Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (8)

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Sport und Buch Strauß, Köln, 2000

M.THEVIS, E.NOLTEERNSTING, G.OPFERMANN, W.SCHÄNZER:
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In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (8). Sport und Buch Strauß, Köln, (2000) 89-98

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Synthesis and GC/MS-Characterization of Glucuronide Conjugates of Anabolic Steroids

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Introduction

The excretion of administered anabolic steroids and endogenous steroid hormones is observed as parent compound, phase-I- and phase-II-metabolites the latter of which can be mainly sulphates or glucuronides [1]. When analysing urine samples for conjugates, glucuronic acid conjugates are usually hydrolysed enzymatically and the aglycones are derivatised and identified by GC/MS [2] but the improvements in interfacing HPLC to mass spectrometry enable more and more the sensitive analysis of underivatised and not hydrolysed phase-II-metabolites [3-4]. Therefore, reference compounds are required which are seldom commercially available and which have to be synthesized and characterized by different analytical techniques including gas chromatography and mass spectrometry. Mass spectral data of underivatised unconjugated steroids were obtained and exploited by Budzikiewicz et al. (1964) [5] and several data were published dealing with unconjugated steroids derivatised for GC/MS analysis [6-9] as well as the characterization of methyl and acetyl derivatives of aryl glucuronic acid conjugates in general [10].

In this study the mass spectrometric behaviour of commercially obtained steroid glucuronides and synthesized reference material (figure 1) as per-TMS and methyl ester-per-TMS derivatives and proposals for the origin of characteristic fragment ions generated by the glycosidic or steroidal moieties are presented.

Experimental

The 3-O-β-glucuronide conjugates of androsterone, d_5-androsterone, 5α-estran-3α-ol-17-one (norandrosterone), 17α-methyl-5β-androstane-3α,17β-diol and the 17-O-β-glucuronides of 1-methyl-5α-androst-1-en-17α-ol-3-one (metenolone), nortestosterone and methyltestosterone were prepared by the modified classical Koenigs-Knorr-synthesis [11] treating the steroids with Methyl-1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate in the presence of Ag_2CO_3 [12] followed by hydrolysis and purification.

Derivatisation

A) Trimethylsilylation

The obtained steroid glucuronide conjugates (5µg) were derivatised to the per-TMS products with 100 µl of a mixture of MSTFA/ethanethiol/NH_4I (100:0.6:0.2, v:v:w) at 60°C for 20 minutes.

B) Methylation of the carboxylic group

The methyl ester per-TMS derivatives of the glucuronides (5µg) were performed by esterification of the glucuronic acid with methyl iodide (20 µl) in a volume of 200 µl of acetonitrile and K_2CO_3 (ca. 50mg) as catalyst. The mixture was heated for 1 h at 60°C and the supernatand was transferred into a reagent tube. The sample was evaporated to dryness and solved in 100 µl of the derivatisation mixture mentioned above (part A).

GC/MS analysis

The analyses were performed on a Finnigan GCQ ion trap GC/MS system equipped with an ATAS Optic 2 injector system. The conditions were as follows:

column:	HP Ultra 1, 14m x 0.2mm i.d., filmthickness 0.11 µm
carrier gas:	helium, 1.5ml/min, split 1:10
injector temp.:	325°C
temp. programm:	0 min 140°C, +20°C/min, 5 min 320°C
interface temp.:	325°C
ionisation:	EI (70 eV), mass range 70-920 amu.

Results

The spectra of the glucuronide conjugates per-TMS derivatives and methyl ester per-TMS derivatives show common and individual fragment ions. In figure 2 the spectrum of androsterone glucuronide per-TMS is presented and general fragmentation ways of the steroid conjugates will be described exemplarily.

General fragment ions of the glucuronic acid moiety

The ions m/z 375, 305, 292, 217, 204 and 169 are found in all spectra of per-TMS derivatives of the steroid glucuronides and additionally m/z 465 and 449 are observed in some cases, the origin of

which is proposed to be the glycosidic moiety. A spectrum of per-trimethylsilylated glucuronic acid is presented in figure 3 showing fragment ions comparable to those obtained from the glucuronide conjugates.

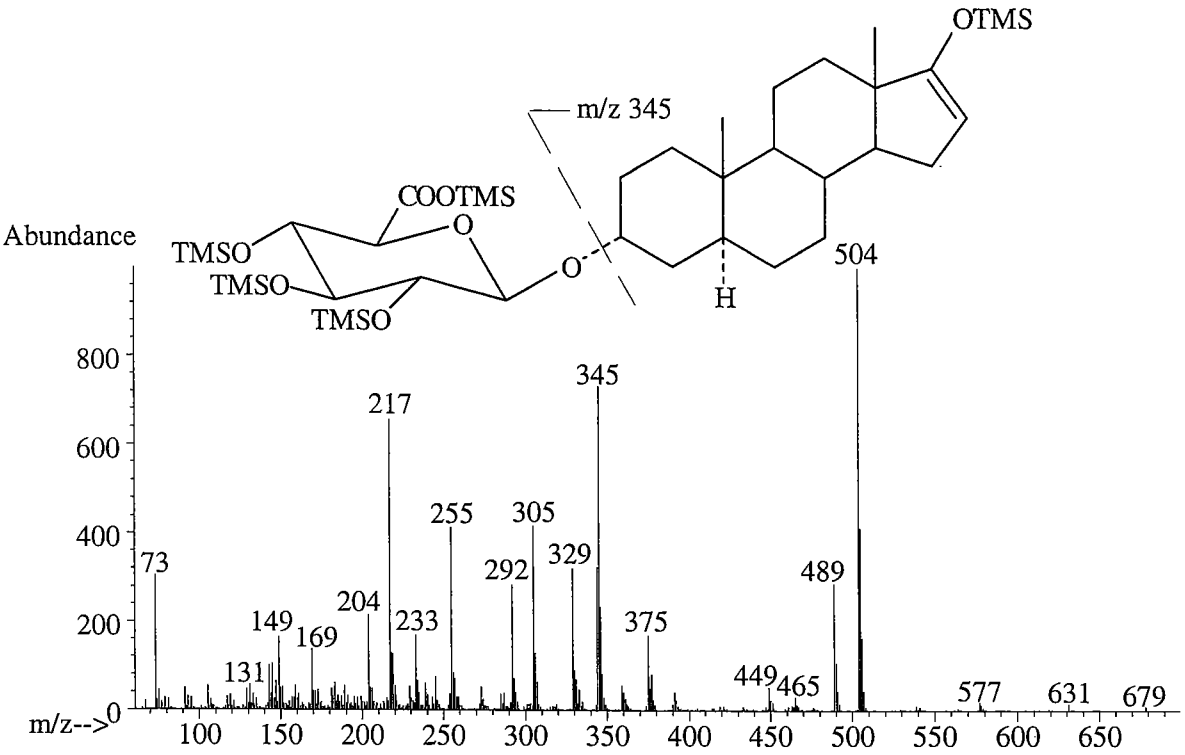

Figure 2: EI mass spectrum of androsterone glucuonide per-TMS, mol wt: 826

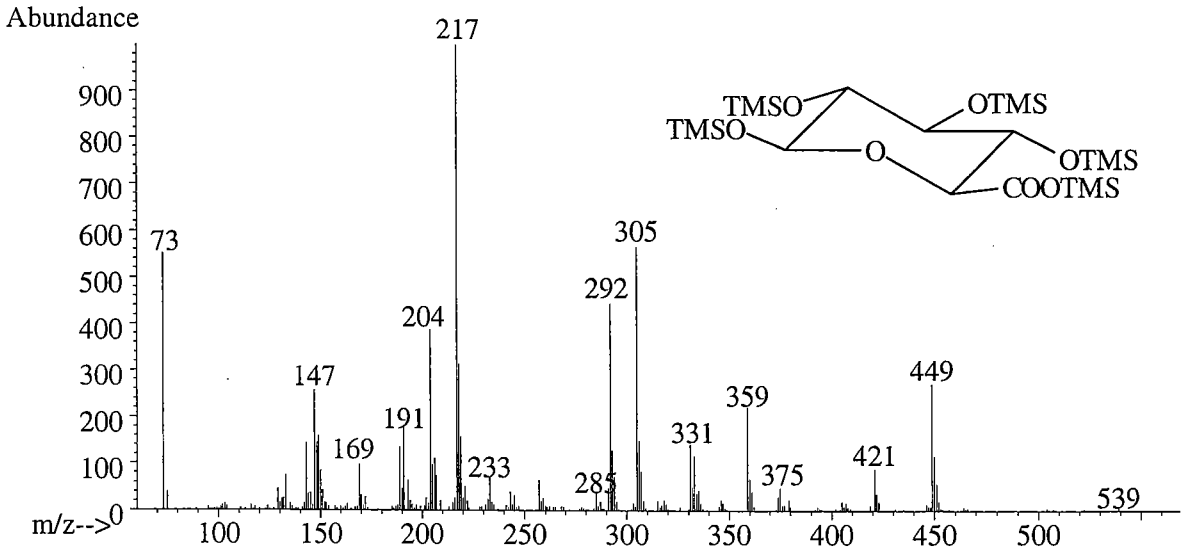

Figure 3: EI mass spectrum of glucuronic acid per-TMS, mol wt: 554

m/z 465 -> m/z 375, m/z 407 -> m/z 317, m/z 449. A fission of the O-glycosidic bond between the steroid oxygen and the glucuronic acid moiety (figure 4) 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). The intensity of the secondary ions (m/z 375, 317) varies with the position of glucuronidation and the general steroid structure.

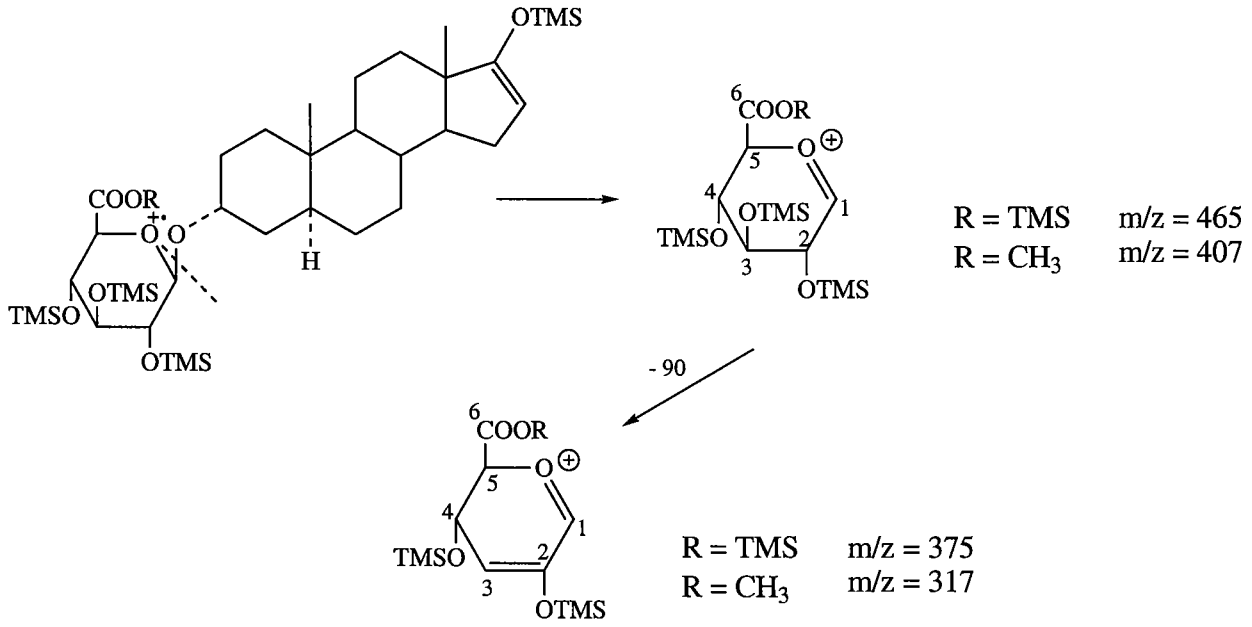

Figure 4: Proposed generation of fragment ions (m/z 465, 375 / m/z 407, 317) originating from the glycosidic moiety.

The loss of the whole steroid from the molecular ion with a hydrogen abstraction produces m/z 464, which loses a methyl radical (-15) forming the fragment m/z 449. The latter is present in the spectrum of glucuronic acid per-TMS too 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, m/z 292, m/z 217, m/z 204, m/z 169. The fragment ions m/z 305 and m/z 292 were not detected in any daughter ion spectrum taken from any ion having an abundance higher than 10% of the base peak. Their structure is not cleared yet but m/z 292 comprises the ester group because spectra of the methyl ester derivatives of steroid glucuronides show a shift of 58 mass units from m/z 292 to m/z 234. The fragments m/z 217 and m/z 204 are known from other sugar derivatives [13] with a proposed structure presented in figure 5. The ion m/z 169 is generated by the glycosidic moiety too as shown in the spectrum of glucuronic acid per-TMS but can also origin from some steroid moieties from the D-ring bearing an O-TMS group after enolization of a 17-keto group.

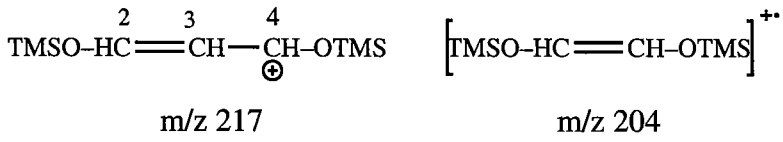

Figure 5: Proposed structure of the fragment ions m/z 217 and m/z 204, which are detected in each glucuronide conjugate spectrum.

Fragmentation of androsteroneglucuronide per-TMS (mol wt: 826)

m/z 504 -> m/z 489 -> m/z 329

The base peak in the spectrum of androsterone glucuronide per-TMS is m/z 504 (figure 2A). Its generation is proposed to start with a fission of the bond between the oxygen and C-1 of the glucuronic acid yielding an oxygen radical. Due to the broken ring structure a subsequent transfer of the TMS-group from C-3 ís possible accompanied by a neutral loss of 322 amu (figure 6). The fact that the ester group is definitely removed with this step is proven by the methyl ester TMS derivative of androsterone glucuronide, the spectrum of which contains the same fragment m/z 504 in a decreased intensity.

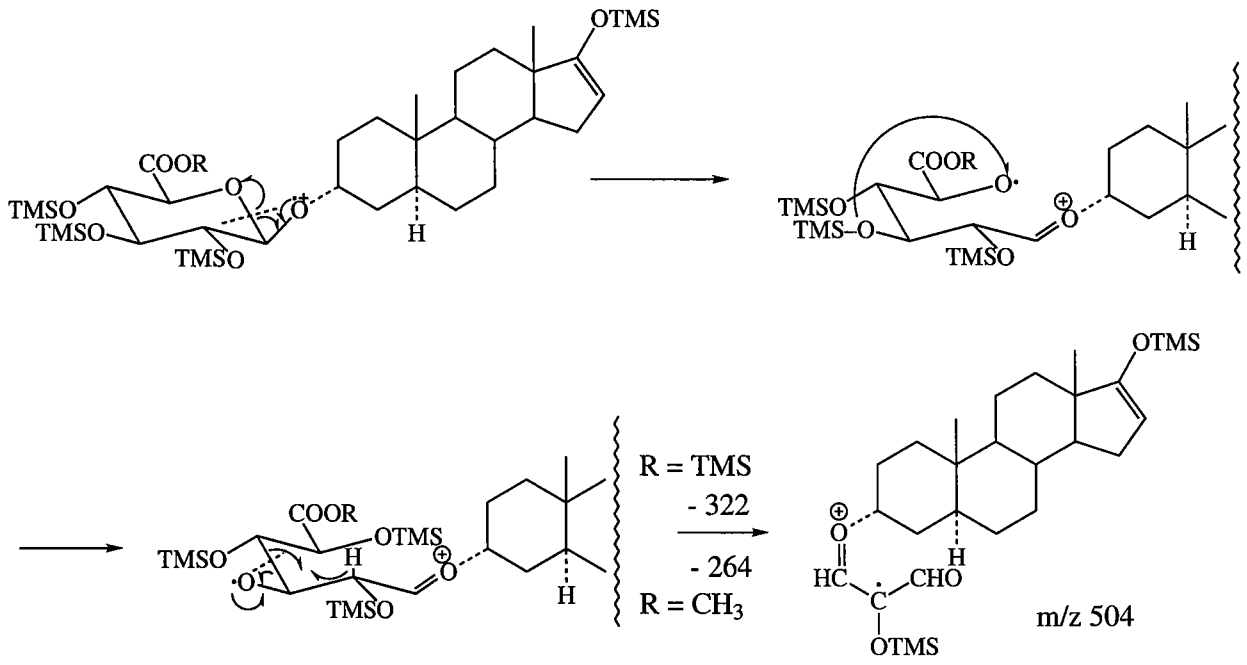

Figure 6: Fragmentation pattern for the generation of m/z 504 of androsterone glucuronide per-TMS and methyl ester TMS.

The main daughter ion of m/z 504 is m/z 489, resulting from a neutral loss of a methyl radical. This is psotulated to be C-18, because of several facts: a) the spectrum of 19-norandrosterone-glucuronide is highly comparable to that of androsterone glucuronide with respect to the difference of 14 amu. The corresponding fragment m/z 475 (489 – 14) is present in spite of the lack of C-19; b) the main daughter ion of m/z 489 is m/z 329, resulting from the neutral loss of the sugar rest (figure 7), so the loss of 15 amu can not result from this part of the molecule; c) studies concerning the loss of a methyl radical from unconjugated norandrosterone confirmed the cleavage of the C-18 bond [7].

The generation of m/z 329 by loss of the glycosidic rest (-160) is described in figure 7. The hydrogen transfer must result from different sources because daughter ion spectra of m/z 494 of d_5-androsterone glucuronide showed secondary ions with mass/charge values of 333 and 334 (table 1).

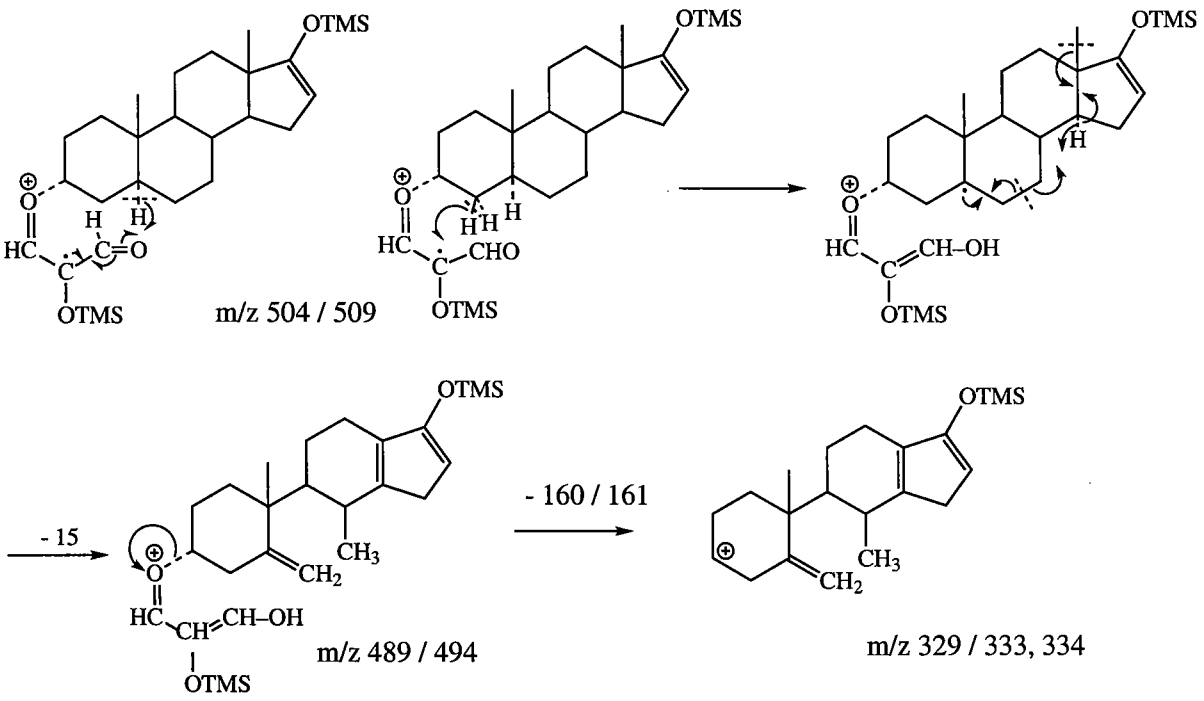

Figure 7: Proposed generation of the fragment ions m/z 489 and m/z 329 of androsterone glucuronide per-TMS.

m/z 375, m/z 345, m/z 255

The fragment m/z 375 is already described to originate from the glycosidic moiety but in case of androsterone glucuronide and its isomers etiocholanolone glucuronide and epiandrosterone glucuronide there is another source of this ion. This is proven by norandrosterone glucuronide and

d_5-androsterone glucuronide where the fragment is shifted to m/z 361 and 380, respectively, in a spectrum still showing an m/z of 375 due to the glycosidic moiety. Further, the spectra of the methyl ester TMS derivatives of androsterone glucuronide and isomers contain the corresponding ion m/z 317 (sugar rest) and still m/z 375. The steroidal fragment m/z 375 is proposed to be the aglycone with a methylenoxy group at C-3 (aglycone + 30, see table 1 and 2).

The aglycone fragment is present with m/z 345 and a neutral loss of TMS-OH (-90) leads to m/z 255.

The described fragmentation pattern of androsterone glucuronide per-TMS and methyl ester per-TMS can partly be transferred to other glucuronide conjugates. The conjugate of 11β-OH-etiocholanolone as well as 17-O-glucuronides like testosterone glucuronide, 19-nortestosterone glucuronide, methyltestosterone glucuronide and metenolone glucuronide or the metabolite of methyltestosterone 17α-Methyl-5β-androstane-3α,17β-diol-3-O-glucuronide suit to the proposed fragmentations with some individual features. The 11β-OH-etiocholanolone glucuronide for example shows the corresponding fragment ions to androsterone gucuronide in compliance with mass differences of +88 (additional O-TMS group) or -2 (loss of the additional O-TMS group as TMS-OH) and an increased intensity of the molecular ion.

Further, the molecular ion is present with high abundances of those conjugates bearing a 1- or 4-en-3-one steroid structure which forms a conjugated π-electron system after enolization of the keto-group, enabling the stabilisation of the ion. Consequently, other fragment ions disappear or have at least decreased intensities compared to androsterone glucuronide, but the fragments belonging to the glycosidic moiety are generally present. The spectrum of nortestosterone glucuronide per-TMS is shown in figure 8.

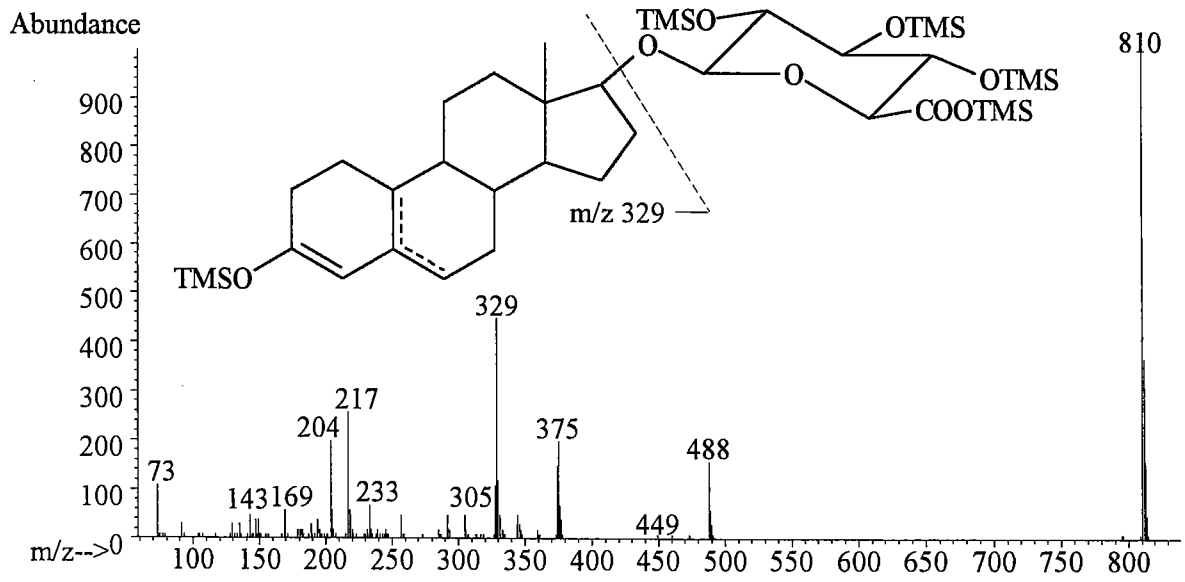

Figure 8: EI mass spectrum of nortestosteroneglucuronide per-TMS, mol wt: 810

Conclusion

The mass spectra of glucuronidated steroids with varying structure and different conjugation sites show common and individual fragment ions. In general fragments originating from the glycosidic moitey (m/z 375, 305, 292, 217, 204 and 169) are present together with the aglycone fragment and, except methyltestosterone glucuronide and the metabolite 17α-methyl-5β-androstane-3α,17β-diol-3-glucuronide, M^+-322 whose generation is proposed above. The molecular ion is present with conjugates bearing a steroid nucleus with a 3-keto-4-ene structure which delocalises the induced charge, while androsterone glucuronide and related compounds generate the fragment M^+-249 which is missing in the group of testosterone glucuronide analogues (table I).

Acknowledgements

We thank the Bundesinstitut für Sportwissenschaft, Cologne, for their financial support.

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Table 1: Selected fragment ions of per-trimethylsilylated steroid glucuronide conjugates

Analyte	M_r	Fragment ions (intensity in % relative to base peak)															
		M^+	M^+ - 249	M^+ - 322	M^+ - 322 -15	m/z 465	m/z 449	m/z 375	aglyc. + 30	aglyc.	M^+ - 322 - 15 - 160	m/z 305	m/z 292	aglyc. - 90	m/z 217	m/z 204	m/z 169
AG per-TMS	826		577 (1.9)	504 (100.0)	489 (30.0)	465 (2.4)	449 (4.3)	375 (18.3)	*	345 (64.1)	329 (33.9)	305 (42.0)	292 (25.8)	255 (36.5)	217 (57.3)	204 (19.2)	169 (12.5)
D_5-AG per-TMS	831		582 (3.1)	509 (100.0)	494 (14.2)	465 (5.2)	449 (3.3)	375 (8.6)	380 (4.8)	350 (30.8)	333 (14.3) 334 (12.1)	305 (46.0)	292 (28.0)	260 (24.7)	217 (64.8)	204 (28.4)	169 (12.4)
NorAG per-TMS	812		563 (3.6)	490 (100.0)	475 (36.0)	465 (2.5)	449 (4.3)	375 (5.3)	361 (8.7)	331 (52.5)	315 (22.8)	305 (40.1)	292 (20.2)	241 (35.4)	217 (53.0)	204 (21.2)	169 (9.6)
EpiAG per-TMS	826		577 (4.3)	504 (42.2))	489 (14.8)		449 (3.2)	375 (14.5)	*	345 (44.8)	329 (13.7)	305 (27.7)	292 (22.6)	255 (35.0)	217 (100.0)	204 (69.0)	169 (26.2)
EG per-TMS	826		577 (2.4)	504 (82.6)	489 (26.4)		449 (4.2)	375 (22.2)	*	345 (100.0)	329 (26.1)	305 (38.7)	292 (22.3)	255 (43.3)	217 (51.7)	204 (9.7)	169 (10.0)
11β-OH-EG per-	914	914 (4.9)	665 (1.0)	592 (65.7)	577 (8.0)	465 (2.3)	449 (22.5)	375 (44.5)	463 (2.5)	433 (30.0)	417 (7.0)	305 (48.1)	292 (38.2)	343 (88.6)	217 (100.0)	204 (59.0)	169 (64.0)
TG per-TMS	824	824 (100.0)		502 (16.1)			449 (2.5)	375 (17.2)	373 (1.4)	343 (34.5)		305 (5.6)	292 (4.6)	253 (2.1)	217 (26.5)	204 (26.3)	169 (9.5)
NorTG per-TMS	810	810 (100.0)		488 (20.5)				375 (25.4)	359 (2.1)	329 (53.1)	313 (1.2)	305 (5.8)	292 (4.9)	239 (2.4)	217 (27.9)	204 (22.5)	169 (6.0)
MTG per-TMS	838	838 (18.9)						375 (69.3)	387 (1.9)	357 (100.0)	341 (11.7)	305 (2.1)		267 (7.7)	217 (16.1)	204 (4.1)	169 (15.7)
MG per-TMS	838	838 (44.1)		516 (31.2)	501 (1.9)	465 (2.0)	449 (2.4)	375 (100.0)		357 (47.3)		305 (10.3)	292 (18.1)	267 (4.3)	217 (90.7)	204 (43.8)	169 (54.5)
MTM1-G per-TMS	842		593 (5.6)		505 (1.6)	465 (3.6)	449 (5.5)	375 (100.0)	391 (6.8)	361 (6.5)	345 (13.9)	305 (21.3)	292 (12.0)	271 (52.7)	217 (57.2)	204 (13.3)	169 (13.9)

* resulting ion is m/z 375, same mass origins from the glycosidic moiety

AG = Androsteroneglucuronide, EG = Etiocholanoloneglucuronide, TG = Testosteroneglucuronide, MTG = Methyltestosteroneglucuronide, MG = Metenoloneglucuronide, MTM1-G = 17a-Methyl-5b-androstane-3a,17b-diol-3-glucuronide

Table 2: Selected Fragment ions of methylated and trimethylsilylated steroid glucuronide conjugates

		Fragment ions (intensity in % relative to base peak)														
Analyte	**M_r**	M^+	M^+ - 15	M^+ - 264	M^+ - 264 -15	m/z 407	aglyc. + 30	aglyc.	M^+ - 264 - 15 - 160	m/z 317	m/z 305	aglyc. - 90	m/z 234	m/z 217	m/z 204	m/z 169
AG Me-TMS	768			504 (10.8)	489 (3.7)	407 (1.9)	375 (7.7)	345 (100.0)	329 (34.3)	317 (8.2)	305 (6.8)	255 (52.8)	234 (28.5)	217 (69.9)	204 (24.0)	169 (12.3)
d_5-AG Me-TMS	773	773 (7.9)	758 (3.3)	509 (19.6)	494 (6.0)	407 (1.3)	380 (17.6)	350 (100.0)	334 (13.9) 333 (25.3)	317 (10.3)	305 (6.9)	260 (9.2)	234 (17.8)	217 (79.4)	204 (48.3)	169 (8.3)
NorAG Me-TMS	754			490 (17.3)	475 (7.3)	407 (2.0)	361 (10.1)	331 (93.0)	315 (37.1)	317 (10.0)	305 (5.1)	241 (59.6)	234 (36.1)	217 (100.0)	204 (37.5)	169 (18.6)
EpiAG Me-TMS	768	768 (1.7)	753 (2.8)	504 (8.9)	489 (2.7)		375 (14.4)	345 (75.6)	329 (14.3)	317 (6.1)	305 (4.6)	255 (6.8)	234 (18.4)	217 (100.0)	204 (70.7)	169 (12.2)
EG Me-TMS	768	768 (6.9)	753 (4.8)	504 (14.4)	489 (3.6)		375 (12.0)	345 (100.0)	329 (17.3)	317 (10.2)	305 (5.1)	255 (9.3)	234 (16.3)	217 (57.7)	204 (39.5)	169 (7.0)
11β-OH-EG Me-	856	856 (9.3)	841 (16.6)	592 (18.2)	577 (2.3)	407 (3.3)		433 (23.8)	417 (4.4)	317 (25.3)	305 (9.4)	343 (58.3)	234 (58.3)	217 (100.0)	204 (44.2)	169 (70.0)
TG Me-TMS	766	766 (100.0)	751 (2.4)	502 (5.8)			373 (2.2)	343 (43.6)	327 (5.5)	317 (14.9)	305 (1.0)	253 (5.2)	234 (3.0)	217 (30.5)	204 (21.5)	169 (4.9)
NorTG Me-TMS	752	752 (100.0)	737 (2.6)	488 (3.6)			359 (1.2)	329 (17.9)	313 (1.4)	317 (7.7)		239 (1.6)	234 (2.2)	217 (13.2)	204 (13.6)	169 (4.7)
MTG Me-TMS	780	780 (4.1)						357 (100.0)	341 (1.8)	317 (35.0)	305 (2.0)		234 (2.2)	217 (18.2)	204 (9.4)	169 (9.2)
MG Me-TMS	780	780 (8.4)	765 (1.4)					357 (18.8)		317 (31.1)	305 (2.8)		234 (3.3)	217 (44.3)	204 (56.7)	169 (4.0)
MTM1-G Me-TMS	784						391 (1.2)	361 (6.6)	345 (1.8)	317 (30.4)	305 (4.7)	271 (70.3)	234 (26.1)	217 (100.0)	204 (55.6)	169 (3.2)

AG = Androsteroneglucuronide, EG = Etiocholanoloneglucuronide, TG = Testosteroneglucuronide, MTG = Methyltestosteroneglucuronide, MG = Metenoloneglucuronide, MTM1-G = 17a-Methyl-5b-androstane-3a,17b-diol-3-glucuronide