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APPLICATION OF AN ION-CLUSTER TECHNIQUE TO THE ANALYSIS OF ENDOGENOUS HYDROXYLATED COMPOUNDS

I. PREPARATION OF A DOUBLET BY USE OF A MIXTURE OF TRIMETHYLSILYLIMIDAZOLE (TSIM) AND TSIM-d_3

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SUMMARY

In order to apply the intensity-matching technique to polyhydroxysteroids in biological fluids, the doublet of $[M]^+$ and $[M+3]^+$ was prepared in the process of derivatization by use of a mixture of trimethylsilylimidazole (TSIM) and TSIM-d_3 and was used for mass fragmentography. The molar ratio of TSIM and TSIM-d_3 was defined by the reciprocal of the number of hydroxyl groups in steroids. This technique may be useful in identifying low levels of the endogenous polyhydroxysteroids of interest in biological fluids.

INTRODUCTION

Mass fragmentography gives more reliable results than gas chromatography (GC), in the qualitative and quantitative analyses of organic compounds because the identification of the compounds of interest is based on the utilization of information on their characteristic fragment ions and their relative intensities in combination with their GC retention times. Even if this highly specific technique is used, however, it cannot be ensured that the peak of the compound of interest in the mass fragmentogram has no unexpected contaminants that have the same GC and mass spectrometric characteristics, because a great many complex endogenous substances exist in biological fluids.

To overcome this problem, Knapp *et al.*[1] developed the so-called artificially created ion-cluster technique, which is based on monitoring the doublet produced from an equimolar mixture of stable isotope-labelled and unlabelled compounds. However, it is difficult to apply this technique to the detection of the endogenous substances in biological samples because a doublet of constant intensity cannot be maintained owing to its dilution *in vivo*.

Chapmann and Bailey[2] utilized the molecular ion cluster derived from the bromomethyldimethylsilyl ether derivative to determine testosterone levels in healthy

human plasma. However, it may be difficult to apply this derivatization to the identification of polyhydroxysteroids because the retention time of the derivative may be markedly prolonged as the number of hydroxyl groups in the molecule increases. In addition, it may be not so easy to introduce this bulky silyl group in the reagent to the highly hindered hydroxyl group in steroids.

In a previous paper[3], the artificially created ion-cluster technique was applied to the micro-determination of bile acids in rat serum. In this technique, the doublet was prepared with a mixture of deuterium-labelled and unlabelled methanol (1:1). Dimethyltrideuteromethylsilylimidazole (TSIM-d_3) was synthesized in order to be able to prepare easily variants containing deuterium atoms in the derivatization process. This paper describes the application of the artificially created ion-cluster technique to endogenous polyhydroxysteroids by use of the above mixture with a definite ratio of TSIM and TSIM-d_3.

EXPERIMENTAL

Gas chromatography–mass spectrometry (GC–MS)

A Shimadzu LKB 9000 GC–MS system equipped with a data processing system and a multiple-ion detector (Shimadzu LKB 9066) was employed. The column was 1.5 m × 2.5 mm glass coil containing 1.5% OV-101 on Gas-Chrom Q. The temperature of the 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°, the ionization source was kept at 290°, the accelerating voltage was 3.5 kV and the ionization energy and trap current were 70 eV and 60 μA, respectively.

Samples and reagents

The steroids used were commercially available. Trimethylsilylimidazole (TSIM) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and TSIM-d_9 from Merck Sharp and Dohme (Japan), (Tokyo, Japan). Dimethyltrideuteromethylsilylimidazole (TSIM-d_3) was synthesized as follows. Dimethyltrideuteromethylchlorosilane was prepared from dimethyldichlorosilane and trideuteromethylmagnesium bromide by the method of Shostakovskii *et al.*[4]. Dimethyltrideuteromethylchlorosilane was converted into the corresponding disilazane by treatment with ammonia according to the method for preparing hexamethyldisilazane[5]. This silazane was then treated directly with imidazole, affording TSIM-d_3 (b.p. 91° at 12 mm Hg).

Derivatizations

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

RESULTS AND DISCUSSION

An equimolar mixture of TSIM and TSIM-d_9 was applied to a monohydroxysteroid for the preparation of an artificially created ion cluster. Fig. 1 shows the mass spectrum of the TMS and TMS-d_9 ether derivatives of 5α-cholestan-3β-ol (I). The molecular ion was accompanied by the ion of *m/e* 469 which was shifted 9 mass units from that of the corresponding TMS ether derivative (*m/e* 460).

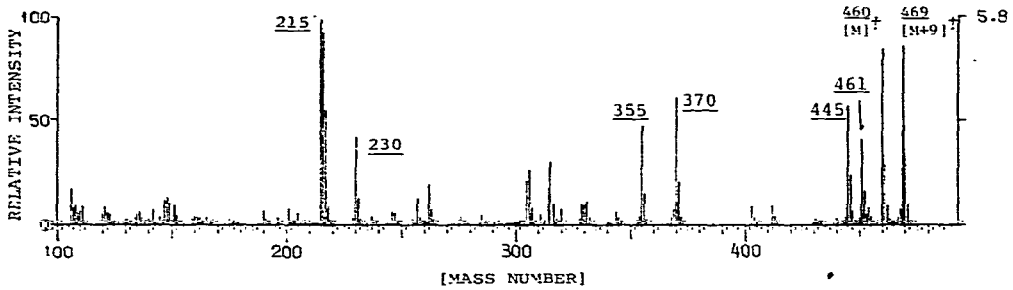


Fig. 1. Mass spectrum of TMS and TMS-d_9 ether derivatives of 5α-cholestan-3β-ol.

The molecular ions of *m/e* 460 and 469 were chosen for mass chromatography. As shown in Fig. 2, almost identical peak heights and peak shapes were obtained by monitoring two fragment ions although the peak of the TMS-d_9 ether was eluted faster than that of the corresponding TMS ether. This result suggests that there is no isotope effect in the silylation process.

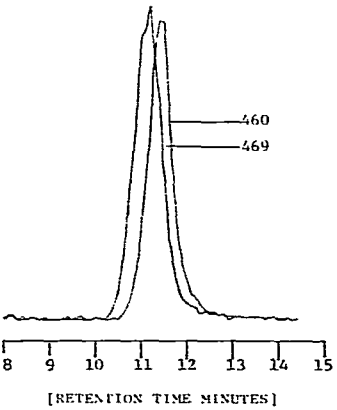


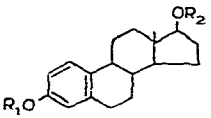
Fig. 2. Chromatogram of TMS and TMS-d_9 ether derivatives of 5α-cholestan-3β-ol monitored at molecular ions of *m/e* 460 and 469.

As described previously[3], the intensity-matching technique in which the doublet created by derivatization is utilized may be useful for monitoring the purity of the peaks of interest. The above mixture was therefore also used for the identification of estradiol (II) as a model of the dihydroxysteroids. The reaction product from II theoretically should consist of an equimolar mixture of four variants: bis-TMS ether (1), 3-TMS-17-TMS-d_9 ether (2), 3-TMS-d_9-17-TMS ether (3) and bis-TMS-d_9 ether (4), the structures of which are summarized in Table I. However, it was very difficult to separate these variants completely by GC.

On the other hand, when this reaction product is measured in a direct inlet system, the ion intensities of the molecular ions of these variants at *m/e* 416, 425 and 434 should be observed in the ratio 1:2:1 because two variants of compounds 2 and 3 have the same molecular ion at *m/e* 425. However, the expected ratio was not ob-

TABLE I

STRUCTURES OF VARIANTS OF ESTRADIOL SILYL ETHERS



Variable	*Compound*							
	E_2	*1*	*2*	*3*	*4*	*5*	*6*	*7*
R_1	H	TMS	TMS	TMS-d_9	TMS-d_9	TMS	TMS-d_3	TMS-d_3
R_2	H	TMS	TMS-d_9	TMS	TMS-d_9	TMS-d_3	TMS	TMS-d_3
Mol.wt.	272	416	425	425	434	419	419	422

tained, indicating that the molecular ion of 1 just coincides with the fragment ion of *m/e* 416 which is produced by the elimination of a trideuteromethyl (CD_3) group from the molecular ion of 4, so that the ion intensity at *m/e* 416 appears to be stronger than that given by the expected ratio.

As all TMS-d_9 ethers always give this fragment of $[M-CD_3]^+$, the use of the molecular ion cluster created with an equimolar mixture of TSIM and TSIM-d_9 should therefore be limited to monohydroxysteroids. In order to apply this technique to polyhydroxysteroids, it is necessary to develop a new silylating agent that not only removes the interference from the $[M-CD_3]^+$ ion in the TMS-d_9 ethers but also minimizes the isotope effect on the retention times of the deuterated variants in mass fragmentography.

TSIM-d_3 was synthesized as the most suitable reagent for this purpose and then an equimolar mixture of TSIM and TSIM-d_3 was used for the identification of polyhydroxysteroids by means of the molecular ion-cluster technique with a definite intensity. Fig. 3 shows the mass spectrum of the reaction product from II obtained with this reagent mixture. It was found that the molecular ion cluster was very similar to that of bromine-containing compounds except for a shift of 3 mass units, and the ion intensities in the molecular ion cluster of M^+, $[M+3]^+$ and $[M+6]^+$ were maintained at the expected ratio of 1:2:1 without interference from the $[M-CD_3]^+$ ion derived from the TMS-d_9 ethers.

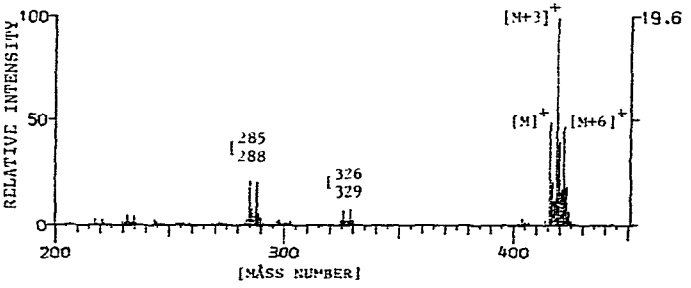


Fig. 3. Mass spectrum of reaction product from estradiol and an equimolar mixture of TSIM and TSIM-d_3.

If the equimolar mixture of TSIM and TSIM-d_3 is used for derivatization of polyhydroxysteroids, the ion intensities of the molecular ion cluster should be calculated theoretically from the inherent coefficients of each term in the following equation:

$$(X+Y)^n = X^n + n\,X^{n-1}\,Y + \ldots + Y^n \qquad (1)$$

where n is the number of hydroxyl groups in the compounds of interest and X^n, $X^{n-r}Y^r$ and Y^n are the molecular ions of the per-TMS, the mixed TMS and TMS-d_3 and the per-TMS-d_3 ethers, respectively. Eqn. 1 indicates that the number of hydroxyl groups in steroids can be estimated by measuring the ratios of the inherent ion intensities in the molecular ion cluster created by use of this reagent mixture.

The GC behaviour of TMS, TMS-d_3 and TMS-d_9 ether derivatives of hydroxysteroids was investigated. The retention times of these derivatives increased in the order TMS-d_9, TMS-d_3 and TMS ethers and corresponded well with the number of deuterium atoms in the molecule. This result can be explained in terms of the physical isotope effect on the affinity of these deuterium species towards the column materials.

As mentioned above, the silylation product of II with the mixture of TSIM and TSIM-d_3 consists of four variants. Consequently, the sum of the molecular ion intensities of the mixed TMS and TMS-d_3 ethers is represented by an ion intensity of M+3 in the mass spectrum. In order to illustrate the doublet peaks with identical peak heights and peak shapes in the mass fragmentogram by use of M and M+3 ions, therefore, it is essential that there should be no isotope effect on the GC column between the above two variants.

The reaction product of II with TSIM and TSIM-d_3 (1:1) was prepared and the isotope effect on the mixed TMS and TMS-d_3 ethers was measured by mass fragmentography.

As the ratio of the ion intensities of the molecular ions of these variants is expected to be 1:2:1 for *m/e* 416, 419 and 422, the intensity-matching technique[6] described previously[7] was applied to the preparation of a doublet with equal intensities; the reciprocals of the theoretical ratios of the ion intensities between the above two ions were used as the coefficients in this technique. Fig. 4 shows the chromatogram

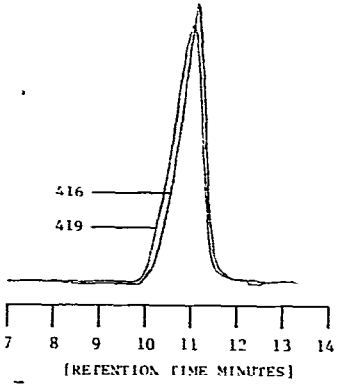


Fig. 4. Chromatogram of bis-TMS ether and mixed TMS and TMS-d_3 ether derivatives of estradiol with an equimolar mixture of TSIM and TSIM-d_3 monitored at molecular ions of *m/e* 416 and 419.

of the bis-TMS ether and the mixed TMS and TMS-d_3 ether derivatives of II monitored at the molecular ions of *m/e* 416 and 419. The peak height of the mixed TMS and TMS-d_3 ethers was slightly lower than that of the bis-TMS ether and the retention times of these peaks were almost identical. This result may indicate indirectly that there is little difference in the retention times between the two variants of compounds 5 and 6.

Fortunately, when the retention times of these variants were reduced by half, the peak shapes and the peak heights agreed well. It was also found that as the M+3 variants in the silyl ether derivatives of II, estriol (III) and estetrol (IV) had little physical isotope effect on deuterium atoms, they did not adversely affect the preparation of the doublet in this technique. It is also noteworthy that there was no isotope effect on the derivatization process used to prepare the doublet.

In mass fragmentography, on the other hand, if the doublet containing ions of identical intensity is selected as the monitoring ions, the artificially created ion-cluster technique can be applied easily without modification, as reported by Knapp *et al.*[1]. However, the utilization of a doublet has the important disadvantage that its detection limit would be decreased because the ion currents of all molecular ion clusters of the mixed TMS and TMS-d_3 ethers, except for the doublet of equal intensities, were neglected and so the losses would be markedly increased as the number of hydroxyl groups in the molecule increased, as shown in Table II. The use of M+3 together with M ions in the intensity-matching technique[6] may also be inadvisable because the loss of the ion currents of all molecular ion clusters is increased with increasing number of hydroxyl groups in the molecule, as in the technique of Knapp *et al.*[1].

TABLE II

COMPARISON OF ION INTENSITIES IN MOLECULAR ION CLUSTER CALCULATED FROM EQN. 1 AND FOUND IN MASS SPECTRA

Number of hydroxyl groups	*Model compound*	*Ratio of TSIM and TSIM-d_3*	*Ion intensity*							
			Calculated				*Found*			
			M	*M+3*	*M+6*	*M+9*	*M*	*M+3*	*M+6*	*M+9*
1	Estrone	1:1	1.00	1.00			1.00	0.96		
2	Estradiol	1:1	1.00	2.00	1.00		1.00	2.02	0.93	
		2:1	1.00	1.00	0.25		1.00	0.97	0.24	
3	Estriol	1:1	1.00	3.00	3.00	1.00	1.00	2.92	2.66	0.90
		3:1	1.00	1.00	0.33	0.04	1.00	1.01	0.37	0.03

Fortunately, it is possible to change the pattern of the molecular ion cluster by varying the molar ratios between TSIM and TSIM-d_3. Thus, if the ratio of mixing of TSIM and TSIM-d_3 could be made to agree with the reciprocal of the number of hydroxyl groups in the steroid, the M and M+3 doublet in the molecular ion cluster should have the most abundant and equal intensities, as shown in Table II. The mass spectrum of the silylated product from II with TSIM and TSIM-d_3 (2:1) is shown in Fig. 5. A doublet with equal intensities was observed at *m/e* 416 and 419. As the ion intensity of *m/e* 422 (the molecular ion of the bis-TMS-d_3

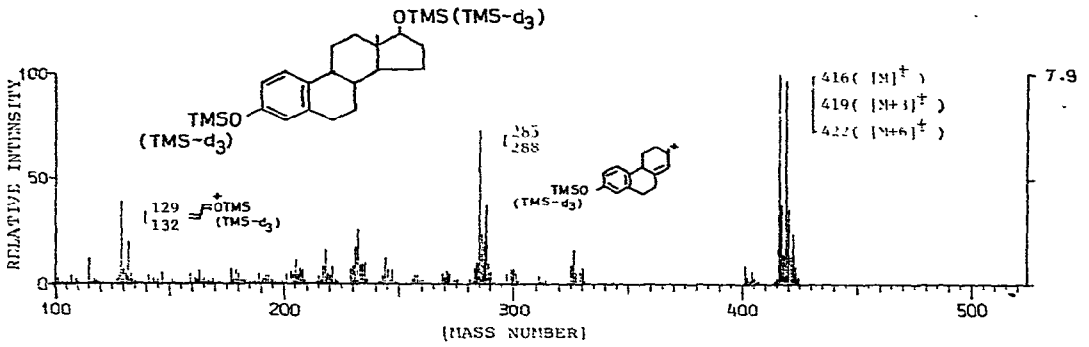


Fig. 5. Mass spectrum of reaction product from estradiol and a mixture of TSIM and TSIM-d_3 (2:1).

ether) was reduced to exactly one quarter of that of the bis-TMS ether (*m/e* 416), the ion current of the doublet should be enhanced by utilizing this mixture. The fragment ions containing the TMS group[8,9] in these derivatives were accompanied by new ions of *m/e* 132 and 288, which were shifted 3 mass units from the corresponding TMS ether derivatives (*m/e* 129 and 285). The ratios of the ion intensities of these doublets were in agreement with those of TSIM and TSIM-d_3, which indicates that these ions contain one TMS group. As shown in Table II, the ratios of the ion intensities in the molecular ion cluster agreed well with the coefficients of each term in the expansion of eqn. 1.

Consequently, the most favourable doublet with equal intensities was obtained easily by mixing TSIM and TSIM-d_3 in a molar ratio identical with the reciprocals listed in Table II according to the number of hydroxyl groups present. This technique is similar to the utilization of the coefficients in the intensity-matching technique described in previously[7]. In this work, the doublet provided for the intensity-matching technique was then used for the molecular ions of the per-TMS and the mixed TMS and TMS-d_3 ethers (M and M+3).

Fig. 6 shows the mass fragmentogram obtained by silylating III with the mixture of TSIM and TSIM-d_3 (3:1) and then by monitoring simultaneously the ions

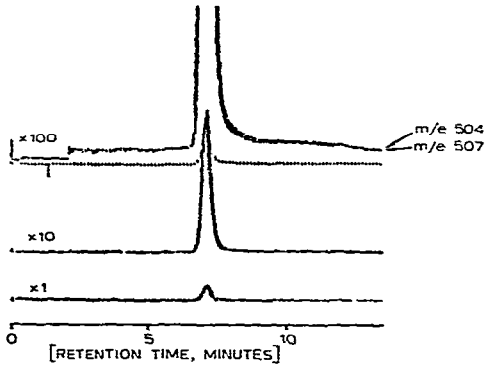


Fig. 6. Mass fragmentogram of reaction product from estradiol and a mixture of TSIM and TSIM-d_3 (3:1) monitored at molecular ions of tris-TMS ether (*m/e* 504) and bis-TMS-mono-TMS-d_3 ether (*m/e* 507) derivatives.

of M and M+3 as in the artificially created ion-cluster technique[1]. Peaks of two ions with almost identical peak heights and peak shapes were recorded. By the observation of a "single peak" created from the mixing ratio of TSIM and TSIM-d_3, it can easily be established whether the peaks on the mass fragmentogram originated from a trihydroxysteroid or not. In addition, the presence and distribution of contaminants in the peaks can be monitored by comparing both the peak height and peak shape. This technique enables accurate analytical results to be presented without overestimation when this mass fragmentographic technique is used for the quantitative analysis of polyhydroxysteroids, especially in extracts from biological fluids. The doublet can be prepared by use of a mixture of TSIM and TSIM-d_3 for steroids with four or less hydroxyl groups, and the technique may be accurate enough for practical applications, as shown in Fig. 6.

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