore, the monomethyl derivative of $E_2$ is the 3-*O*-methyl ether.

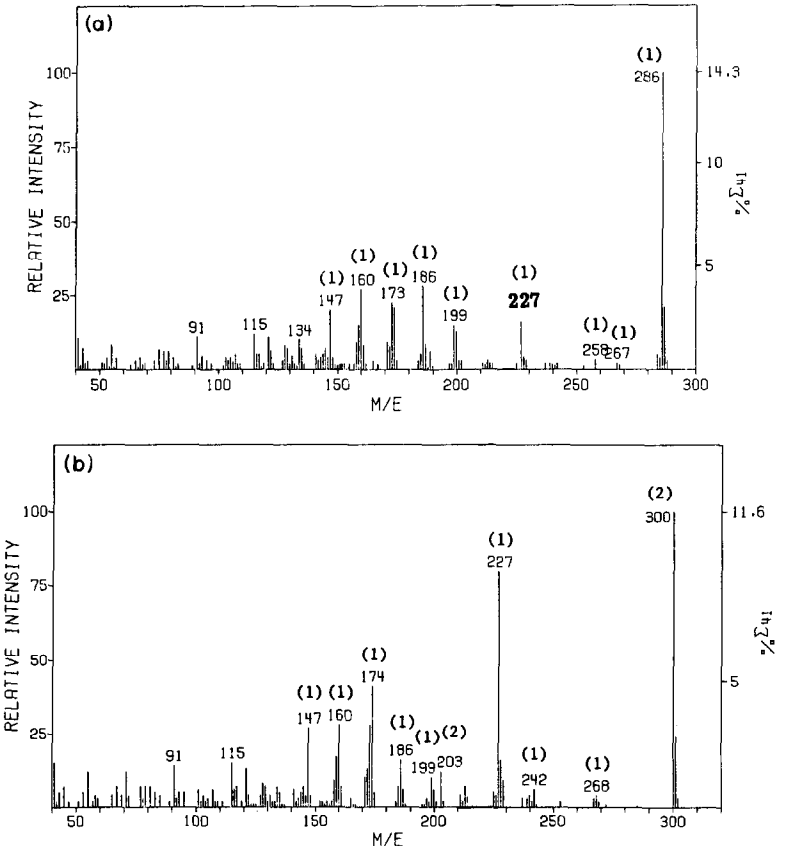



Fig. 3a (top). Mass spectrum of estradiol-3-*O*-methyl ether. The numbers in parenthesis indicate the number of added methyl groups.

Fig. 3b (bottom). Mass spectrum of estradiol-3,17$\beta$-di-*O*-methyl ether. The numbers in parenthesis indicate the number of added methyl groups.

In the case of $E_3$, 1 $\mu$l of anion was approx. an equivalent amount needed to permethylate 100 $\mu$g of sample. A mixture of three products was obtained, however: $E_3$-Me, 60.3%; $E_3$-diMe, 29.2%, and $E_3$-triMe, 10.5%. When 30 $\mu$l or more anion was used (>thirty-fold excess), the trimethyl derivative was essentially the only product formed (>98% of the product mixture). It was interesting that the amount of the dimethyl derivative increased initially with 1 to 3 $\mu$l anion and then gradually decreased to a negligible level.

Significant ions at *m/e* 147, 174, and 227 in the mass spectra of $E_3$-Me, $E_3$-diMe, and $E_3$-triMe (Figs. 4a, 4b, and 4c respectively), containing one added methyl group each and also present in the spectra of $E_2$-Me and $E_2$-diMe (Figs. 3a and 3b), indicate that $E_3$-Me is the 3-O-methyl ether (mol. wt. = 302). The dimethyl derivative of $E_3$ (mol. wt. = 316) is probably the 3,16$\alpha$-di-O-methyl ether, because of the appearance of the significant ion at *m/e* 229 which likely contains carbon-16 and the methoxyl substituent as interpreted below.

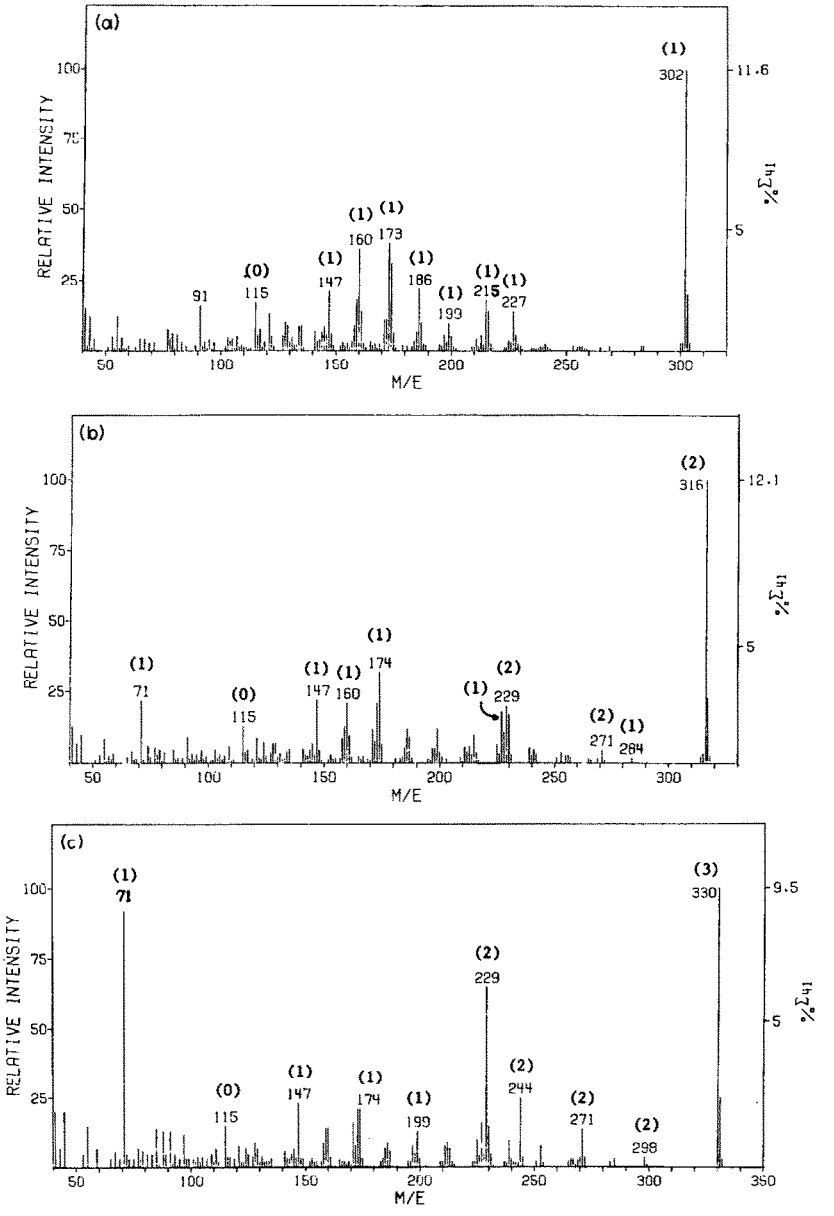



Fig. 4a (top). Mass spectrum of estriol-3-*O*-methyl ether. The numbers in parenthesis indicate the number of added methyl groups.

Fig. 4b (middle). Mass spectrum of estriol-3,16$\alpha$-di-*O*-methyl ether. The numbers in parenthesis indicate the number of added methyl groups.

Fig. 4c (bottom). Mass spectrum of estriol-3,16$\alpha$,17$\beta$-tri-*O*-methyl ether. The numbers in parenthesis indicate the number of added methyl groups.

The mass spectrum of $E_3$-triMe (Fig. 4c) closely parallels that of the pertrimethylsilyl (TMS) derivative of $E_3$ [14, and in this laboratory], allowing for the mass difference between methyl and TMS-groups (58 a.m.u.). The molecular ion at *m/e* 330 is the most intense ion in the spectrum. It loses one molecule of methanol (32 a.m.u.) to give the ion at *m/e* 298. Using the interpretation of the mass spectrum of the TMS-derivative of $E_3$ presented previously [14] as a guide, other characteristic ions in Fig. 4c are: *m/e* 71, carbons 15, 16, and 17 less one methoxyl group; *m/e* 229 (referred to above), M-(carbons 11, 12, 13, 17, and 18 + $CH_3OH$); *m/e* 244, M-(carbons 12, 13, 17, and 18 + $CH_3OH$); and *m/e* 271, M-(carbons 11 and 12 + $CH_3OH$).

Under all conditions, estrone yielded multiple products which were not easily separated and identified. This result was not unexpected because of previous experience with the permethylation of keto-steroids [15] and keto-steroid glucuronides [10]. Therefore, permethylation was deemed not to be a useful method for the derivatization of estrone and estrone-3-glucuronide for GC and GC–MS analysis and these compounds were not studied further.

It was somewhat surprising that $E_2$ and $E_3$ were not permethylated under the less rigorous conditions (1–5 μl anion) and that a thirty-fold or greater excess of anion was necessary to completely derivatize the 17-hydroxyl group. The methylsulfinylmethide carbanion is a rather powerful base but its attack on this hydroxyl is apparently sterically hindered. Similarly, more rigorous reaction conditions are required in order to silylate the 17-hydroxyl than the phenolic hydroxyl group at C-3.

*Estrogen glucuronides.* In order to determine what level of anion was necessary to permethylate the five glucuronides of $E_2$ and $E_3$, 100 μg samples of each (1.17 μequiv of $E_2$-3G1 and $E_2$-17G1 and 1.29 μequiv of $E_3$-3G1, $E_3$-16G1, and $E_3$-17G1) were methylated with amounts of anion ranging from 3 to 50 μl. $E_2$-17G1, $E_3$-16G1, and $E_3$-17G1 all yielded single permethyl derivatives with 5 μl or more anion.

There were two minor components in the product mixture from $E_2$-3G1, both having longer retention times than the permethyl derivative and one of which disappeared when the level of anion was 20 μl or greater. The other minor component ranged from 7 to 13% of the total product mixture with no real trend observed. From the relative retention times and methylene unit data, this second minor component was likely $E_2$-17G1 present as about a 10% impurity in the commercial sample of $E_2$-3G1.

The product mixture from the permethylation of $E_3$-3G1 with less than 20 μl of anion also contained two minor components with retention times longer than the hexamethyl derivative. The second one also disappeared above 20 μl. The first minor component was present in all product mixtures up to 14% of the total, but was not identified. It was also probably an impurity in the sample of $E_3$-3G1.

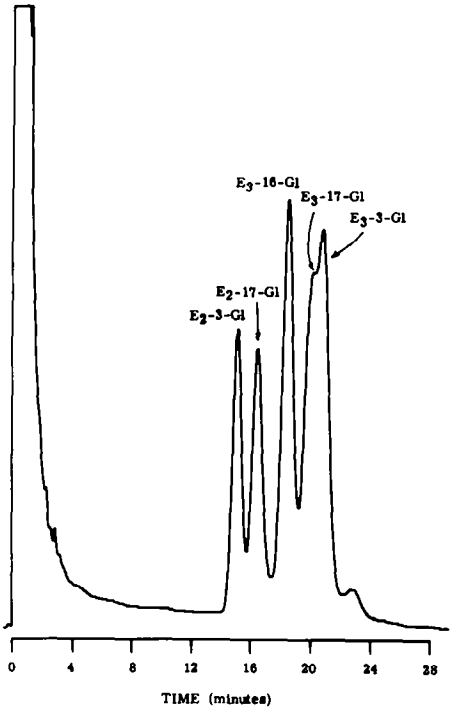



Fig. 5. Isothermal GC separation of the five permethylated glucuronides of estradiol and estriol. See text for abbreviations. Conditions: same as in Fig. 1 except that the column oven was maintained at 285°C.

The isothermal GC separation (OV-17) of a mixture of five permethylated estrogen glucuronides ($E_2$-3G1 and $E_2$-17G1 as the pentamethyl and $E_3$-3G1, $E_3$-16G1, and $E_3$-17G1 as the hexamethyl derivatives) is shown in Fig. 5. The resolution of all components except $E_3$-17G1 and $E_3$-3G1 is quite satisfactory. A temperature programmed separation at 2°C/min from a lower temperature such as 200°C was very similar. These five compounds were not well separated on SE-30 columns either isothermally or with temperature programming. Methylene unit values for these compounds are presented in the Table.

One highly unusual phenomenon observed here was that while permethyl $E_2$-3G1 had a shorter retention time than permethyl $E_2$-17G1, permethyl $E_3$-3G1 had the longest retention time of the three permethylated $E_3$ glucuronides. Apparently, the two substituents at carbons 16 and 17 of hexamethyl $E_3$-16G1 and $E_3$-17G1 interact in some manner which reduces the polarity of the entire molecules, thus shortening their relative retention times from what would normally have been expected.

Figures 6 (a and b) and 7 (a, b, and c) are the mass spectra of the two pentamethyl $E_2$ glucuronides and the three hexamethyl $E_3$ glucuronides respectively. The spectra are all quite different and are characteristic of permethylated glucuronides. Each is discussed separately below.

The mass spectrum of pentamethyl $E_2$-3G1 (Fig. 6a), a phenolic ether glucuronide, contains a relatively

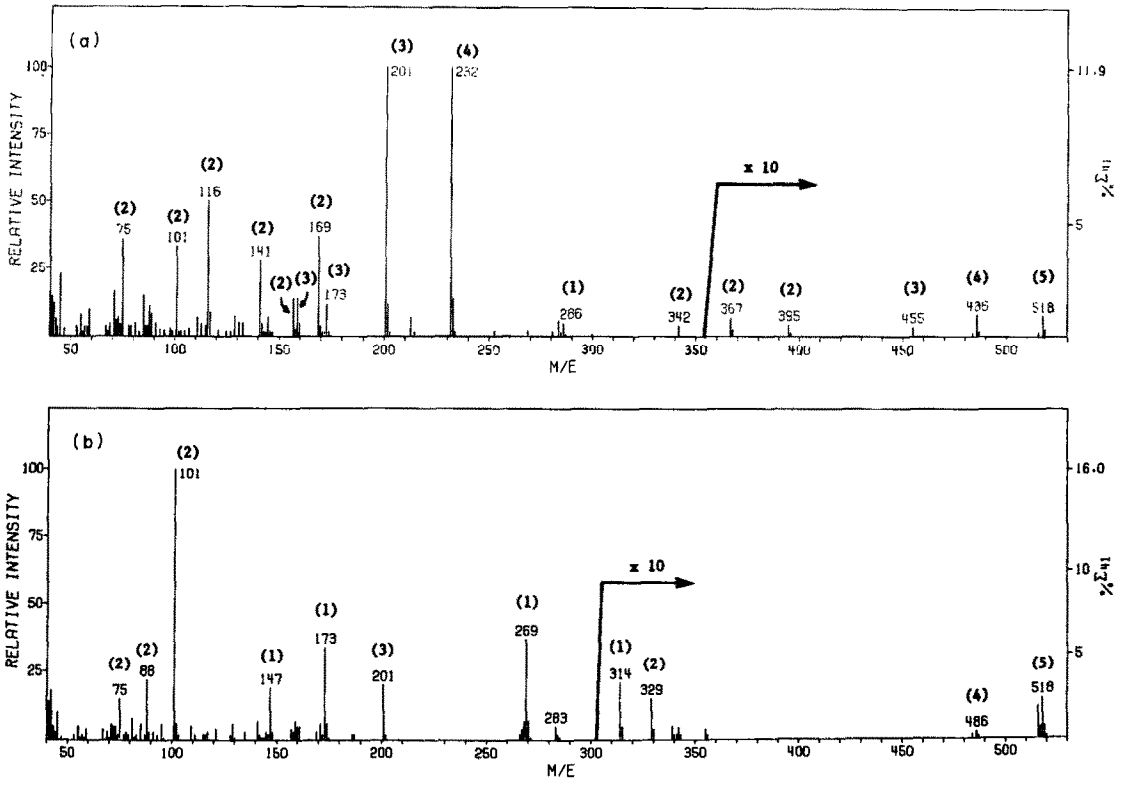
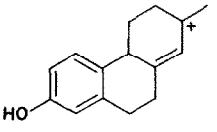



Fig. 6a (top). Mass spectrum of pentamethyl estradiol-3-glucuronide. The numbers in parenthesis indicate the number of added methyl groups.

Fig. 6b (bottom). Mass spectrum of pentamethyl estradiol-17$\beta$-glucuronide. The numbers in parenthesis indicate the number of added methyl groups.

small molecular ion (0.8%) at *m/e* 518 with losses of methanol (*m/e* 486) and methoxyl (*m/e* 455). Ions due to the permethylated glucuronic acid moiety are present at *m/e* 75, 101, 141, 169, 173, and 201. Two other previously recognized ions due to the fragmentation of the glucuronic acid moiety and characteristic of phenolic ether glucuronides are *m/e* 116 and *m/e* 232 [9]. The latter arises from cleavage of the glycosidic bond on the glucuronic acid side with transfer of a hydrogen to the aglycone to give also the ion at *m/e* 286, which is equivalent to $E_2$-17-*O*-methyl ether. The other ion which is probably characteristic of the aglycone is at *m/e* 213. This ion contains no added methyl groups and is similar to the ion at *m/e* 227 in Fig. 3b, the mass spectrum of $E_2$-diMe:




*m/e* 213

Figure 6b, the mass spectrum of pentamethyl $E_2$-17G1, an aliphatic ether glucuronide, also contains a small molecular ion at *m/e* 518 (1.5%) with loss of one molecule of methanol (*m/e* 486). The ions at *m/e* 75, 88, 101, and 201 are due to the permethylated glucuronic acid moiety. Whereas, in the spectrum of permethyl $E_2$-3G1 the *m/e* 201 ion is more intense than that at *m/e* 101, typical of permethylated phenolic ether glucuronides, the *m/e* 101 ion is the more intense in Fig. 6b, the usual case for permethylated aliphatic ether glucuronides. The ion at *m/e* 269 is due to cleavage of the glycosidic bond on the steroid side, the charge remaining on the steroid fragment. Other ions due to fragmentation of the aglycone are at *m/e* 147 and 173, each containing one added methyl group and having the same identity as in Fig. 3b.

The mass spectrum of hexamethyl $E_3$-3G1 (Fig. 7a) is also characteristic of a permethylated phenolic ether glucuronide: ions at *m/e* 75, 101, 116, 141, 169, 173, 201, and 232 due to the glucuronic acid moiety; a small molecular ion at *m/e* 548; successive losses of two molecules of methanol (*m/e* 516 and 484); and the ion at *m/e* 316 corresponding to glycosidic bond cleavage with hydrogen transfer to the aglycone. Other ions due to the aglycone are at *m/e* 71 (as in Figs. 4b and 4c) and at *m/e* 215, which contains one added methyl group and is probably the same as the *m/e* 229 ion in Fig. 4c less the methyl ether at C-3.

Figs. 7b and 7c, the mass spectra of the two hexamethyl aliphatic ether glucuronides, $E_3$-16G1 and $E_3$-17G1, are somewhat similar but also contain significant differences, mostly in terms of relative intensities. Both contain molecular ions at *m/e* 548 and show losses of methanol (*m/e* 516). Both also have ions at *m/e* 75, 88, 101, 141, 173 (partially), and 201

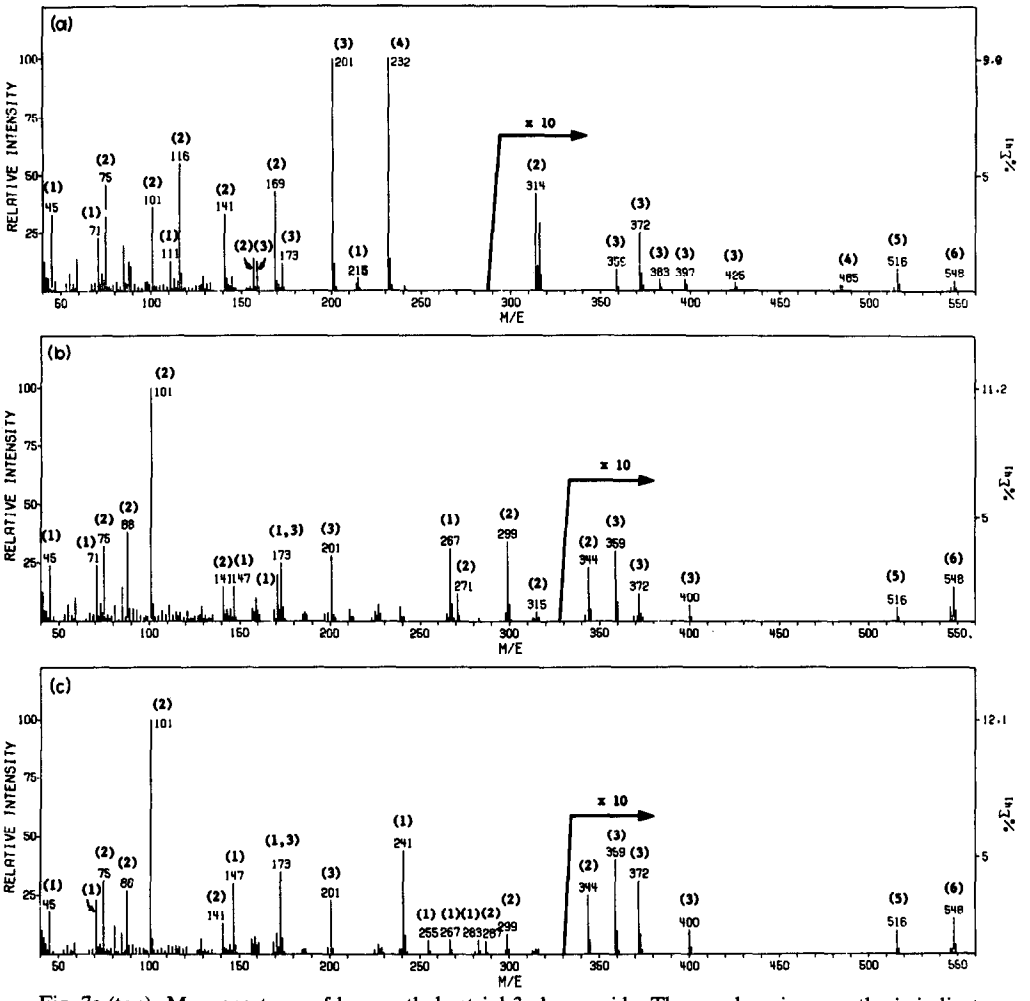


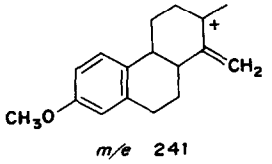

Fig. 7a (top). Mass spectrum of hexamethyl estriol-3-glucuronide. The numbers in parenthesis indicate the number of added methyl groups.

Fig. 7b (middle). Mass spectrum of hexamethyl estriol-16α-glucuronide. The numbers in parenthesis indicate the number of added methyl groups.

Fig. 7c (bottom). Mass spectrum of hexamethyl estriol-17β-glucuronide. The numbers in parenthesis indicate the number of added methyl groups.

due to fragmentation of the glucuronic acid moiety and ions at *m/e* 299 from glycosidic bond cleavage on the steroid side. However, the relative intensities of the *m/e* 299 ions are quite different. Both lose one molecule of methanol, yielding the ions at *m/e* 267. The ions at *m/e* 71, 147, and 173 (partially), common to both spectra, are due to fragmentation of the aglycone as described above.

The ion at *m/e* 241 in Fig. 7c, containing one added methyl group, is unusual and not easily interpreted by comparison with Fig. 4c, but is characteristic of permethylated $E_3$-17G1. This ion could have arisen by the loss of carbons 16 and 17 with both substituents and a hydrogen from the remaining fragment:




*m/e* 241

One unusual feature of all of the mass spectra of the permethylated $E_2$ and $E_3$ glucuronides is the ion at M-2 due to the loss of two hydrogens. This phenomenon is not a normal one for either permethylated glucuronides [9] or permethylated estrogens (Figs. 3b and 4c). The M-2 ion is reduced markedly or absent in spectra taken at an ionizing potential of 16 eV.

From the above discussion it is clear that one could determine intact estrogen glucuronide profiles (except estrone) in body fluids by analysis of the permethyl derivatives by GC and GC–MS. The isothermal GC separation shown in Fig. 5 is sufficient for the quantitative determination of $E_2$-3G1, $E_2$-17G1, and $E_3$-16G1. A mass spectrometric technique must be used to quantify $E_3$-3G1 and $E_3$-17G1 if both are present because they are not well separated by GC but have very different and characteristic mass spectra.

Current studies in this laboratory include the development of isolation methods to obtain the glucur-

onide mixtures for quantitative analysis. Column chromatography, solvent extraction, including continuous extraction, and simple permethylation of the residue obtained from the evaporation of untreated urine (50–100 $\mu$l), as was carried out previously with bile [9], are all being tested. Preliminary results clearly indicate differences in urines from males and females and between pregnant and nonpregnant females. These differences will be determined, quantified, and related to total $E_2$ and $E_3$ values as the methodology becomes established in the future.

*Acknowledgements*—The author is grateful for financial support from the Public Health Service (NIH AM 17665) and for the technical assistance of Mr. Francis E. Wilkinson.

## REFERENCES

1. Dolphin R. J.: *J. Chromatog.* **83** (1973) 421–429.
2. Nugent D. W. and Fottrell P. F.: *Clin. chim. Acta* **46** (1973) 201–203.
3. Adessi G., Eichenberger D., Goutte Ch. and Jayle M. F.: *Clin. chim. Acta* **45** (1973) 369–374.
4. Adessi G., Eichenberger D., Nhuan T. Q. and Jayle M. F.: *Steroids* **25** (1975) 553–564.
5. Adlercreutz H. and Hunneman D. H.: *J. steroid Biochem.* **4** (1973) 233–237.
6. Adlercreutz H., Tikkanen M. J. and Hunneman D. H.: *J. steroid Biochem.* **5** (1974) 211–217.
7. Miklosi S., Biggs J. S. G., Selvage N., Canning J. and Lythal G.: *Steroids* **26** (1975) 671–681.
8. Tikkanen M. J. and Adlercreutz H.: *J. steroid Biochem.* **3** (1972) 807–818.
9. Thompson R. M., Gerber N., Seibert R. A. and Desiderio D. M.: *Drug Metab. Dis.* **1** (1973) 489–505.
10. Thompson R. M. and Desiderio D. M.: *Biochem. biophys Res. Commun.* **48** (1972) 1303–1310.
11. Soares J. R., Zimmermann E. and Gross S. J.: *FEBS Lett.* **61** (1976) 263–266.
12. Adlercreutz H., Lehtinen T. and Tikkanen M.: *J. steroid Biochem.* **7** (1976) 105–107.
13. Leclercq P. A. and Desiderio D. M.: *Analyt. Lett.* **4** (1971) 305–316.
14. Luukkainen T. and Adlercreutz H.: *Ann. med. exp. Fenn.* **45** (1967) 264–268.
15. Leclercq P. A.: *Biomed. Mass. Spectrom.* **1** (1974) 109–114.
16. Miyazaki H., Ishibashi M., Itoh M., Morishita N., Sudo M. and Nambara T.: *Biomed. Mass Spectrom.* **3** (1976) 55–59.