Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (7)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1999

T. KUURANNE, M. VAHERMO, A. LEINONEN, K. KUOPPASALMI, J. TASKINEN, E. ELOVAARA, R. KOSTIAINEN:
ESI-MS and MS/MS Detection of the Glucuronide Conjugate of 17□-methyl-5□-androstan-3□,17□-diol and its D3-labelled Analogue
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (7). Sport und Buch Strauß, Köln, (1999) 323-327

Kuuranne, T.[1,2], Vahermo, M.[1], Leinonen, A.[2], Kuoppasalmi,K.[2,3], Taskinen, J.[1], Elovaara, E.[4], Kostiainen, R.[1]

ESI-MS and MS/MS detection of the glucuronide conjugate of 17α-methyl-5β-androstan-3α,17β-diol and its d_3-labelled analogue

[1]Division of Pharmaceutical Chemistry, Department of Pharmacy, University of Helsinki, Finland, [2]Doping Control Laboratory, United Laboratories Ltd, Helsinki, Finland, [3]KTL, National Public Health Institute, Helsinki, Finland, [4]Finnish Institute of Occupational Health, Helsinki, Finland

INTRODUCTION

Androgenic anabolic steroids are the most commonly abused drugs in human sports [1]. During the last decades knowledge of steroid metabolism has dramatically increased and analytical methods to detect steroid abuse have been developed. It is known that most synthetic anabolic steroids are metabolised very extensively in human body by both phase I and phase II metabolic reactions [2]. Accordingly, in most case glucuronide conjugated metabolites should be used as target compounds in doping analysis. The doping analysis of anabolic steroids is traditionally performed by gas chromatographic separation coupled to mass spectrometric detection (GC/MS), which demands solid phase extraction, enzymatic hydrolysis of steroid conjugates, liquid-liquid extraction and further derivatization of free steroids. The methods used are very often laborious and time-consuming. For method validation and confirmation purposes the use of conjugated standards is recommended but unfortunately, only few conjugated reference substances are commercially available. In this study the glucuronide conjugate of 17α-methyl-5β-androstan-3α,17β-diol (metabolite of methyltestosterone, methandriol and metandienone) and its 17α-deuterated methyl analogue were synthesised in milligram amounts and characterised directly by ESI-MS and ESI-MS/MS. Suitable conditions for liquid chromatographic separation with MS detection (LC/MS) were also examined together with steroid glucuronide fragmentation.

EXPERIMENTAL

17α-methyl-5β-androstan-3α,17β-diol and its d_3-labelled analogue were synthesised chemically according to Shinohara et al. [3]. Further, the corresponding 3α-O-glucuronides were synthesised by enzyme-assisted synthesis using rat liver microsomal preparation as a source of UDP-glucuronosyltransferase enzymes, which catalyse conjugation with glucuronic acid [4]. After purification the analytes were dissolved in a mixture of methanol and water (50:50) with 7,5 mM ammonium acetate. For the positive ion mode the pH was adjusted to 4,1 to enhance the ionisation process, but in negative ion mode the pH was neutral. The samples were introduced by direct injection using 10 µl loop.

Mass spectrometric analysis was performed with Perkin-Elmer Sciex API 300 Triple quadrupole LC/MS/MS instrument (Sciex; Toronto, Canada) with electrospray ion source. The collision gas in MS/MS studies was nitrogen and the optimal collision energies in negative and positive ion mode MS/MS were determined at 45 eV and 10 eV, respectively. The LC used in the development of LC/MS method was Perkin-Elmer LC-200 series HPLC. The column was Waters Symmetry C18 (3,9 x 150 mm, particle size 5 µm). The mobile phase was a mixture of 20 mM ammonium acetate in water (A) and 90 % methanol in water (B). Isocratic flow (A:B=30:70) of 0,5 ml/min was split at the ratio of 1:100 before the inlet to the ion source. Injection volume was 20 µl.

RESULTS AND DISCUSSION

Enzyme-assisted synthesis with rat liver microsomal enzyme preparation was found as a suitable method to produce glucuronide conjugate of 17α-methyl-5β-androstan-3α,17β-diol and its d_3-labelled analogue in milligram amounts. On the basis of the literature [5] and our own glucuronidation studies with parent 17α-methyltestosterone, having only sterically hindered tertiary 17β-hydroxy group, the site of glucuronidation can be concluded to be a secondary 3α-hydroxy group in 17α-methyl-5β-androstan-3α,17β-diol.

Negative ion MS yielded deprotonated molecule ion $[M-H]^-$ and weak loss of water $[M-H-H_2O]^-$. In positive ion mode, formation of intensive adducts of sodium $[M+Na]^+$ and ammonium $[M+NH_4]^+$ were detected (Table I). In positive ion MS some fragmentation

occurred already with very low orifice voltage (10 V), mainly losses of water and ammonium adduct. The most intensive ions $[M-H]^-$ and $[M+NH_4]^+$ were selected as the parent ions in negative and positive ion MS/MS, respectively. In negative ion mode MS/MS the parent ion $[M-H]^-$ was very stable, only weak losses of water and losses of 118 and 178 amu were detected with the collision energy of 45 eV (Table II). Increasing the collision energy led to the domination of unspecific fragments from glucuronide moiety. However, positive ion MS/MS produced intense and structure characteristic product ions formed by loss of glucuronide moiety and losses of water from both conjugate and aglycone structure (Table III, Figures 2a and 2b).

LC/MS-analysis of steroid glucuronides could be performed in both negative and positive ion modes alike. In these preliminary studies significant difference between sensitivities of different polarities was not observed. For the steroid glucuronide under examination reasonable retention time of 7,4 minutes was achieved with good peak shape.

CONCLUSION

Enzyme-assisted synthesis is method worth while to consider in production of glucuronide conjugates of anabolic steroids.

Both negative and positive ion MS and MS/MS can be used in order to describe the fragmentation pattern of conjugated steroids. In positive ion mode the glucuronide conjugates of 17α-methyl-5β-androstan-3α,17β-diol and its deuterium labelled analogue have a strong tendency to form specific and moderately stabile fragments by cleaving glucuronide moiety and one or two water molecules. Raising the collision energy enhances the formation of unspecific fragments from glucuronide moiety.

Negative ion MS gives additional information about the steroid conjugate, but positive ion mode would be, however, a method of choice for screening purposes in the future. Positive ion MS/MS seems promising method for confirmation analysis if the orifice voltage and the collision energy are low enough to ensure the appearance of specific fragments. Good retention and reasonable analysis time could be achieved in LC/MS under optimised conditions.

REFERENCES

[1] Cowan, D. A. and Kickman, A. T., Doping in sport: Misuse, analytical tests, and legal aspects, *Clin. Chem.* 43:7 (1997) 1110-1113

[2] Schänzer, W. and Donike, M., Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites, *Anal. Chim. Acta* 275 (1993) 23-48

[3] Shinohara, Y. et al., Synthesis of deuterium labelled 17-methyl-testosterone, *Steroids* 44 (1984) 253-260

[4] Luukkanen, L. et al., Enzyme-assisted synthesis and structural characterization of nitrocatechol glucuronides, *Bioconjugate Chem.* 10 (1999) 150-154

[5] Schänzer, W., Metabolism of anabolic androgenic steroids, *Clin. Chem.* 42:7 (1996) 1001-1020

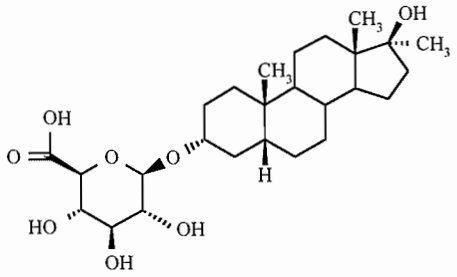

Figure 1. Structure of 17α-methyl-5β-androstan-17β-ol-3α-O-glucuronide.

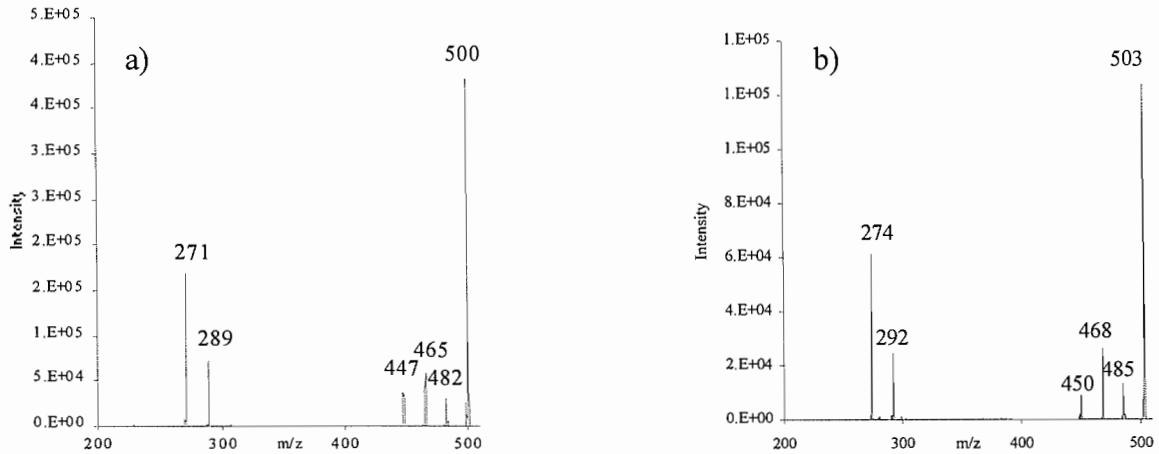

Figure 2. Positive ion ESI MS/MS spectra of a) 17α-methyl-5β-androstan-17β-ol-3α-O-glucuronide and b) its d_3-labelled analogue.

Table I. Negative and positive MS of 17α-methyl-5β-androstan-17β-ol-3α-O-glucuronide and its d_3-labelled analogue (relative abundance in parenthesis).

	5β-MTG	5β-LMTG
Negative ion MS		
$[M-H]^-$	481 (100)	484 (100)
$[M-H_2O]^-$	463 (27)	466 (26)
Positive ion MS		
$[M+Na]^+$	505 (22)	508 (77)
$[M+NH_4]^+$	500 (100)	503 (100)
$[M+NH_4-H_2O]^+$	482 (16)	485 (16)
$[M+H-H_2O]^+$	465 (4)	468 (9)
$[M+H-2H_2O]^+$	447 (5)	450 (6)

Tables II and III. Negative and positive MS/MS of 17α-methyl-5β-androstan-17β-ol-3α-O-glucuronide and its d_3-labelled analogue (relative abundance in parenthesis). Collision energy 45 eV in negative and 10 eV in positive ion mode.

II

	5β-MTG	5β-LMTG
Parent ion		
$[M-H]^-$	481 (13)	484 (10)
$[M-H-H_2O]^-$	463 (1)	466 (1)
$[M-H-118]^-$	363 (3)	366 (2)
$[M-H-178]^-$	303 (4)	306 (4)
Other ions	175 (4)	175 (4)
	157 (8)	157 (10)
	113 (63)	113 (81)
	95 (16)	95 (12)
	85 (94)	85 (92)
	75 (100)	75 (100)
	57 (15)	57 (14)

III

	5β-MTG	5β-LMTG
Parent ion		
$[M+NH_4]^+$	500 (100)	503 (100)
$[M+NH_4-H_2O]^+$	482 (9)	485 (9)
$[M+H-H_2O]^+$	465 (15)	468 (17)
$[M+H-2H_2O]^+$	447 (8)	450 (7)
$[M+H-Glu-H_2O]^+$	289 (17)	292 (14)
$[M+H-Glu-2H_2O]^+$	271 (41)	274 (45)