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USE OF SILYLATING AGENTS FOR THE IDENTIFICATION OF HYDROXYLATED STEROIDS BY GAS CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

DISCRIMINATION BETWEEN PHENOLIC AND ALCOHOLIC HYDROXYL GROUPS

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SUMMARY

A phenolic trimethylsilyl (TMS) group was selectively exchanged for a dimethylalkylsilyl (DMAS) group on a gas chromatographic column by use of sandwich injection technique with DMAS-imidazole, and a TMS ether derivative of a phenolic steroid was converted into a DMAS ether or a mixed TMS and DMAS ether derivative with over 95% recovery. The selective exchange reaction seemed to be caused by the difference in lability between the ethereal TMS linkages to phenolic and alcoholic hydroxyl groups. This selectivity was found to be useful for discriminating gas chromatographically between the phenolic and alcoholic hydroxyl groups in steroids.

INTRODUCTION

Gas chromatography–mass spectrometry (GC–MS) has been widely used in the field of drug metabolism because it provides considerable information on the chemical structures of metabolites. Trimethylsilylation is particularly useful for the preparation of thermostable and volatile derivatives of drug metabolites or biochemically important compounds that have polar functional groups.

It is known that the trimethylsilyl (TMS) group substituted at a certain position in the compound to be analyzed is exchanged for the perdeuterotrimethylsilyl (TMS-d_9) or acyl group, producing a mixed TMS and TMS-d_9 or a mixed TMS and acyl derivative[1–7]. The preparation of the mixed TMS and TMS-d_9 derivatives was accomplished by utilizing the higher lability of acidic compared with ethereal TMS groups in the exchange reaction on a gas chromatographic column[4–7].

Hence the lability of the TMS group, observed in the preparation of the mixed

TMS and TMS-d_9 derivatives, appeared to be promising for the discrimination of phenolic and alcoholic hydroxyl groups by GC–MS. This paper deals with the GC identification of the phenolic hydroxyl group in a compound by use of an exchange reaction of the TMS derivative with dimethylalkylsilylating agents, namely dimethylethyl- and dimethyl-*n*-propylsilylimidazoles.

EXPERIMENTAL

Gas chromatography

A Shimadzu GC-5AP gas chromatograph equipped with a flame-ionization detector (FID) was employed. The column was 1.5 m × 3 mm glass tube packed with 1.5% OV-17 (Ohio Valley Co., Marietta, Ohio, U.S.A.) on Gas-Chrom Q, 80–100 mesh (Applied Science Labs., State College, Pa., U.S.A.). The temperature of column oven was maintained at 250° and that of the injection port and detector was 270°. The flow-rate of the carrier gas (nitrogen) was 40 ml/min.

Mass spectrometry

An LKB-9000S GC–MS system equipped with a data processing system was employed. The column was a 1.5 m × 3 mm glass coil packed with 1.5% OV-17 on Gas-Chrom Q. The temperature of column oven was maintained at 210–250°. The flow-rate of the carrier gas (helium) was 30 ml/min. The temperature of the injection port and separator was 270° and the ionization source was maintained at 290°. The accelerating energy and trap current were 70 eV and 60 μA, respectively.

Samples and reagents

Estrone, estradiol (E_2), estriol, estetrol and 2-hydroxyestrone were commercially available. E_2 bis-TMS ether was obtained as follows; E_2 was dissolved in N,O-bistrimethylsilyltrifluoroacetamide (BSTFA), allowed to stand for 1 h at room temperature and the resulting solution was evaporated to dryness *in vacuo* in order to remove BSTFA and its principal reaction by-product. The residue was confirmed to be gas chromatographically pure from the fact that it contained neither E_2 nor E_2 mono-TMS ether.

BSTFA, TMS-imidazole (TSIM) and TMS-d_9-imidazole (TSIM-d_9) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Merck Sharp & Dohme (Tokyo, Japan). DMES-imidazole (DMESI) and DMPS-imidazole (DMPSI) were synthesized in our laboratory by the method described in a previous paper[8].

Derivatization

Silylation was carried out in a microvial. To 0.1–0.2 mg of a steroid sample 100 μl of a silylating agent were added and the mixture was allowed to stand for 30 min at room temperature.

Exchange reaction by a sandwich injection technique was carried out by dissolving 100 μg of each steroid in 20 μl of a silylating agent, allowing the mixture to stand for 30 min at room temperature and then injecting it into the gas chromatograph by a sandwich injection technique with another silylating agent. Successive samples were drawn into a syringe in the following order, for example: 1.0 μl of DMESI, 0.4 μl of E_2–TSIM solution and 1.0 μl of DMESI.

Quantitative exchange reactions were carried out by dissolving 100 μg of each steroid in 20 μl of TSIM or BSTFA. After silylation, the excess of silylating agent was removed by evaporation to dryness *in vacuo*. The residue was then dissolved in dry pyridine and an aliquot of the solution was injected into the gas chromatograph by the sandwich injection technique with DMESI (or DMPSI) containing 5% of imidazole.

RESULTS AND DISCUSSION

Selective labelling with a perdeuterotrimethylsilyl (TMS-d_9) group on a gas chromatograph[7] was applied to E_2 bis-TMS ether. Contrary to our expectations, GC–MS revealed that the formation of a mixed TMS and TMS-d_9 ether derivative took place to a small extent.

It has previously been reported that E_2 bis-TMS ether was converted into a mixed TMS and TMS-d_9 ether with TSIM-d_9 on a gas chromatograph by means of the "sandwich injection" technique[9]. This technique was applied to the E_2–TSIM solution with DMESI instead of TSIM-d_9, as shown in Fig. 1a. The reaction product showed two peaks due to E_2 bis-TMS ether and a new product on the gas chromatogram.

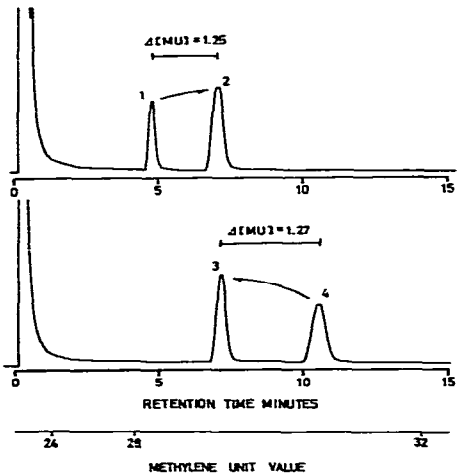

Fig. 1. Gas chromatograms of reaction products from the exchange reaction of estradiol bis-TMS ether with DMESI (above) and estradiol bis-DMES ether with TSIM (below). Peaks: 1 = estradiol bis-TMS ether; 2 = 3-O-DMES-17-O-TMS ether; 3 = 3-O-TMS-17-O-DMES ether; 4 = bis-DMES ether.

The retention time of the new product was greater than that of E_2 bis-TMS ether, the difference in the methylene unit values between the two substances being 1.25. This value was in good agreement with the mean value of $\Delta[MU]_E$ (ref. 10), an increment of the methylene unit value between the TMS and DMES ether derivatives obtained with 18 monohydroxysteroids[8]. Therefore, this difference suggested that the new product was formed by exchange of the TMS group at C-3 or C-17 in E_2 bis-TMS ether with the DMES group.

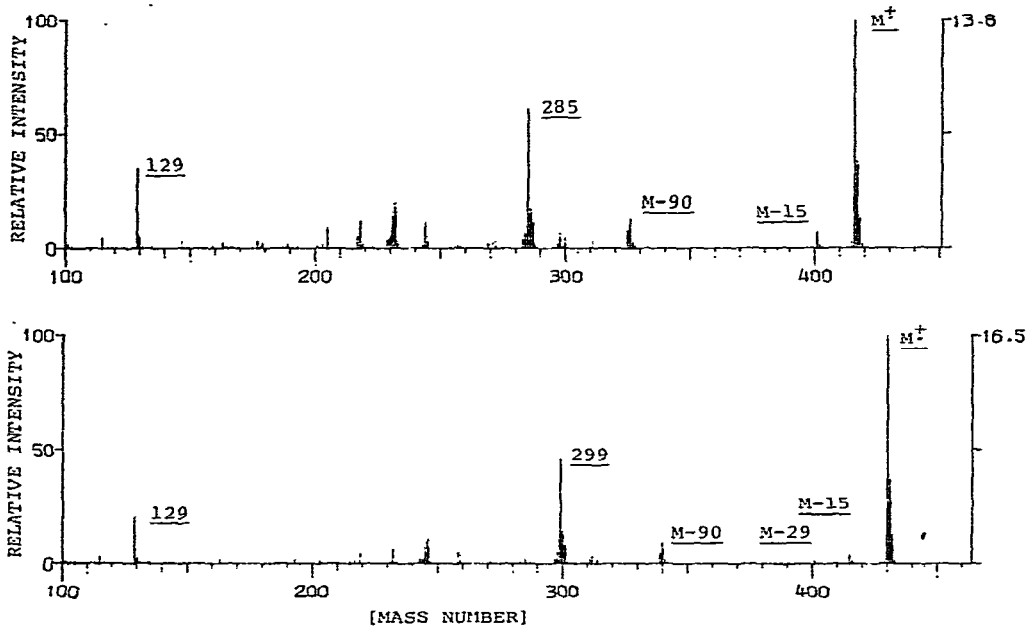

Fig. 2. Mass spectra of estradiol mixed TMS and DMES ether (below) obtained by exchange reaction of bis-TMS ether (above) with DMESI.

Fig. 2 shows the mass spectra of E_2 bis–TMS ether and the new product obtained by the exchange reaction between the TMS and DMES groups and the structures of the fragment ions are shown in Fig. 3. The shift of the molecular ion from *m/e* 416 to 430 indicates that one of TMS groups in E_2 bis-TMS ether was substituted for the DMES group. The characteristic loss of ring D together with hydrogen abstraction and elimination of silanol from the molecular ion[11] exhibited no mass shift in the mass spectrum of the new product; the presence of the fragment ion of *m/e* 129 (I) indicates that the TMS group at C-17 in the new product was not exchanged with DMESI. The shift of 14 mass units for the fragment ion containing ring A (IV)[12] from *m/e* 285 to 299 suggests that the TMS group at C-3 in the new product was exchanged for the DMES group. The occurrence of these fragment ions verifies that the phenolic TMS group in E_2 bis-TMS ether was exchanged selectively for the DMES group while the alcoholic TMS group at C-17 remained unchanged (Fig. 4).

(I) R_1=TMS *m/e* 129 (III) R_2=DMES *m/e* 299

(II) R_1=DMES *m/e* 143 (IV) R_2=TMS *m/e* 285

Fig. 3. Structures of fragment ions.

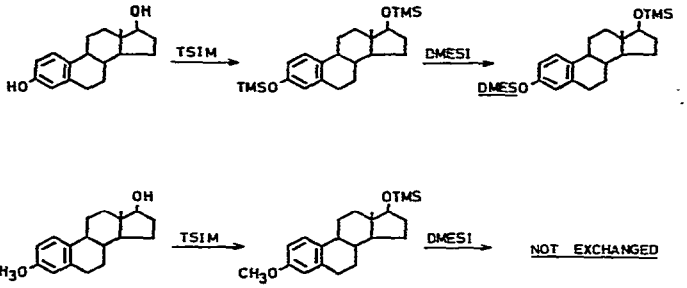

Fig. 4. Exchange reactions of E_2 and E_2 3-methyl ether.

Consequently, the new product was identified as E_2 3-O-DMES-17-O-TMS ether. The selectivity of this exchange reaction was further supported by the fact that E_2 3-O-methyl-17-O-TMS ether did not undergo any alteration in this reaction.

E_2 3-O-TMS-17-O-DMES ether could be obtained from E_2 bis-DMES ether by the exchange reaction with TSIM, as shown in Fig. 1b, the difference in the methylene unit values between these two derivatives being 1.27. Fig. 5 shows the mass spectra of E_2 3-O-TMS-17-O-DMES ether and E_2 bis-DMES ether. The structure of the product was confirmed by the presence of a molecular ion at *m/e* 430 and fragment ions at *m/e* 143 (II) and 285 (IV).

This selective exchange reaction with DMESI was applied to TMS ether derivatives of some typical estrogens. GC–MS also revealed that the phenolic TMS group was selectively replaced by the DMES group to provide the new derivatives by this reaction.

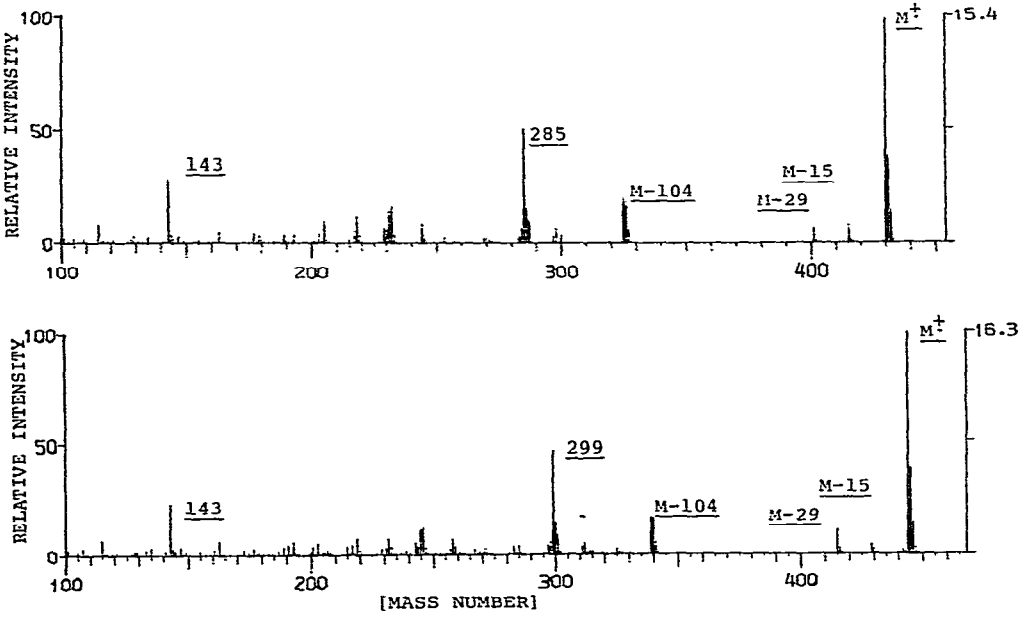

Fig. 5. Mass spectra of estradiol mixed TMS and DMES ether (above) obtained by exchange reaction of bis-DMES ether (below) with TSIM.

The formation of the mixed TMS and DMES ether derivatives seemed to be dependent on the ratio of the amount of TSIM to that of DMESI. The pure E_2 bis-TMS ether was dissolved in the mixture of TSIM and DMESI in various proportions. The exchange rate showed a tendency to increase in proportion to the increased percentage of DMESI. Pure E_2 bis-TMS ether was then dissolved in dry pyridine and injected into the gas chromatograph by the sandwich injection technique with DMESI.

The mixed TMS and DMES ether was formed in a yield of more than 85%, but it was difficult to obtain it quantitatively with satisfactory reproducibility. From a series of experiments it was found that this exchange reaction is catalyzed by a small amount of imidazole derived from the silylating agent. This catalytic effect was examined by use of DMESI containing imidazole in various proportions. Addition of imidazole to DMESI at a level of 5–10% afforded the mixed TMS and DMES ether in a yield of more than 95%.

When the pure E_2 bis-TMS ether was dissolved directly in DMESI, the mixed TMS and DMES ether was accompanied by the bis-DMES ether and the proportion of this undesirable by-product increased with increase in the ratio of imidazole to DMESI. In the sandwich injection technique, however, no by-product was formed even if DMESI containing imidazole was used. Thus, only this technique proved to be effective for selective labelling for the identification and determination of the phenolic hydroxyl group in the TMS ether derivative. Fig. 6 shows a gas chromatogram of the reaction product obtained from E_2 bis-TMS ether by this procedure.

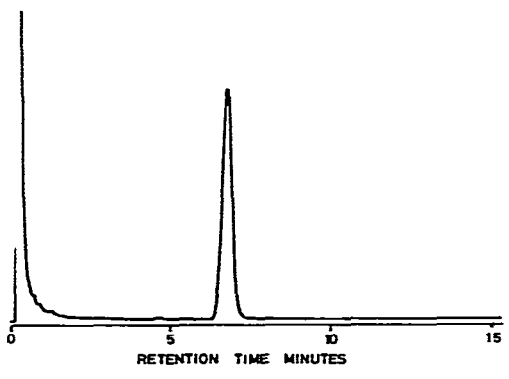

Fig. 6. Gas chromatogram of estradiol mixed TMS and DMES ether obtained by quantitative exchange reaction of estradiol bis-TMS ether.

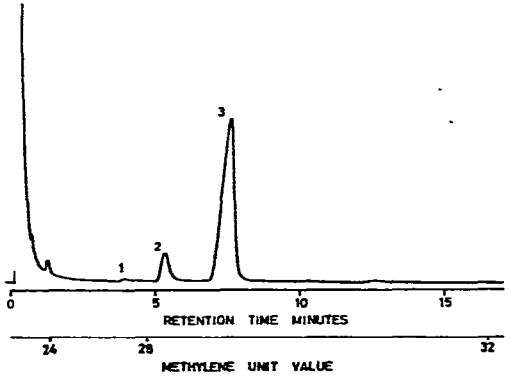

Fig. 7. Gas chromatogram of the reaction products from the exchange reaction of 2-hydroxyestrone bis-TMS ether with DMESI. Peaks: 1 = 2-hydroxyestrone bis-TMS ether; 2 = mixed TMS and DMES ether; 3 = bis-DMES ether.

Table I lists the Δ[MU] values between various persilylated derivatives of each estrogen and their products formed by the exchange reaction. These values were closely related to the mean of $\Delta[MU]_E$ and $\Delta[MU]_P$ obtained with 18 monohydroxysteroids[8]. Further, when the mass spectra of the persilylated derivative and the new product were compared, the molecular ion peak of the latter was shifted 14 or 28

TABLE I

DIFFERENCE IN METHYLENE UNIT VALUES (Δ[MU]) OBTAINED BY EXCHANGE REACTION

Silylated derivative	*Δ[MU]*		
	TSIM	*DMESI*	*DMPSI*
Estrone-TMS	—	1.24	2.02
Estradiol-TMS	—	1.25	2.07
Estriol-TMS	—	1.29	2.02
Estetrol-TMS	—	1.23	1.96
Estrone-DMES	1.17	—	0.81
Estradiol-DMES	1.27	—	0.84
Estriol-DMES	1.21	—	0.79
Estetrol-DMES	1.17	—	0.72
Estrone-DMPS	1.92	0.73	—
Estradiol-DMPS	1.96	0.73	—
Estriol-DMPS	2.03	0.76	—
Estetrol-DMPS	1.93	0.72	—

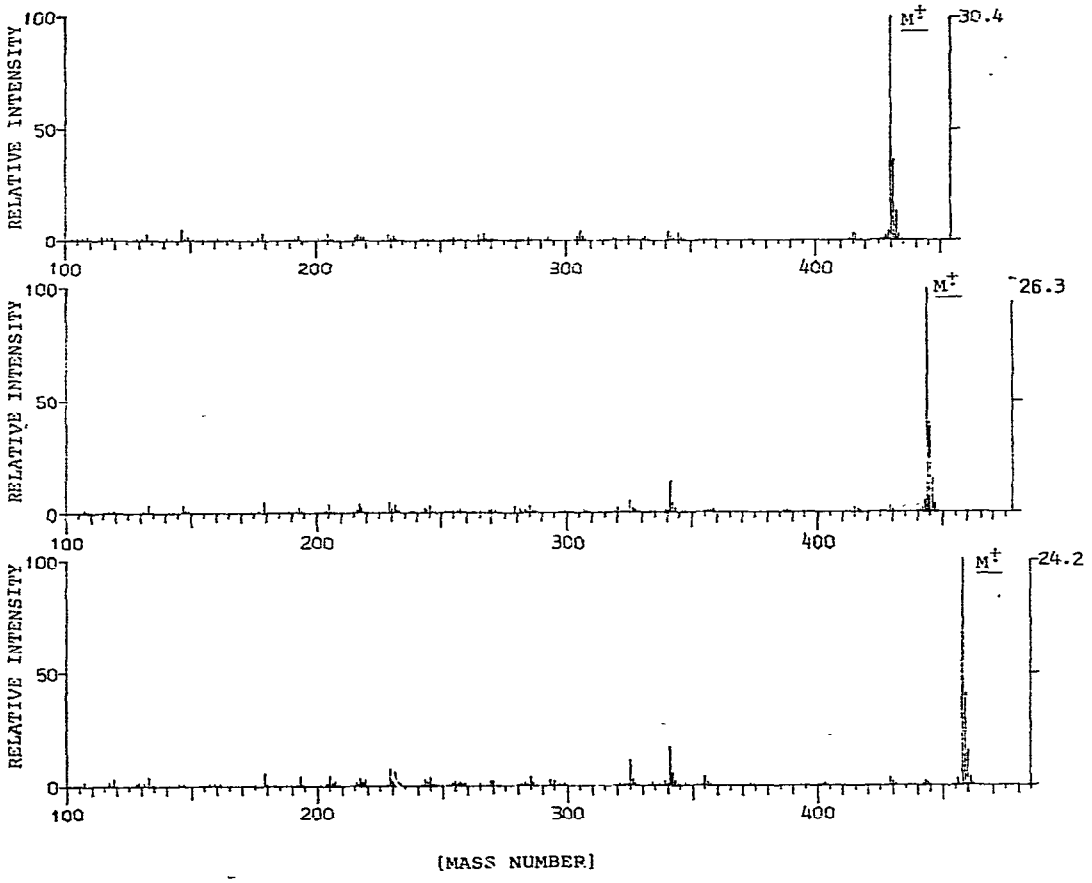

Fig. 8. Mass spectra of 2-hydroxyestrone bis-TMS (above), mixed TMS and DMES ether (centre) and bis-DMES ether (below).

mass units, equivalent to one or two methylene groups. These results lent support to the conclusion that the phenolic group in persilylated steroids is selectively displaced by another silyl group in this exchange reaction.

2-Hydroxyestrone (2-OHE_1) was also submitted to the exchange reaction. A solution of 2-OHE_1 dissolved in TSIM was injected on to the GC column together with DMESI. As shown in Fig. 7, the reaction product exhibited two peaks, the retention times of which were greater than that of 2-OHE_1 bis-TMS ether, the differences in the methylene unit values between the TMS ether and the new product being 1.25 and 2.25, respectively. These values suggest that peaks 2 and 3 in Fig. 7 correspond to the mixed TMS and DMES ether and bis-DMES ether derivatives of 2-OHE_1, respectively. This assignment was supported by the GC–MS data, which indicated that the molecular ions of peaks 2 and 3 were shifted from *m/e* 430 to 444 and 458, as shown in Fig. 8. However, which of two TMS ethers was replaced by the DMES group still remains unclear.

The bis-DMES ether derivative of 2-OHE_1 corresponding to the latter peak in Fig. 7 was formed quantitatively by the procedure described under Experimental. These results indicate that the exchange reaction is also applicable to polyhydroxylated steroids containing two or more phenolic hydroxyl groups.

In conclusion, the utilization of this selective exchange reaction permits one to determine by GC and GC–MS the number of phenolic hydroxyl groups in hydroxysteroids from the difference in the methylene unit values between the persilyl ether and the mixed silyl ether derivative.

APPENDIX

Although Δ[MU] has been used as a novel index in ref. 10 and in this paper, the authors would like to adopt Δ[Um][8] instead of Δ[MU] in order to discriminate Δ[Um] from Δ[MU] calculated by the conventional methylene unit value.

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