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MASS-SPECTROMETRY OF STEROID GLUCURONIDES*

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SUMMARY

By using N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) or N-methyl-N-trimethylsilyl heptafluorobutyramide (MSHFBA) in the presence of 5% trimethylchlorosilane (TMCS) as catalyst, the trimethylsilyl ether esters of the following steroid glucuronides (G) were formed: oestrone 3-G, oestradiol-17β 3-G, oestriol 3-G, 2-hydroxyoestradiol-17β 2-G, 2-methoxyoestradiol-17β 3-G, 2-hydroxyoestradiol-17β 3-methyl ether 2-G, oestradiol-17β 17-G, oestriol 16α-G, 17-epi-oestriol 16α-G, oestriol 17β-G, testosterone 17-G, dehydroepiandrosterone 3-G, and pregnanediol 3-G. These derivatives proved to be most suitable for direct mass spectrometry. All steroid glucuronide derivatives investigated exhibited characteristic mass spectra. The spectra showed molecular ion peaks with different intensities. Relatively high intensities were observed, when an aliphatic hydroxyl group was substituted by the glucuronyl residue. Lower intensities were found in steroid glucuronides, in which an aromatic hydroxyl group was conjugated.

The main fragment of the trimethylsilylated glucuronic acid moiety of "aliphatic" glucuronides was *m/e* 465 and that of "aromatic" glucuronides *m/e* 464. Furthermore, "aromatic" glucuronides yielded a fragment of M-392 which represents the aglycon moiety and one additional TMS group from glucuronic acid. In contrast, "aliphatic" glucuronides showed a fragment of M-481, due to the loss of the glucuronic acid moiety together with the glycosidic oxygen. The fragment M-322 which results from the loss of a part of the glucuronic acid moiety was found only in "aliphatic" glucuronides.

INTRODUCTION

Mass spectrometry (MS) in combination with gas–liquid chromatography has been increasingly used during recent years for the specific determination of steroids in body fluids. So far, only free steroids (or suitable derivatives) were investigated by MS, whereas steroid conjugates had first to be hydrolysed by acid or enzymes before the aglycon could be subjected to MS. The last procedure excluded the possibility of assigning the exact structure of the original steroid conjugate.

To overcome this difficulty, it was decided to develop a technique by which steroid glucuronides as such could be investigated directly by MS, following derivative formation with suitable reagents. In the present paper, a method will be described which allows the preparation and the subsequent MS of derivatives of C_{18}-, C_{19}- and C_{21}-steroid glucuronides. Furthermore, details will be given about the discrimination between "aliphatic" and "aromatic" glucuronides of the C_{18}-steroids.

EXPERIMENTAL

Steroids. The following steroid glucuronides (G) were synthesized: oestrone 3-G, oestradiol-17β 3-G, oestriol 3-G, oestradiol-17β 17-G, oestriol 16α-G, 17-epi-oestriol 16α-G, oestriol-17β-G, 2-hydroxyoestradiol-17β 2-G, 2-methoxyoestradiol-17β 3-G and 2-hydroxyoestradiol-17β 3-methyl ether 2-G; for details see [2, 3]. Testosteron 17β-G, dehydroepiandrosterone 3-G and pregnanediol 3-G were obtained from Ikapharm, Ramat-Gan, Israel. The purity of glucuronides was checked by thin layer and gas–liquid chromatography.

Chemicals. All solvents (A.R. grade when available) were obtained from Merck (Darmstadt F.R. Germany) and distilled before use. N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA), N-methyl-N-trimethylsilyl heptafluorobutyramide (MSHFBA) and trimethylchlorosilane (TMCS) were purchased from Macharey & Nagel (Düren, F.R. Germany).

Formation of derivatives. Trimethylsilyl (TMS) ether esters of the steroid glucuronides were prepared by dissolving the steroid containing sample (100–200 μg) in 95 μl of MSTFA or MSHFBA and 5 μl TMCS and by heating the mixture for 2 h at 80°. The mixture was allowed to cool down at room temperature and stored over a desiccant at −18° until further use.

Mass spectrometry. The instrumentation consisted of the combined gas chromatograph–mass spectrometer LKB 9000 (LKB-produkter, Bromma, Sweden). The samples, containing the glucuronide derivatives dissolved in the silylyting reagent, were evaporated to dryness. The residues were introduced *via* the direct inlet system into the mass spectrometer and subsequently evaporated into the ion source at a temperature of 80–120°. Experimental conditions:

* Part of this investigation has been presented at the 1st European Congress of Clinical Chemistry, München, April 1974 [1].

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Table 1. Molecular weights (M), relative intensities of the molecular ions (relative intensities %) and the number of trimethylsilyl groups per molecule of steroid glucuronides. Ionisation energy was 30 eV; for further details, see Experimental. G = glucuronide

Steroid glucuronide	M	Relative intensity %	TMS
Oestrone 3-G	734	0.1	4
Oestradiol-17β 3-G	808	0.04	5
Oestriol 3-G	896	0.03	6
2-Hydroxyoestradiol-17β 2-G	896	0.3	6
2-Methoxyoestradiol-17β 3-G	838	0.1	5
2-Hydroxyoestradiol-17β 3-methyl ether 2-G	838	0.35	5
Oestradiol-17β 17-G	808	5	5
Oestriol 16α-G	896	6	6
Oestriol 17β-G	896	4	6
17-Epioestriol 16α-G	896	3	6
Testosterone 17-G	826	2	5
Dehydroepiandrosterone 3-G	752	0.1	4
Pregnanediol 3-G	856	0.03	5

temperature of the ion source 270°, trap current 60 μAmp., ionisation energy 30 eV, accelerating voltage 3500 V, slits 0.10–0.14 mm, scan speed *m/e* 70–1000 in 10–60 s, filter 20 or 60 s^{-1}, resolution ($R_{10\%\ \text{valley}}$) 1000–1200.

RESULTS AND DISCUSSION

The trimethylsilyl ether esters of steroid glucuronides are compounds of high thermal stability; due to their high volatility, they evaporate in the mass spectrometer between 80 and 120° without decomposition. These properties make the TMS-ether esters of the steroid glucuronides particularly suitable for mass spectrometry. Gas chromatographic investigation revealed that the formation of the TMS ether esters is quantitative [4] which facilitates the interpretation of the mass spectra obtained. Whereas the mass spectra of carbohydrate derivatives rarely disclose pronounced molecular ions, the steroid glucuronide derivatives studied here showed distinct peaks of the molecular ions.

The molecular weights, the relative intensities of the molecular ions and the number of the trimethylsilyl groups per molecule of the various steroid glucuronides are summarised in Table 1. High relative intensities of the molecular ions were observed, when the glucuronic acid was attached by a glycosidic bond to the aliphatic ring D. In contrast, low relative intensities, ranging from 0.03 to 0.35, were found in those glucuronides, in which the glucuronic acid was bound to ring A of the steroid molecule. It should be noted, however, that the differences in the relative intensities appear not to depend on the nature of the binding hydroxyl group but on the position in the steroid molecule (ring A vs ring D); this observation is supported by the low relative intensities of the glucuronides of dehydroepiandrosterone and pregnanediol.

In principle, three pathways of fragmentation may be considered: (1) Fragmentation of the steroid glucuronide with the glycosidic linkage intact, (2) fragmentation of the aglycon and (3) fragmentation of the glucuronic acid moiety.

The characteristic fragmentation of other trimethylsilylated hydroxy compounds is also observed in steroid glucuronides. Thus, fragment ions M^+-15 (loss of a methyl group), M^+-90 (loss of trimethylsilanol), M^+-(15 + 90) and M^+-(90 + 90) are formed. Fragment ions of M^+-249 and M^+-322 arise by partial cleavage of the glucuronic acid moiety. The possible structures of the two latter fragments are shown in Fig. 1. Whereas M^+-249 is not found regularly, M^+-322 is observed with relative high intensity (14–44%) in all aliphatic glucuronides. In contrast, M^+-322 does not occur in aromatic glucuronides.

Fragmentation of the aglycon requires first the complete cleavage of the glucuronic acid moiety. The kind of cleavage depends on the nature of the binding oxygen. If the glucuronic acid is bound to the oxygen of a phenolic hydroxyl group, a fragment ion of M^+-392 is obtained. The formation of this ion may be explained by a rearrangement process: The glucuronic acid is split off from the molecular ion and replaced by a TMS group, which may be transferred from the glucuronic acid TMS ether ester moiety. If, however, the glucuronic acid is bound to the oxygen of an alcoholic hydroxyl group, cleavage occurs also between the aglycon and the glycosidic oxygen, but

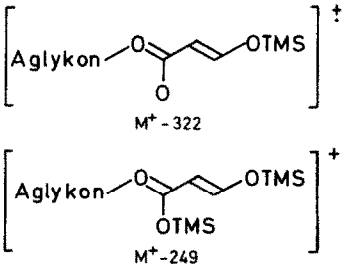

Fig. 1. Possible structures of fragments M^+-249 and M^+-322. TMS = trimethyl silyl group.

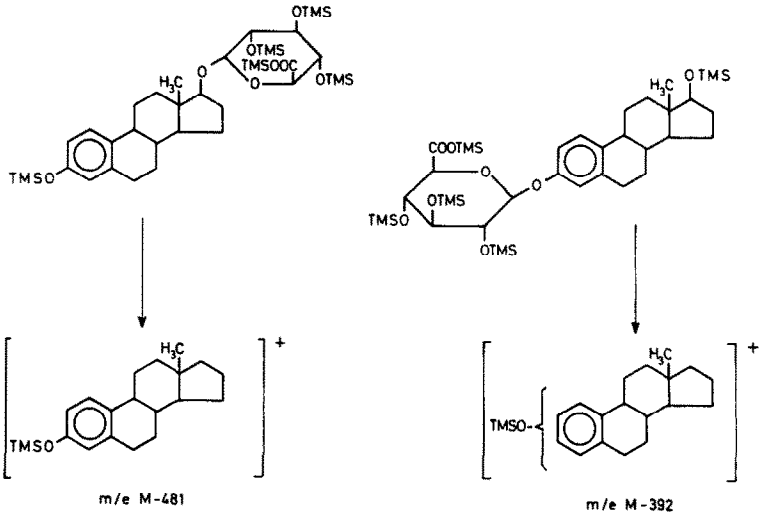

Fig. 2. Fragmentation pattern of oestradiol-17β 17-glucuronide and oestradiol-17β 3-glucuronide. TMS = trimethylsilyl group.

without rearrangement of a silyloxy group, thus leading to fragment ions M^+-481 which represent the steroid moiety without the binding oxygen (Fig. 2).

Billets *et al.*[5] explain the formation of the fragment ion M^+-392 by cleavage of the binding between the glycosidic oxygen and glucuronic acid with simultaneous rearrangement of a trimethylsilyl group from the glucuronic acid to the binding oxygen, as shown in Fig. 3. On the other hand, a mechanism involving the transition state of a six membered ring is possible, by which a silyloxy group is transferred from the glucuronic acid to the other position of the glycosidic oxygen of the steroid. It should be mentioned that two further fragment ions are detectable in measurable intensities. Fragment M^+-464 is found only in aromatic glucuronides, M^+-465 only in aliphatic glucuronides. Both fragments arise by cleavage of the glucuronic acid whereby the binding oxygen remains at the steroid molecule. In aromatic glucuronides, this reaction is accompanied by a transfer of a proton.

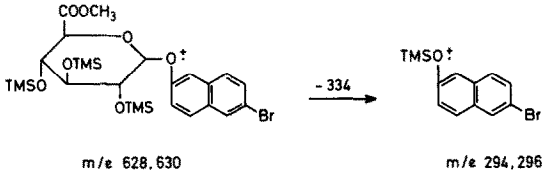

Fig. 3. Mechanism of fragmentation according to Billets *et al.*[5], demonstrated on the example of 6-bromonaphthol 2-glucuronide.

As demonstrated by Billets *et al.*[5], the fragmentation of the glucuronic acid moiety leads to the production of a number of fragments which were found

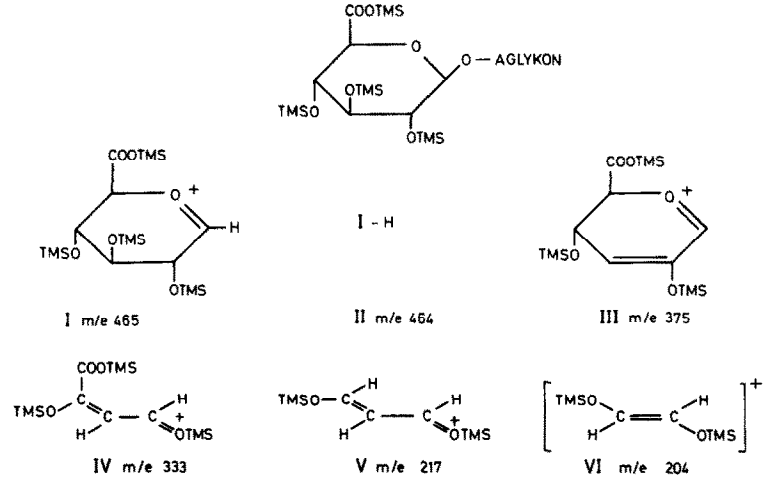

Fig. 4. Fragmentation pattern of glucuronic acid moiety of steroid glucuronides. TMS = trimethylsilyl group.

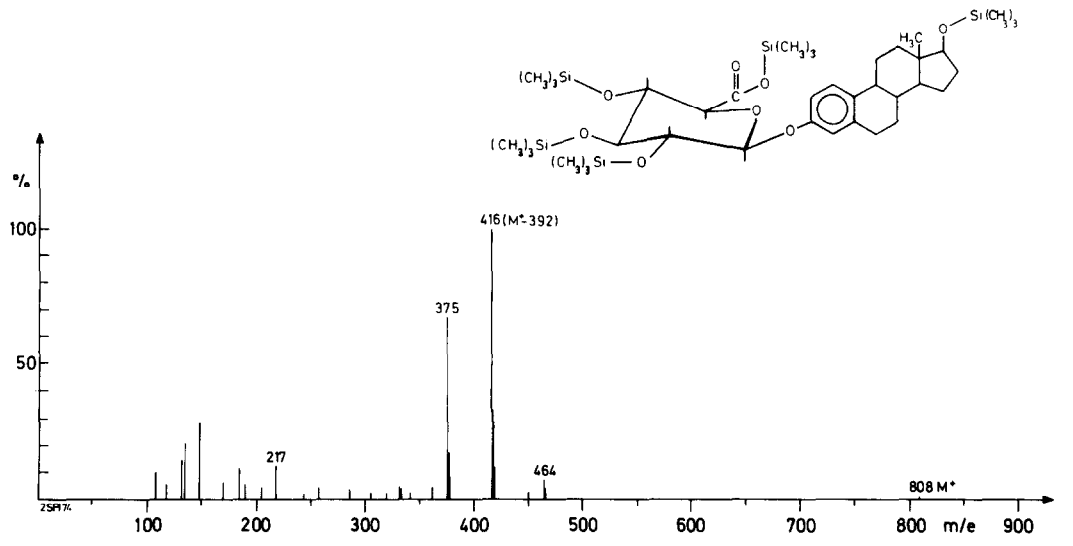

Fig. 5. Mass spectrum of TMS ether ester of oestradiol-17β 3-glucuronide.

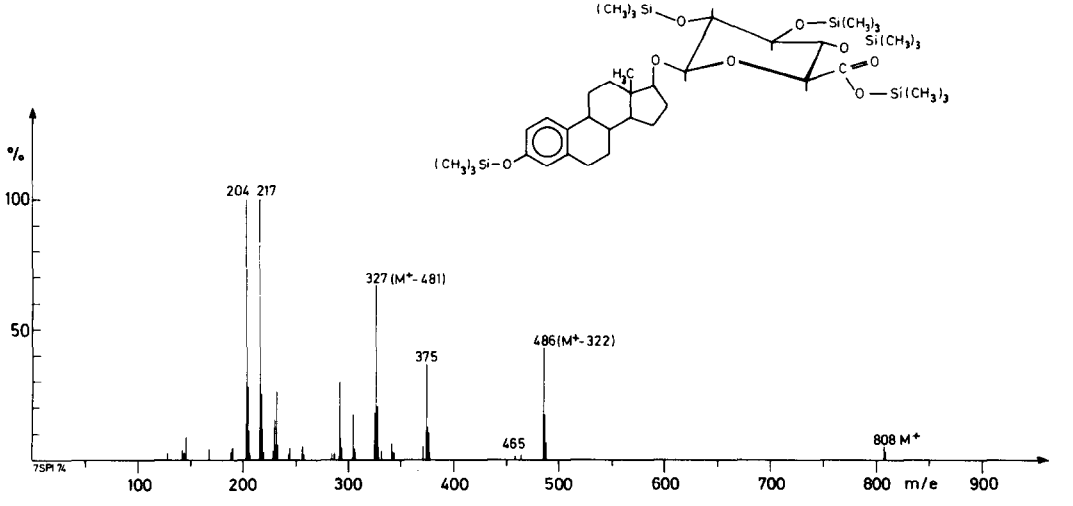

Fig. 6. Mass spectrum of TMS ether ester of oestradiol-17β 17-glucuronide.

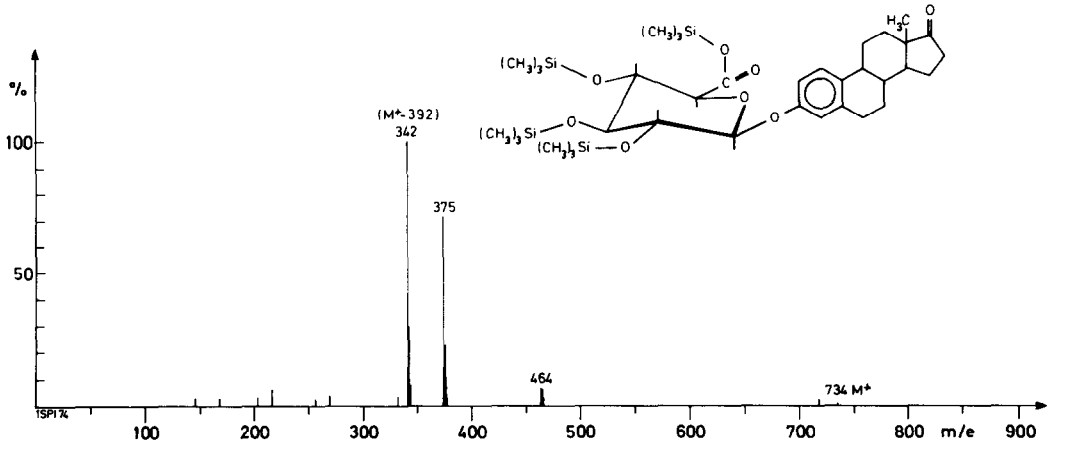

Fig. 7. Mass spectrum of TMS ether ester of oestrone 3-glucuronide.

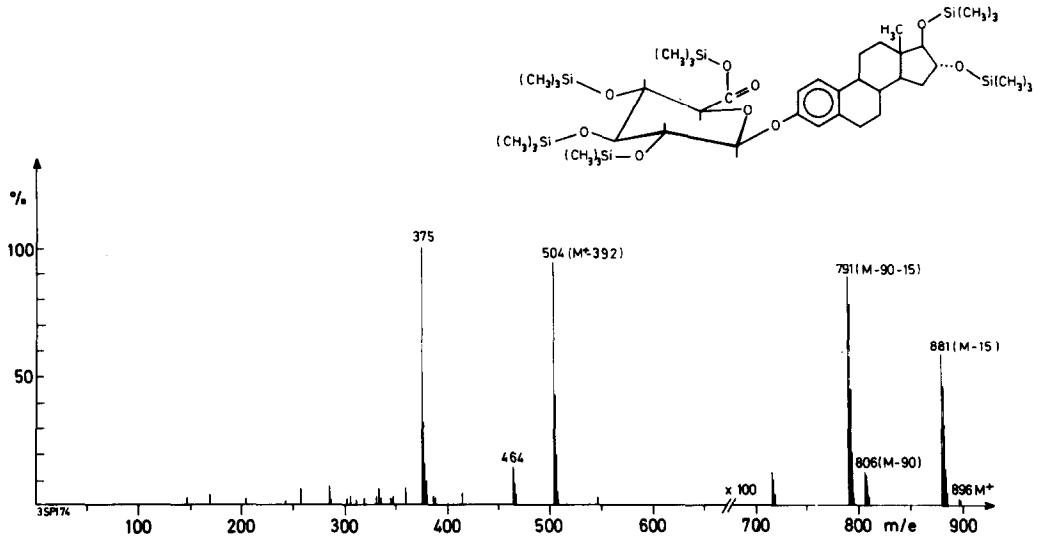

Fig. 8. Mass spectrum of TMS ether ester of oestriol 3-glucuronide.

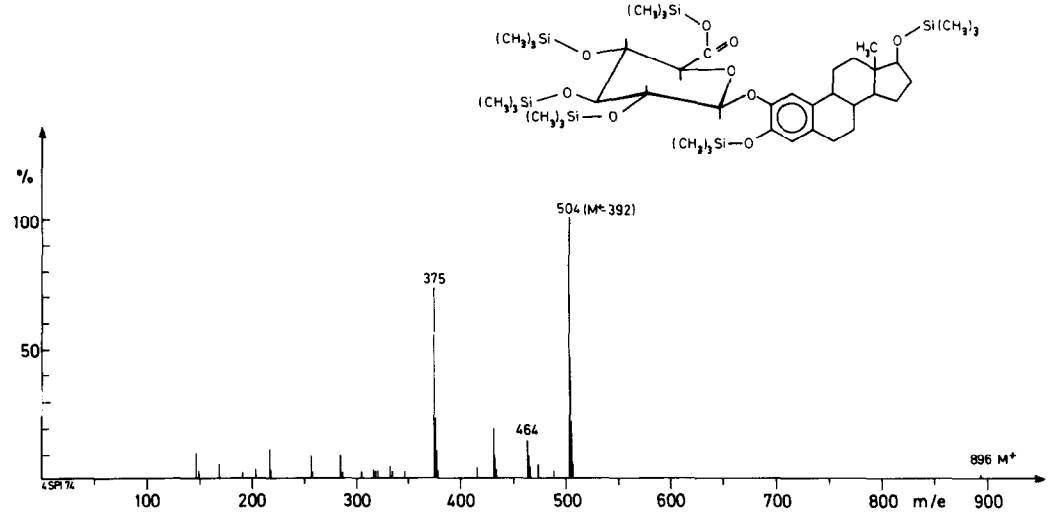

Fig. 9. Mass spectrum of TMS ether ester of 2-hydroxyoestradiol-17β 2-glucuronide.

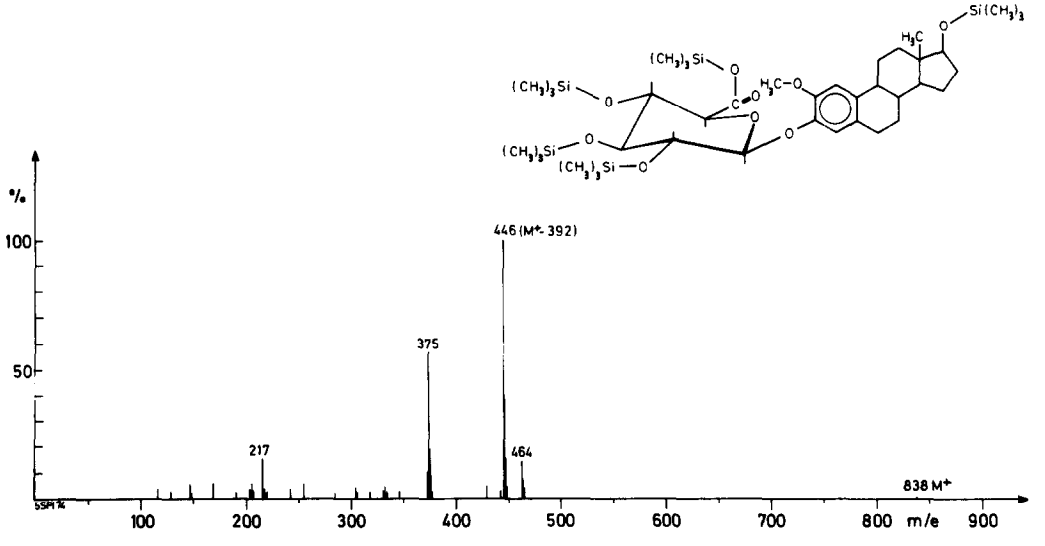

Fig. 10. Mass spectrum of TMS ether ester of 2-methoxyoestradiol-17β 3-glucuronide.

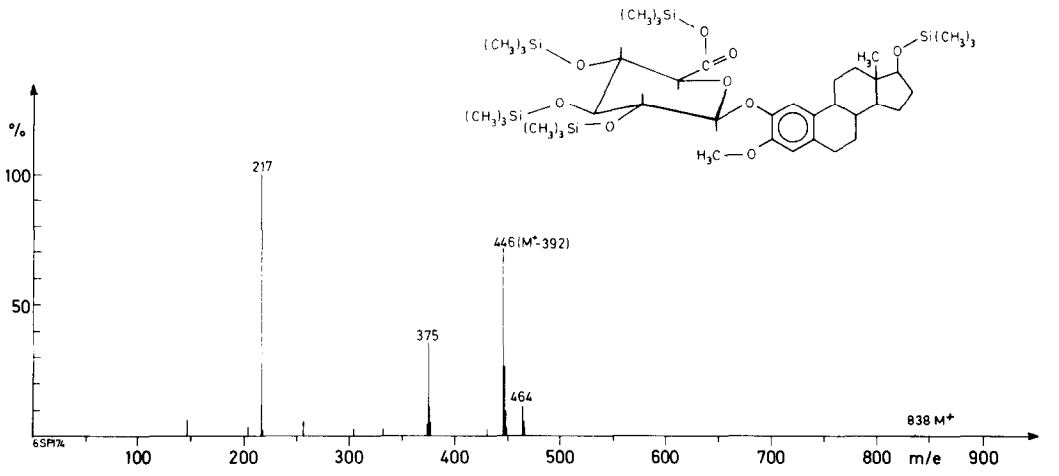

Fig. 11. Mass spectrum of TMS ether ester of 2-hydroxyoestradiol-17β 3-methyl ether 2-glucuronide.

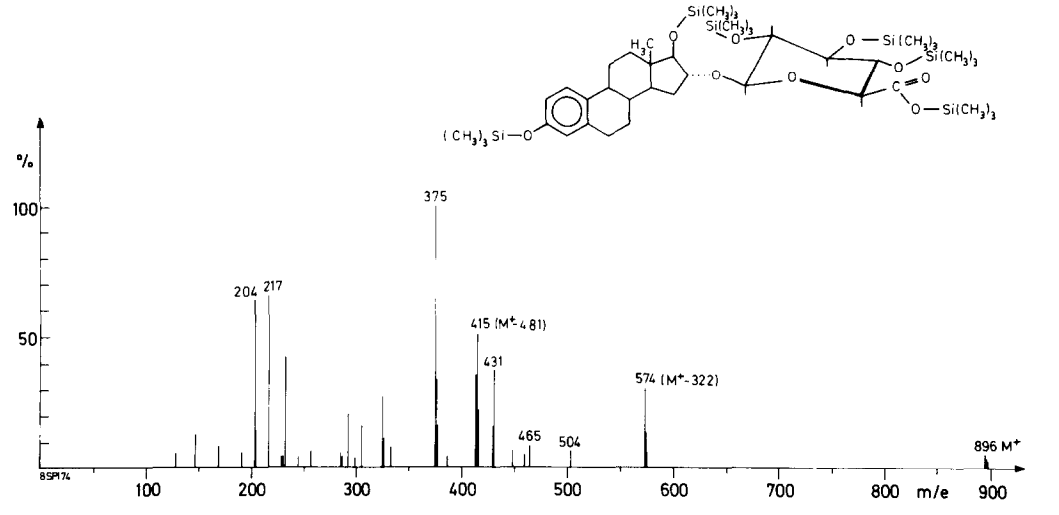

Fig. 12. Mass spectrum of TMS ether ester of oestriol 16α-glucuronide.

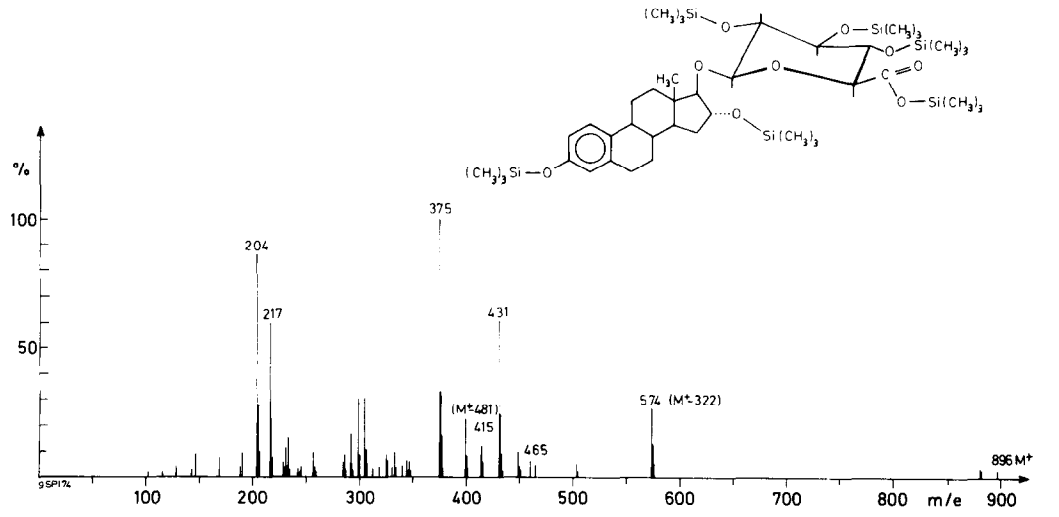

Fig. 13. Mass spectrum of TMS ether ester of oestriol 17β-glucuronide.

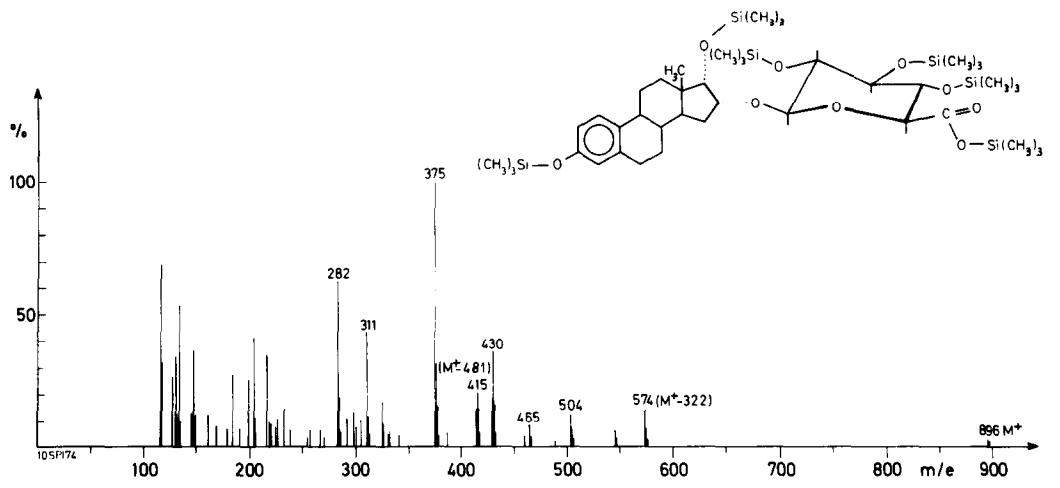

Fig. 14. Mass spectrum of TMS ether ester of 17-epioestriol 16α-glucuronide.

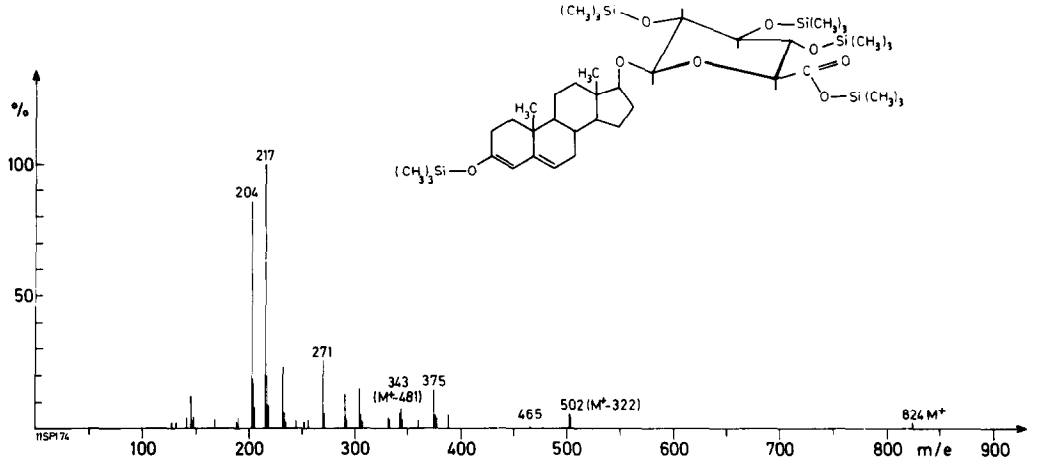

Fig. 15. Mass spectrum of TMS ether ester of testosterone 17-glucuronide.

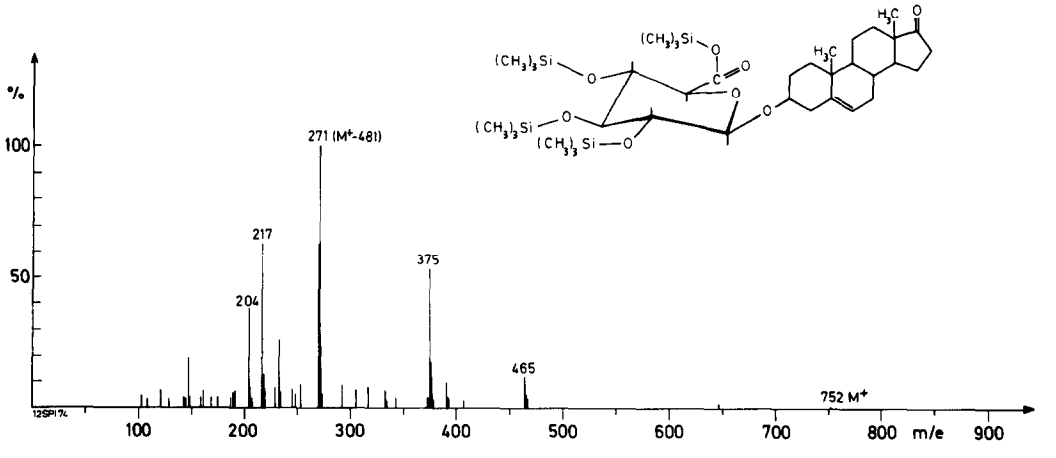

Fig. 16. Mass spectrum of TMS ether ester of dehydroepiandrosterone 3-glucuronide.

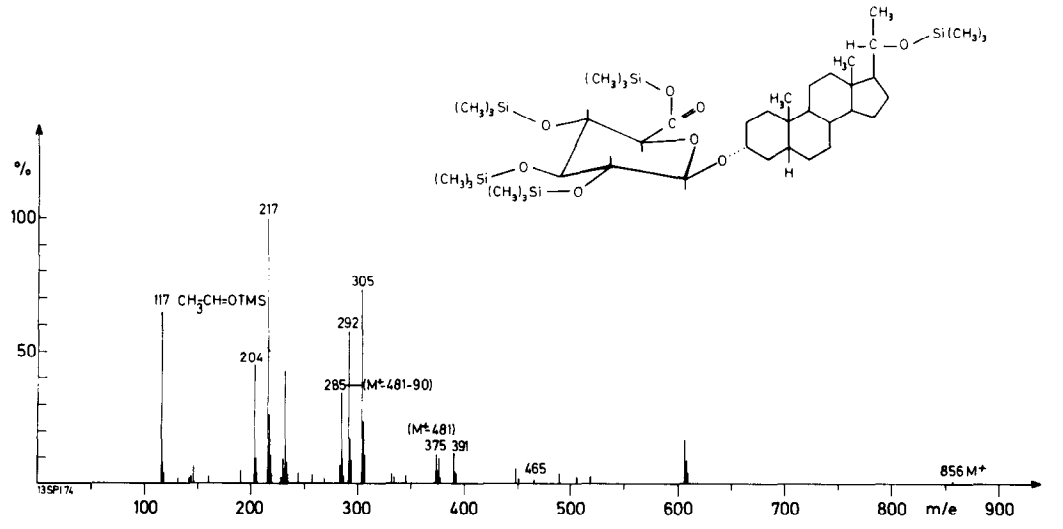

Fig. 17. Mass spectrum of TMS ether ester of pregnanediol 3-glucuronide.

Table 2. Main fragments of trimethylsilyl ether esters of steroid glucuronides. Relative intensities (%) are given in brackets. For details, see Experimental. G = Glucuronide

Steroid glucuronide	M-322	M-392	M-464	M-465	M-481	464	217	204
Oestrone 3-G	402 (0)	342 (110)	270 (4)	—	253 (0)	(7)	(6)	(3)
Oestradiol-17β 3-G	486 (0)	416 (100)	344 (0.5)	—	327 (0.7)	(7)	(12)	(4)
Oestriol 3-G	574 (0)	504 (95)	432 (0.5)	—	415 (1.6)	(14)	(8)	(3)
2-Hydroxyoestradiol-17β 2-G	574 (0)	504 (100)	432 (20)	—	415 (1.3)	(14)	(14)	(6)
2-Methoxyoestradiol-17β 3-G	516 (0)	446 (100)	374 (10)	—	357 (0.5)	(14)	(15)	(4)
2-Hydroxyoestradiol-17β 3-methyl ether 2-G	516 (0)	446 (71)	374 (4)	—	357 (0)	(12)	(100)	(3)
Oestradiol-17β 17-G	486 (44)	416 (1.3)	—	343 (5)	327 (52)	(0)	(100)	(100)
Oestriol 16α-G	574 (32)	504 (7)	—	431 (37)	415 (50)	(0)	(66)	(64)
Oestriol 17β-G	574 (27)	504 (5)	—	431 (60)	415 (12)	(0)	(60)	(86)
17-Epioestriol 16α-G	574 (14)	504 (12)	—	431 (35)	415 (20)	(0)	(34)	(40)
Testosterone 17-G	502 (5.7)	432 (0)	—	459 (3)	343 (6)	(0)	(100)	(86)
Dehydroepiandrosterone 3-G	430 (0.8)	360 (0)	—	—	271 (100)	(0)	(63)	(38)
Pregnanediol 3-G	534 (0.3)	464 (0.6)	—	391 (11)	375 (10)	(0.5)	(100)	(45)

in all glucuronides investigated. Figure 4 summarises those with the highest intensities. The ions *m/e* 465, 375 and 333 still contain the carboxy group; by means of these ions, a compound can be identified as a glucuronide. *m/e* 375 is particularly suitable because it is formed in intensities between 10 and 100%. The ions *m/e* 217 and 204 exhibited higher intensities in aromatic glucuronides than in aliphatic glucuronides. It is noteworthy that *m/e* 464 is found only when aromatic glucuronides are fragmented; it corresponds to the glucuronic acid radical minus one mass unit.

The mass spectra of the steroid glucuronides, studied in the present investigation, are shown in Figs. 5–17.

The main fragment ions, formed by the three different pathways of fragmentation, are summarised in Table 2. M^+-322 arises from fragmentation of the steroid glucuronide with the glycosidic linkage intact. M^+-392, M^+-464, M^+-465 and M^+-481 are fragment ions which are characteristic for the aglycon. The fragment ions *m/e* 464, 217 and 204 derive from fragmentation of the glucuronic acid moiety. As may be seen from the relative intensities, pronounced differences exist between aromatic and aliphatic glucuronides.

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