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Mass spectrometry of steroid glucuronide conjugates. I. Electron impact fragmentation of 5α-/5β-androstan-3α-ol-17-one glucuronides, 5α-estran-3α-ol-17-one glucuronide and deuterium-labelled analogues

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Owing to the developments of analytical instruments and interfaces (e.g. coupling high-performance liquid chromatography to mass spectrometry), there has been increased interest in new reference materials, for example in doping analysis with steroid glucuronide conjugates. The synthesized reference material has to pass several characterization steps including the use of gas chromatography/mass spectrometry (GC/MS) for its structure confirmation. In the present study, the fragmentation and mass spectrometric behaviour of several steroid glucuronide conjugates of endogenous and anabolic steroids after derivatization to pertrimethylsilylated products and to methyl ester pertrimethylsilylated products were investigated using GC/MS ion trap and GC/MS quadrupole instruments. The mass spectra of the derivatives of androsterone glucuronide, d_5-androsterone glucuronide, epiandrosterone glucuronide, etiocholanolone glucuronide, 11β-hydroxy etiocholanolone glucuronide, 19-norandrosterone glucuronide, d_4-19-norandrosterone glucuronide and 1α-methyl-5α-androstan-3α-ol-17-one glucuronide are presented and the origin of typical fragment ions of the glycosidic and steroidal moieties is proposed, based on different derivatization techniques including derivatization with d_{18}-bistrimethylsilylacetamide, methyl ester and trimethylsilyl ester derivatization and selected reaction monitoring. Typical fragmentation patterns which are related to the steroid structure are discussed. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: anabolic steroids; Koenigs–Knorr synthesis; gas chromatography; mass spectrometry; structure confirmation; mass spectra interpretation; trimethylsilyl derivatives

INTRODUCTION

The detection of endogenous and anabolic steroid metabolites in human urine is one of the main tasks in doping analysis. These compounds can be excreted as phase I (not conjugated) and/or phase II (conjugated) metabolites, of which the latter can be sulphates or glucuronides.[1] Usually hydrolysis of these conjugates is performed, yielding the free steroids which are derivatized and then identified by means of gas chromatography/mass spectrometry (GC/MS).[2] The improvements in interfacing high-performance liquid chromatography (HPLC) with mass spectrometry (e.g. electrospray ionization, atmospheric pressure chemical ionization) allow the sensitive detection and quantification of non-hydrolysed and underivatized metabolites[3,4] by avoiding several sample preparation steps. In the future, LC/MS and LC/MS/MS analyses of conjugates may be an alternative for the qualitative and quantitative determination of anabolic steroids in doping analysis but the confirmation of results obtained by measurements with these techniques requires reference materials which are only seldom commercially available.

The synthesized reference substances have to pass different characterization steps (e.g. NMR and MS), which can partly be performed by GC/MS. The interpretation of the mass spectra of unconjugated and underivatised steroids was described in detail by Budzikiewicz *et al*.[5] Several reports dealing with unconjugated steroids derivatized for GC/MS analysis[6–10] and the characterization of methyl and acetyl derivatives of arylglucuronic acid conjugates have been published.[11]

In this study, the mass spectrometric behaviour of commercially available steroid glucuronides and synthesized reference material (Fig. 1) as pertrimethylsilyl (per-TMS) and methyl ester per-TMS derivatives is presented and proposals for the origin of characteristic fragment ions generated by the glycosidic or steroidal moieties are described. These data contain different information to those obtained by e.g. LC/MS analyses, and complete identification and structural

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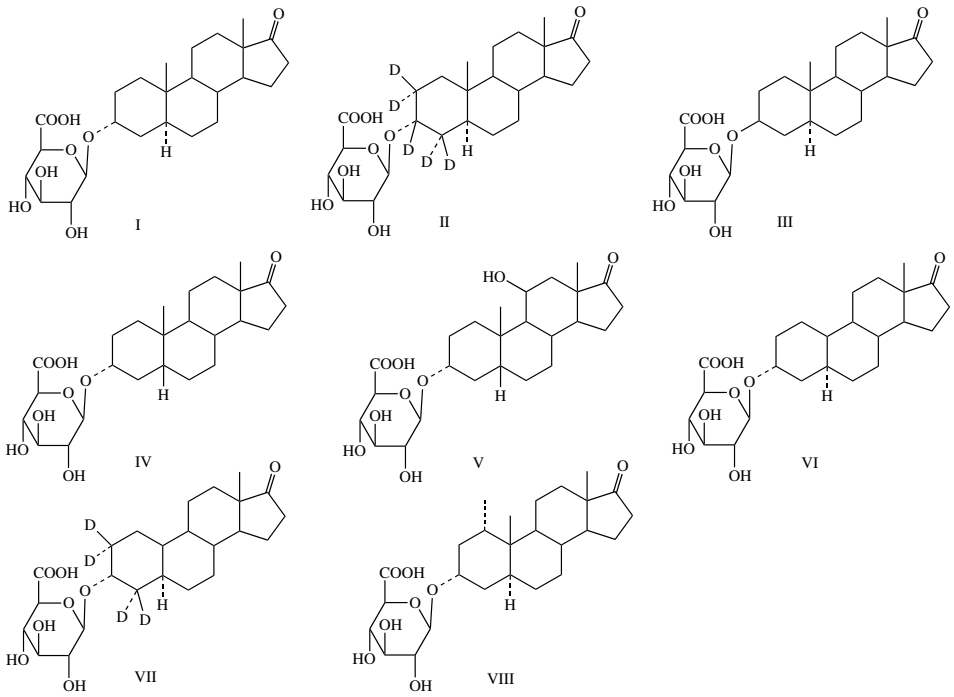

Figure 1. Structural formulae of (**I**) androsterone glucuronide, (**II**) d_5-androsterone glucuronide, (**III**) epiandrosterone glucuronide, (**IV**) etiocholanolone glucuronide, (**V**) 11β-hydroxy etiocholanolone glucuronide, (**VI**) 19-norandrosterone glucuronide, (**VII**) d_4-19-norandrosterone glucuronide and (**VIII**) 1α-methyl-5α-androstan-3α-ol-17-oneglucuronide.

characterization of synthesized steroid glucuronide conjugates by the generation of abundant and typical ions based on the presence of the glucuronic acid moiety in addition to those originating from the steroid.

EXPERIMENTAL

Chemicals and steroids

N-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Chemische Fabrik Karl Bucher (Waldstetten, Germany) and distilled before use, ammonium iodide (purum, p.a.), silver carbonate (purum, p.a.) and toluene (puriss., absolute over molecular sieve) from Fluka (Buchs, Switzerland), sodium hydroxide (p.a.), potassium carbonate (p.a.), glacial acetic acid (p.a.) and iodomethane (for synthesis) from Merck (Darmstadt, Germany), ethanethiol (97%) from Aldrich (Deisendorf, Germany), acetonitrile (p.a.) from Roth (Karlsruhe, Germany) and *tert*-butyl methyl ether and ethyl acetate (distilled before use) from Kraemer & Martin (St. Augustin, Germany). Methyl-1-bromo-1-deoxy-2,3,4-triacetylglucopyranuronate was synthesized by the procedure of Bollenback *et al*.[12] The reference substances epiandrosterone glucuronide, etiocholanolone glucuronide and 11β-hydroxyetiocholanolone glucuronide were purchased from Sigma (St. Louis, MO, USA) and the steroid metabolites norandrosterone, d_4-norandrosterone, 1α-methyl-5α-androstan-3α-ol-17-one and d_5-androsterone were synthesized in our laboratory.[13,14]

Synthesis of steroid glucuronide conjugates

The synthesis of steroid glucuronides is performed by means of a modification of the Koenigs–Knorr reaction,[15–17] treating 75 mg of a steroid in absolutely dry toluene (over molecular sieve) with 500 mg (1.26 mmol) of freshly prepared methyl-1-bromo-1-deoxy-2,3,4-triacetylglucopyranuronate in the presence of 280 mg (1 mmol) of silver carbonate as catalyst.[18] After consumption of the steroid, depending on the position of glucuronidation and steric circumstances, the catalyst is removed by filtration through a medium-porosity frit and the solution is evaporated to dryness by means of a rotary evaporator under reduced pressure. The residue is resolved in 10 ml of methanol, 1 ml of 1 M aqueous NaOH is added and, by frequent control of the pH, the hydrolysis of the methyl and acetyl groups at the glycosidic moiety is achieved. The solution is evaporated to dryness, a volume of 20 ml of doubly distilled water is added and the solution is extracted twice with 40 ml of *tert*-butyl methyl ether to remove the remainder of the unconjugated steroid. The aqueous layer is then adjusted to pH 2.5 with 6 M aqueous HCl and extracted three times with 50 ml of ethyl acetate. The combined organic phases are evaporated to dryness, the residue is dissolved in 2 ml of glacial acetic acid and the steroid glucuronide is purified by semipreparative HPLC fractionation.

Purification of synthesized steroid glucuronide conjugates

HPLC fractionation

A glacial acetic acid solution of the steroid glucuronides is injected in portions of 150 µl into an HP 1090 liquid chromatograph system (Hewlett-Packard) equipped with a 250 µl syringe and a VP 250/10 Nucleosil 100-7 C_{18} preparative column (Macherey–Nagel, Düren, Germany). The mobile phase is a mixture of (A) phosphoric acid in water (pH 2.5) and (B) acetonitrile starting with 30% solvent B increasing to 100% in 10 min and the column is washed for another 5 min with acetonitrile. Three wavelengths were

detected (198, 208 and 228 nm) and, because of the unknown elution time of the desired products, the elutates of all detected signals of the first injection (50 µl) are collected and tested by LC/MS or GC/MS for the glucuronide conjugate. All subsequent injections are fractionated only in the expected time range.

Solid-phase extraction

The combined fractions are diluted with a 10-fold amount of doubly distilled water and the steroid glucuronides are adsorbed on Amberlite XAD_2–polystyrene resin. The XAD_2 columns (pipettes of 0.8 cm i.d. closed with a glass pearl, bed height 5 cm) are finally washed with 5 ml of doubly distilled water and then eluted twice with 5 ml of methanol. The methanolic eluate is evaporated to dryness, yielding the pure steroid glucuronide conjugate with amounts of 22.0–40.1 mg (17.6–31.8% of theory).

The structures of most glucuronides were additionally confirmed by two-dimensional ^1H and ^{13}C NMR spectroscopy.

Derivatization for GC/MS analysis

The steroid glucuronides are derivatized to per-TMS-products (including a TMS-ester group at the glycosidic moiety) by step B (see below), and to methyl ester per-TMS-products by steps A and B, allowing a comparison of the mass spectra and providing information about fragment ion generation. Further, perdeuterotrimethylsilylation is performed without enolization of the 17-keto group by means of d_{18}-bistrimethylsilylacetamide (d_{18}-BSA, step C). The enolization of the 17-keto group was additionally achieved by evaporation of the d_{18}-BSA solution and derivatisation with the same procedure described with step B.

Step A. Methylation of the carboxylic group

5 µg of the steroid glucuronide are solved in a test tube in 200 µl of acetonitrile, 20 µl of iodomethane and 0.5 mg of potassium carbonate are added and the sample is carefully closed and heated for 60 minutes at 60 °C. After cooling to ambient temperature the organic layer is transferred to a fresh tube, evaporated to dryness by means of a rotary evaporator and the residue is trimethylsilylated as described below.

Step B. Trimethylsilylation

A 5 µg amount of the steroid glucuronide is dissolved in a test-tube in 100 µl of a mixture of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, ammonium iodide and ethanethiol (100 : 0.2 : 0.6, v/w/v) and heated for 20 min at 60 °C. The presence of NH_4I in the reaction mixture is used for the *in situ* preparation of trimethyliodosilane, a highly sensitive and unstable compound owing to its tremendous reactivity, which enables a rapid per-trimethylsilylation with enolization of keto groups.

Step C. Perdeuterotrimethylsilylation

A 5 µg amount of the steroid glucuronide is dissolved in 100 µl of a mixture of d_{18}-bistrimethylsilylacetamide, acetonitrile, ethanethiol and ammonium iodide (1000:4000:20:0.1, v/v/v/w) and the sample is heated for 20 min at 60 °C. With this procedure no enolization of the 17-keto group is observed but all hydroxy groups are derivatized to d_9-trimethylsilyl ethers, resulting in increments of fragments by steps of nine. The additional enolization is performed by evaporation of the d_{18}-BSA solution and derivatization as described in step B. Here, a partial exchange of a d_9-TMS goup by an unlabelled TMS group was observed with the TMS ester derivatives; the methyl ester derivatives were stable under the conditions of step B.

GC/MS parameters

All the spectra presented were generated with a Finnigan GCQ ion trap (for molecular masses >800) and with a Hewlett-Packard HP 6890/5973A GC/MSD system with the following parameters: injector, ATAS Optic 2, 300 °C (ion trap only); column, HP Ultra1 (Hewlett-Packard), film thickness 0.11 µm, i.d. 0.2 mm, length 14 m; carrier gas, helium at 1.5 ml min^{-1}; injection volume; 2 µl (100 ng of analyte); split ratio, 1:10; oven temperature, increased from 180 °C at 20 °C min^{-1} to 320 °C, maintained for 5 min at 320 °C; interface temperature, 320 °C; ion source temperature, 225 °C; mass range (full scan), 70–920 (ion trap) and 70–800 (5973A MSD); ionization, electron impact ionization (EI) (70 eV).

High-resolution mass spectrometry parameters

The exact masses of fragment ions were determined with a Hewlett-Packard HP 5890 gas chromatograph coupled to a Finnigan MAT 95 instrument with the following parameters: column, HP Ultra1 (Hewlett-Packard), film thickness: 0.11 µm, i.d. 0.2 mm, length 17 m; carrier gas, helium at 1.5 ml min^{-1}; injection volume, 2 µl (100 ng of analyte); split ratio, 1:10; oven temperature, increased from 185 °C at 5 °C min^{-1} to 260 °C then at 25 °C min^{-1} to 310 °C; interface temperature, 300 °C; ion source temperature, 240 °C; ionization, EI (65 eV); scan mode, Escan; scan range, 285–325; resolution, 5000; scan rate, 0.3 s per scan; calibration gas, perfluorophenanthrene; calibration masses, 292.9824 and 316.9824.

Nuclear magnetic resonance (NMR) analysis

NMR analyses were performed on Bruker DPX 300 and Bruker DRX 500 instruments. Amounts of 5 mg of the glucuronides of androsterone, d_5-androsterone, 19-norandrosterone and d_4-19-norandrosterone were dissolved in CD_3OD and ^1H, ^{13}C (DEPT, distortionless enhancement by polarization transfer), H,H-COSY (homonuclear correlated spectroscopy), HQMC (heteronuclear multiple quantum correlation) and NOESY (nuclear overhauser effect spectroscopy) were performed to confirm the structures of the synthesized substances.

RESULTS AND DISCUSSION

With the derivatization of steroid glucuronide conjugates to methyl ester-TMS and per-TMS products, stable compounds are obtained suitable for GC/MS analysis which are not thermally degradated during the measurements. The EI

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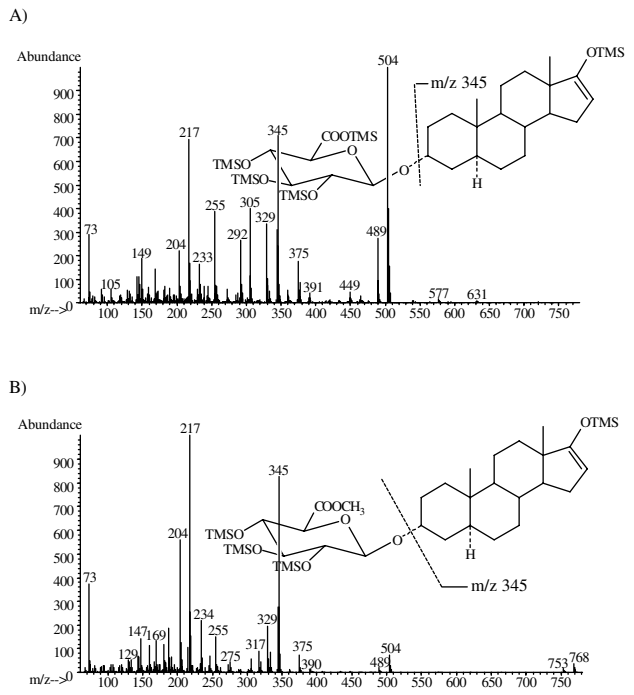

Figure 2. EI mass spectra of (A) androsterone glucuronide per-TMS ($M_r = 826$) and (B) androsterone glucuronide methyl ester per-TMS ($M_r = 768$).

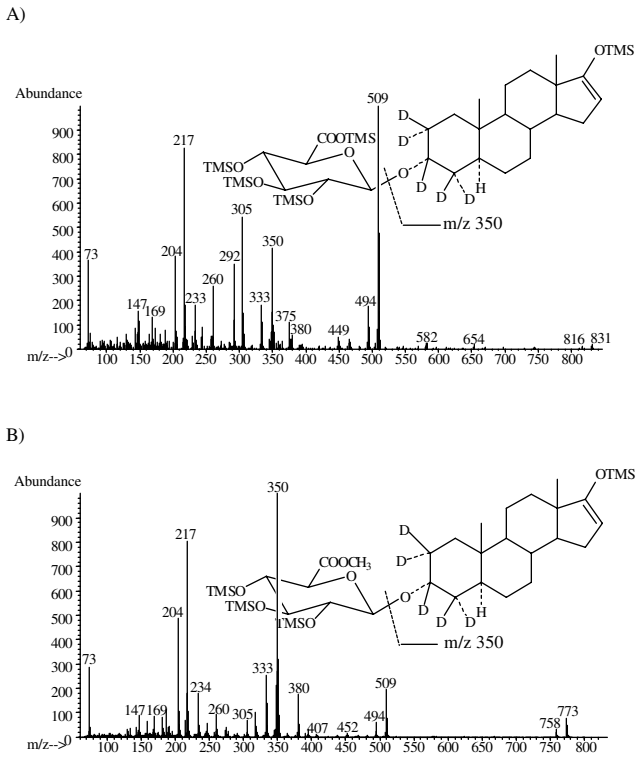

Figure 3. EI mass spectra of (A) d_5-androsterone glucuronide per-TMS ($M_r = 831$) and (B) d_5-androsterone glucuronide methyl ester per-TMS ($M_r = 773$).

mass spectra of the prepared and commercially obtained analytes show common and very specific fragmentations, as shown in the Figs 2–7. The basic fragmentation pattern is presented with androsterone glucuronide (Fig. 2) and is

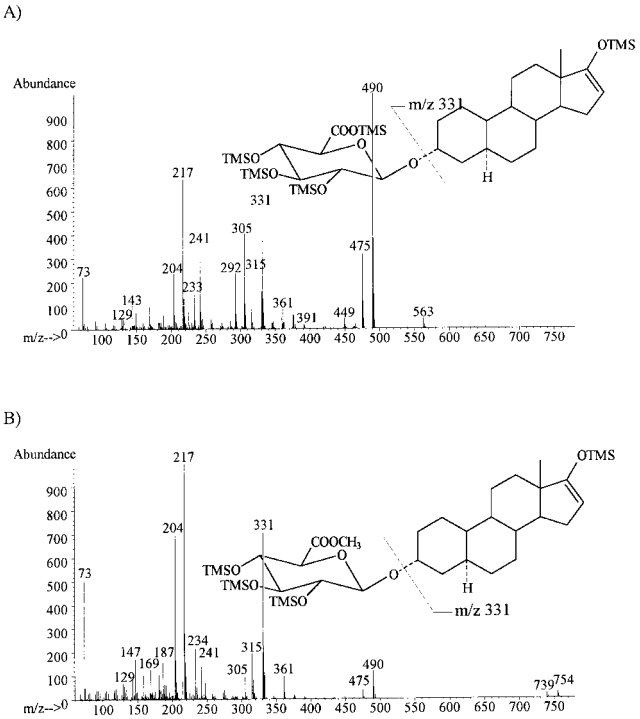

Figure 4. EI mass spectra of (A) 19-norandrosterone glucuronide per-TMS ($M_r = 812$) and (B) 19-norandrosterone glucuronide methyl ester per-TMS ($M_r = 754$).

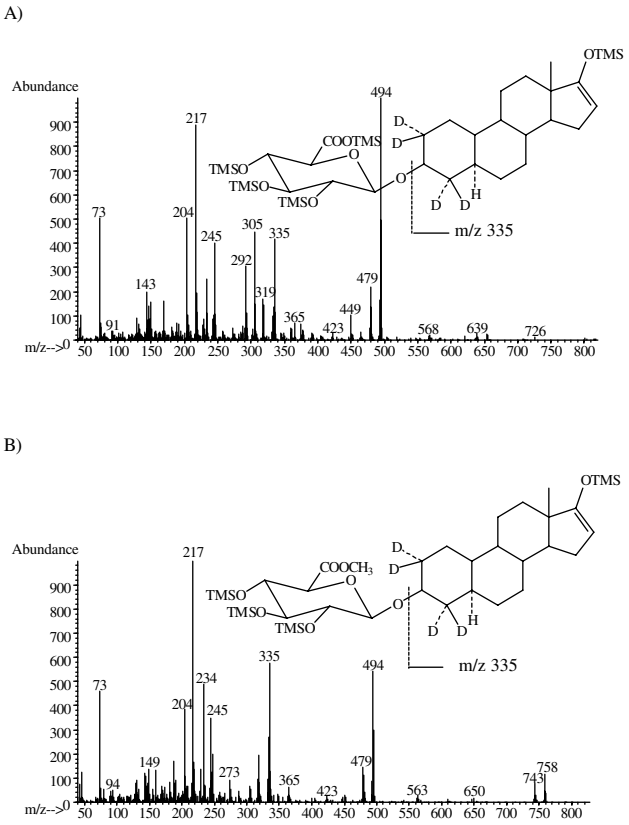

Figure 5. EI mass spectra of (A) d_4-19-norandrosterone glucuronide per-TMS ($M_r = 816$) and (B) d_4-19-norandrosterone glucuronide methyl ester per-TMS ($M_r = 758$).

applied to the spectra of the other substances. All glucuronide conjugates were analysed with both instruments (ion trap

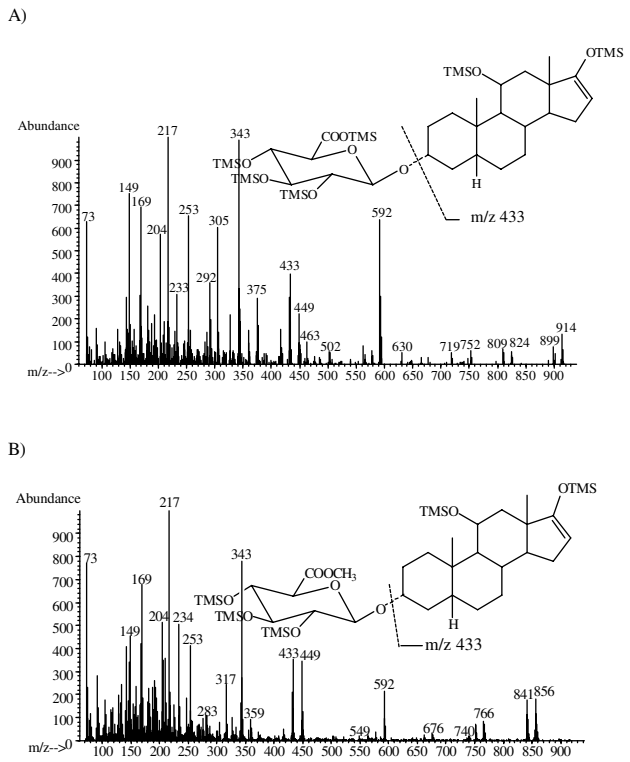

Figure 6. EI mass spectra of (A) 11β-hydroxy etiocholanolone glucuronide per-TMS ($M_r = 914$) and (B) 11β-hydroxy etiocholanolone glucuronide methyl ester per-TMS ($M_r = 856$).

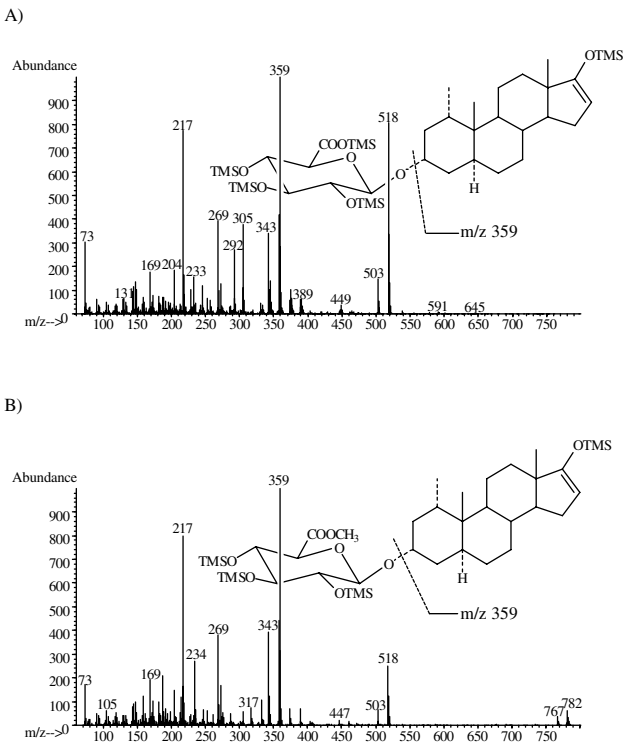

Figure 7. EI mass spectra of (A) 1α-methyl-5α-androstan-3α-ol-17-one glucuronide per-TMS ($M_r = 840$) and (B) 1α-methyl-5α-androstan-3α-ol-17-one glucuronide methyl ester per-TMS ($M_r = 782$).

and quadrupole) but no significant differences due to the analysis techniques were observed in the spectra.

General fragment ions of the glucuronic acid moiety

The ions of m/z 375, 305, 292, 217, 204 and 169 are found in all spectra of per-TMS derivatives of the investigated steroid glucuronides and additionally m/z 465 and 449 are observed in many cases the origin of which is proposed to be the glycosidic moiety. A spectrum of pertrimethylsilylated glucuronic acid is presented in Fig. 8 showing fragment ions comparable to those obtained from the glucuronide conjugates.

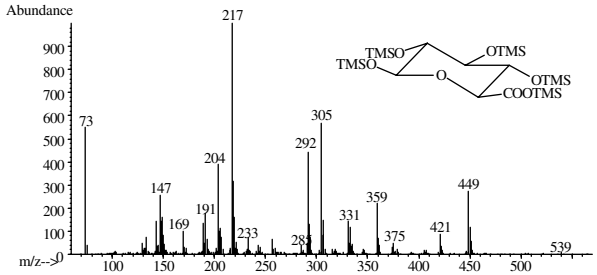

Figure 8. EI mass spectrum of glucuronic acid per-TMS ($M_r = 554$).

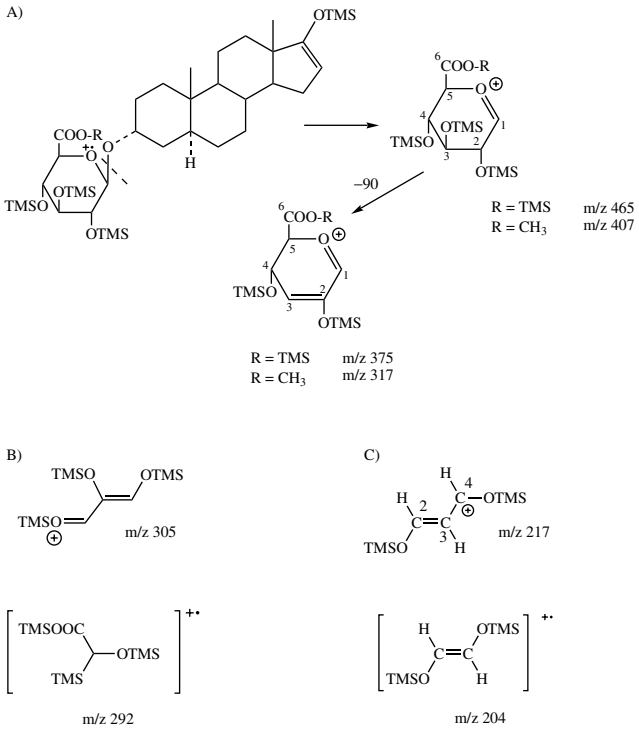

Figure 9. (A) Proposed generation of *m/z* 465 of per-TMS glucuronide conjugates by fission of the *O*-glycosidic bond. Consecutive loss of TMS-OH (−90) leads to *m/z* 375. The substitution of the TMS ester by a methyl ester produces *m/z* 407 and 317, respectively. (B) Proposed composition and structure of the fragment ions of *m/z* 292 and 305. Masses calculated (m_{cal}) and found (m_{exp}): $m_{cal} = 292.135$, $m_{exp} = 292.136$, $m_{cal} = 305.142$, $m_{exp} = 305.144$. (C) Postulated structure of the fragment ions of *m/z* 217 and 204.

m/z 465 → 375, m/z 407 → 317, m/z 449

A fission of the *O*-glycosidic bond between the steroid oxygen and the glucuronic acid moiety [Fig. 9(A)] results in a leaving group consisting of a steroid radical and in a fragment of *m/z* 465, which generates *m/z* 375 by a neutral loss of TMS-OH (−90). Their counterparts in spectra of the methyl ester derivatives are detected with *m/z* 407 (465−58) and *m/z* 317 (375−58). Evidence for the presence of three and two TMS groups, respectively, in these fragments is obtained with the spectra of TMS-deuterium-labelled glucuronide conjugates, where *m/z* 375 shifts to *m/z* 402 (+27) and *m/z* 317 to *m/z* 335 (+18). The abundance of the secondary ions (*m/z* 375, 317) varies with the general steroid structure.

The loss of the whole steroid from the molecular ion with a hydrogen abstraction produces *m/z* 464, which loses $CH_3^{\bullet}$ a radical (−15) forming the fragment of *m/z* 449. The latter is also present in the spectrum of glucuronic acid per-TMS where the same ion can be generated by the loss of TMS-OH from C-1 and a subsequent loss of $CH_3^{\bullet}$.

m/z 305, 292, 217, 204, 169

The fragment ions of *m/z* 305 and 292 were not detected in any product ion spectrum taken from any ion having an abundance higher than 10% of the base peak. Proposals for their generation and structure are based on the following data: (a) *m/z* 292 comprises the ester group because spectra of the methyl ester derivatives of steroid glucuronides show a shift of 58 u from *m/z* 292 to 234, owing to an exchange of a TMS group by a methyl group; (b) the fragment is incremented by 27 u owing to three TMS groups in deuterium-labelling experiments (Table 1); (c) *m/z* 305 does not include the ester group and is also detected in methyl ester derivatives with decreased intensity; (d) the ion shifts to *m/z* 332 due to three deuterium-labelled TMS groups (Table 1); and (e) high-resolution mass spectrometry confirmed the postulated composition of $C_{11}H_{28}Si_3O_3$ and $C_{12}H_{29}Si_3O_3$ for *m/z* 292 and 305, respectively. Their proposed structures are presented in Fig. 9B. The fragments of *m/z* 217 and 204 are known from other sugar derivatives[19] and their postulated structures are shown in Fig. 9(C). These are consistent with the labelling experiments of this study and the fragment increment with respect to the introduced deuteria (Table 1). The ion of *m/z* 169 is also generated by the glycosidic moiety as shown in the spectrum of glucuronic acid per-TMS but can also originate from some steroid moieties from the D-ring bearing an *O*-TMS group after enolization of a keto group (Fig. 1).

Proposed fragmentation pattern of androsterone glucuronide per-TMS (M_r = 826)

The spectrum of androsterone glucuronide-per-TMS (Fig. 2) contains the typical ions originating from the glucuronic acid

Table 1. Selected fragment ions of androsterone glucuronide (AG) derivatives and their counterparts in deuterium labelling experiments

Fragment ions of AG-per-TMS			Fragment ions of AG-methyl ester-tetrakis-TMS		
(*m/z*)	A[a]	B[a]	(*m/z*)	C[a]	D[a]
—[b]	—	—	768	773	795
—	—	—	753	758	780
577	582	595	—	—	—
504	509	513	504	509	513
489	494	498	489	494	498
375[c]	375	402	—	—	—
375[d]	380	375	375[d]	380	375
345	350	345	345	350	345
329	333/334	329	329	333/334	329
—	—	—	317	317	335
305	305	332	—	—	—
292	292	319	—	—	—
255	260	255	255	260	255
—	—	—	247	247	265
—	—	—	234	234	252
233	233	251	—	—	—
217	217	235	217	217	235
204	204	222	204	204	222
			187	187	196

[a] A = 2,2,3,4,4-d_5-androsterone glucuronide-per-TMS; B = androsterone glucuronide-17-enol-TMS-tetrakis-d_9-TMS; C = 2,2,3,4,4-d_5-androsterone glucuronide methyl ester tetrakis-TMS; D = androsterone glucuronide methyl ester 17-enol-TMS-tris-d_9-TMS.

[b] Dashes indicate fragment peak intensity <5% of base peak or not detected.

[c] Originating from the glycosidic moiety.

[d] Originating from the steroid moiety.

moiety but also fragments of steroid-specific mass/charge values.

m/z 577

The cleavage of the glycosidic ring structure is the starting point for several fragmentation steps initialized by the ionization of the *O*-glycosidic bond between the steroid and the glucuronic acid. The loss of 249 u from the molecular ion ($M_r = 826$) leads to m/z 577 as presented in Fig. 10. In case of 2,2,3,4,4-d_5-androsterone glucuronide per-TMS, this fragment shifts to m/z 582, and deuterated TMS groups increment m/z 577 of androsterone glucuronide per-TMS to m/z 595, due to two labelled and one unlabelled (enol ether) TMS groups (Table 1).

m/z 504 → 489 → 329

The base peak of this spectrum is m/z 504, the generation of which is proposed to start identically with m/z 577 by the fission of the ring structure, but followed by a transfer of the TMS group from C-3 to the obtained oxygen radical (Fig. 11). Owing to the cleavage of a ring bond, the molecular conformation can be changed, enabling a transfer of the 6-positioned TMS group and a subsequent loss of 322 u comprising the ester group. Support for this proposal is given by the spectrum of the methyl ester derivative of androsterone glucuronide which also contains the fragment of m/z 504 because of the removal of the substituted molecule part (ester group). Further, the spectra of d_5-androsterone glucuronide show the counterpart with m/z 509, and one deuterated TMS group shifts the fragment of m/z 504 to m/z 513 (Table 1). The main product ions of m/z 504 and 509 are m/z 489 and 494, respectively. The loss of a methyl group responsible for the difference of 15 u can not be located at the sugar residue because a subsequent loss of the remaining glycosidic moiety is observed. Second, the loss of the methyl group cannot originate from C-19 because measurements of 19-norandrosterone glucuronide per-TMS show highly comparable spectra in agreement with the mass difference (Fig. 4) of 14 u. In the literature the removal of a methyl group from norandrosterone-bis-TMS[7] and from estrone[20] was proved to originate from C-18, which is proposed in this case also as shown in Fig. 12.

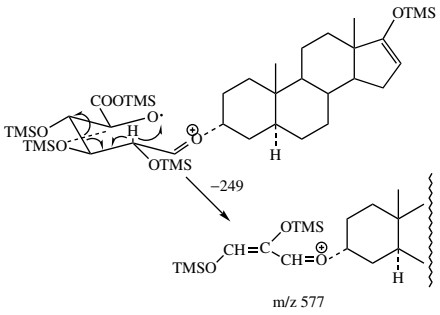

Figure 10. Possible mechanism of the loss of 249 u from the molecular ion of androsterone glucuronide.

The loss of the remaining sugar moiety accompanied by a hydrogen transfer is observed from m/z 489 and 504, generating the ions of m/z 329 and 344 in the case of unlabelled androsterone glucuronide. These are also observed with TMS-labelled derivatives (Table 1) because in this step the last deuterated TMS group is removed. The transferred hydrogen must be located at different carbons of the steroid because the product ion spectra of d_5-labelled androsterone glucuronide show fragments of m/z 333 and 334 (Table 1) originating from a loss of 160 and 161 u, respectively, from m/z 494 (Fig. 12).

m/z 375 → 345 → 255 (m/z 380 → 350 → 260)

The generation of m/z 375 was initially described from the glycosidic moiety, but the appearance of this ion in spectra of the methyl ester derivatives of androsterone glucuronide and its stereoisomers epiandrosterone glucuronide and etiocholanolone glucuronide besides m/z 317 suggested another source for this ion in the case of this steroid structure. This was proved with d_5-androsterone glucuronide, the spectra of which show m/z 375 and 380 as the per-TMS

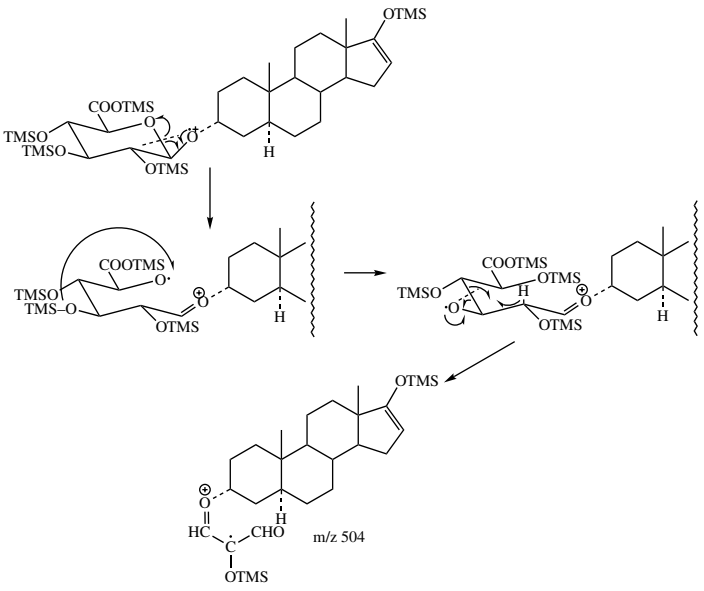

Figure 11. Proposed generation of *m/z* 504 of androsterone glucuronide per-TMS.

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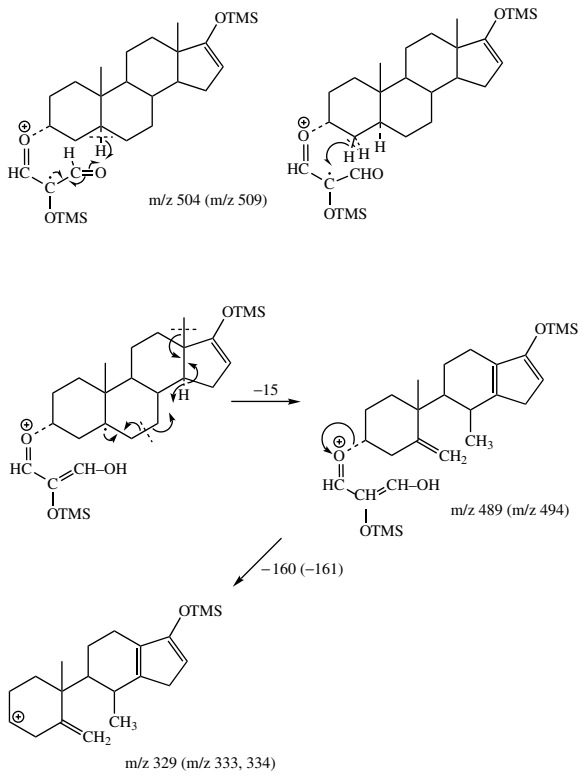

Figure 12. Possible mechanism of the elimination of C-18 and the consecutive loss of the glycosidic moiety after hydrogen (deuterium) transfer generating the fragment ions of *m/z* 489 (494) and *m/z* 329 (333, 334), respectively.

derivative and m/z 317 and 380 as the methyl ester product (Table 1). Further, TMS labelling experiments yielded m/z 375 (aglycone) and m/z 402 (glycosidic residue), and MS/MS experiments on m/z 375 and 380 of androsterone glucuronide and d_5-androsterone glucuronide generated m/z 345 and 350, respectively, by the loss of 30 u. The subsequent loss of TMS-OH from the remaining steroid fragments (m/z 345 and 350) led to m/z 255 and 260.

These basic fragmentation patterns can be applied to the other steroid glucuronides derivatized as described with respect to their individual structural difference. The spectra of androsterone glucuronide and its stereoisomers (epiandrosterone glucuronide and etiocholanolone glucuronide) are identical with only minor differences in ion abundance ratios so their mass spectrometric behaviour is not described separately.

19-Norandrosterone glucuronide per-TMS (M_r = 812)

Comparable to androsterone glucuronide per-TMS (Fig. 2), all fragment ions of 19-norandrosterone glucuronide per-TMS (Fig. 4) comprising the steroid moiety are detected with respect to a difference of 14 u due to the missing methyl group with C-19. The ion of m/z 563 corresponds to m/z 577, m/z 490 to 504, m/z 475 to 489, m/z 361 to 375 (steroid origin), m/z 331 to 345 (aglycone), m/z 315 to 329 and m/z 241 to 255. The glucuronic acid-specific fragment ions (m/z 375, 305, 292, 217, 204 and 169) are present with unchanged mass/charge values.

2,2,4,4-d_5-19-Norandrosterone glucuronide per-TMS (M_r = 816)

The spectrum of deuterium-labelled 19-norandrosterone glucuronide (Fig. 5) contains many fragment ions incremented by 4 u in comparison with the unlabelled nandrolone metabolite. The ion of m/z 563 is shifted to m/z 567, m/z 490 to 494, m/z 475 to 479, m/z 361 to 365, the aglycone m/z 331 to 335 and the product ion, generated by a neutral loss of TMS-OH (−90), is present with m/z 245. Further, the above-proposed loss of 160 and 161 u in case of d_5-androsterone glucuronide (Fig. 12) is also observed with d_4-norandrosterone glucuronide per-TMS, resulting in the fragment ions of m/z 318 (m/z 479 − 161) and m/z 319 (m/z 479 − 160). This indicates that the deuterium, transferred to the leaving group, must not originate from C-3. The fragments originating from the glycosidic moiety (m/z 375, 305, 292, 217, 204 and 169) are not influenced by the deuteration.

11β-Hydroxyetiocholanolone glucuronide per-TMS (M_r = 914)

The fragmentation of the 11β-hydroxylated etiocholanolone glucuronide as the per-TMS derivative (Fig. 6) and methyl ester pentakis-TMS derivative conforms to the described fragmentations in compliance with the increment of 88 u or the subtraction of 2 u which result from the additional 11-*O*-TMS group or its loss as TMS-OH (−90), respectively.

m/z 914 → 899 → 809, m/z 914 → 824 → 809

The molecular ion of the per-TMS derivative is observed with m/z 914 and also the consecutive losses of 15 and 90 u generating the ions m/z 899, 824 and 809.

m/z 592 → 577, m/z 502

The fragments of m/z 592 and 502 correspond to m/z 504 of androsterone glucuronide (incremented by 88 u and reduced by 2 u) and m/z 577 is the counterpart to m/z 489 resulting from a loss of a methyl group from m/z 592.

m/z 433 → 343 → 253

An abundant aglycone fragment is present with m/z 433, which subsequently loses TMS-OH (−90) twice to give m/z 343 and 253. This is also observed with TMS-labelled 11β-hydroxyetiocholanolone glucuronide, where m/z 433 is shifted to 442, due to one d_9-TMS and one unlabelled TMS group (enol ether).

The glucuronide specific ions (m/z 375, 305, 292, 217, 204 and 169) resulting from the glycosidic moiety are also observed with high abundances.

1α-Methyl-5α-androstan-3α-ol-17-one glucuronide per-TMS (M_r = 840)

The per-TMS and methyl ester tetrakis-TMS derivatives of the phase II metabolite of mesterolone (1α-methyl-5α-androstan-17β-ol-3-one) fragments very much like androsterone glucuronide, bearing an extra methyl group at C-1 resulting in a difference of 14 u. The molecular ion is observed weakly with m/z 840 and the −15 u product ion of m/z 825 (Fig. 7).

Table 2. Chemical shifts of carbons and selected hydrogens of the androsterone glucuronide and 19-norandrosterone glucuronide (19-NorAG) in ^{13}C and ^{1}H NMR experiments

Steroid carbons	AG chemical shift (ppm)	19-NorAG
C-1	33.64	25.11
C-2	26.46[a]	30.51[a]
C-3	75.62[a]	75.35
C-4	35.30[a]	40.28[a]
C-5	40.44	37.40
C-6	29.33	34.55
C-7	32.07	31.02
C-8	36.37	42.07
C-9	55.70	49.60
C-10	37.04	48.17
C-11	21.16	26.03
C-12	32.87	32.83
C-13	49.17	49.23
C-14	52.92	52.09
C-15	22.71	22.59
C-16	36.69	36.70
C-17	224.21	224.29
C-18	14.20	14.23
C-19	11.85	
Glucuronic acid carbons		
C-1′	103.03	103.07
C-2′	74.83	74.83
C-3′	77.64	77.62
C-4′	73.21	73.19
C-5′	76.57	76.57
C-6′	172.72	172.68
Glucuronic acid hydrogens		
H-1′	4.36	4.37 } $^{3}J = 7.8$ Hz
H-2′	3.28	3.23 }
H-3′	3.37	3.37
H-4′	3.52	3.52
H-5′	3.75	3.76

[a] Labelled positions in deuterated counterparts.

m/z 591, m/z 518 → 503 → 343

The fragment of m/z 591 corresponds to m/z 577, m/z 518 to 504, m/z 503 to 489 and m/z 343 to 329 of androsterone glucuronide (Fig. 2).

m/z 359 → 269, m/z 389

The base peak is performed by the aglycone with m/z 359, which generates m/z 269 by the neutral loss of TMS-OH (−90) and m/z 389 represents the aglycone fragment plus 30 mass units comparable to m/z 375 in the case of androsterone glucuronide per-TMS. The glucuronic acid fragment ions (m/z 375, 305, 292, 217, 204 and 169) are also present with equivalent abundances.

The NMR data for labelled and unlabelled androsterone glucuronide and 19-norandrosterone glucuronide, obtained by different experiments, as described above, are listed in Table 2. The coupling constant $^{3}J = 7.8$ Hz proved an axial–axial configuration of the hydrogens located at C-1′ and C-2′ of the glucuronide moiety and thus the β-configuration of the analysed steroid glucuronides because of an equatorial bond to the glycosidic oxygen. The chemical shifts of carbons of the deuterated and the unlabelled conjugates in ^{13}C experiments were identical except those bearing the ^{2}H atoms, which appeared as quaternary carbons and were not detected in the DEPT spectrum. A comparison of these data with ^{13}C data for unconjugated androsterone found in the literature[21] indicates the position of glucuronidation by deviation of the chemical shifts mainly of the carbons C-2, C-3 and C-4. The influence of the C-19 methyl group is obviously present on carbons C-1 to C-11, where the chemical shifts differ significantly between androsterone glucuronide and 19-norandrosterone glucuronide, but C-12 to C-18 have nearly identical values.

CONCLUSION

The GC/MS data obtained from 5α-/5β-androstan-/estran-17-one glucuronide conjugates present common fragments and their individual changes are due to structural peculiarities of the steroids. With the combination of two derivatives

(methyl ester per-TMS, per-TMS), structurally related compounds and deuterated analogues, information concerning the origin of distinct fragment ions, and possible ways of their generation are provided. This allows the completion of characteristic studies on synthesized reference materials. The generally present fragment ions of the glycosidic moiety (m/z 375, 305, 292, 217, 204 and 169) facilitate the identification of steroids bearing glucuronic acid with a saturated A-ring structure in combination with expected aglycone fragments, and those resulting from a common fission through the glycosidic part of the molecule (neutral loss of 322 u). The consecutive loss of C-18 (−15) and the remaining sugar residue (−160) produces a typical fragmentation pattern indicating partly the structure of the conjugates. The benefit of derivatization and GC/MS analysis is the presence of abundant fragment ions of both parts of the molecule, the steroid and glycosidic moiety which may be missing with other mass spectrometric and ionization techniques, e.g. LC/MS, where mainly fragments of the steroidal moiety are generated.

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