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Steroid glucuronides – synthesis and development of liquid chromatographic/ mass spectrometric analysis in the detection of doping in sport

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Introduction

The use of anabolic androgenic steroids (AAS) is a growing problem among athletes who wish to improve their performance in sports, and nowadays even among adolescents who wish to improve their appearances. Apart from the ethical points, the use of doping substances presents a serious health problem. Therefore, it is of utmost importance to improve the testing methods to enhance the detection of banned substances. In addition to receptor binding, metabolism and excretion are important for the action of AAS. As for most non-polar compounds, AAS are primarily modified by phase-I and phase-II metabolic reactions prior to their excretion in urine. In human metabolism of AAS, the major excreted metabolites are most often glucuronide conjugates [1,2]. Combination of liquid chromatography (LC) with mass spectrometry (MS) allows direct analysis of intact phase-II metabolites and along the improved methods also the use of solvents in the analytical process will be decreased and the strain on the environment diminished. This European Community -funded project, GRD2-2000-30023, belonged to scope of the Fifth Framework, under the program "Competitive and Sustainable Growth". The project was joint research between four partners from three EU-countries: three doping control laboratories (Athens, Cologne and Helsinki) and one division of a university (University of Helsinki). The project included 41 working months during the time period from August 1, 2001 to December 31, 2004. The scientific and technical objectives of this work were the development and comparison of chemical and enzymatic synthesis methods in the production of glucuronide conjugated anabolic androgenic steroids (AAS), and the use of them as reference material in analytical LC–MS/MS method development. The target compounds are shown in Figure 1.

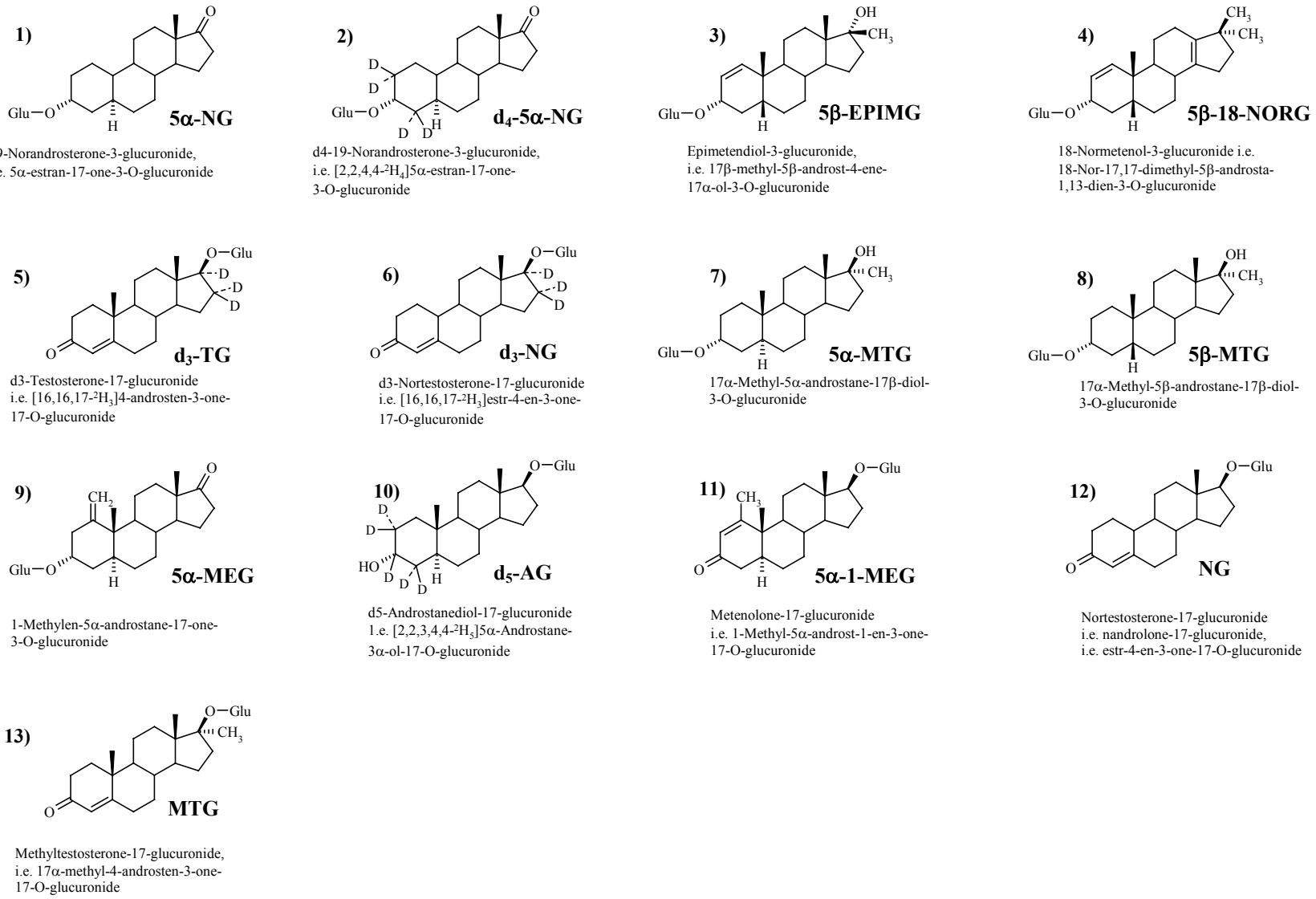

Figure 1. Structures and nomenclature of the target compounds of the project.

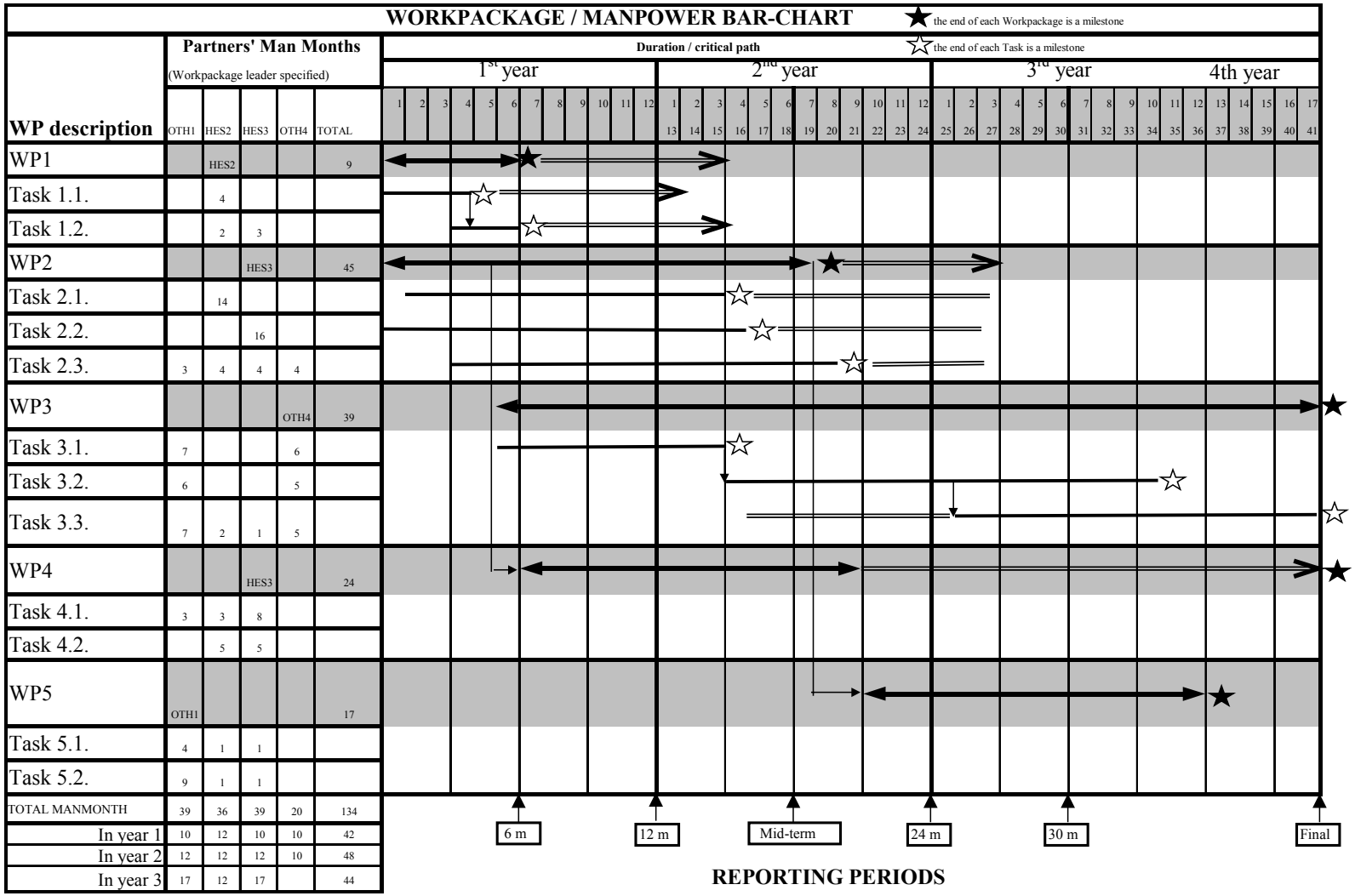

Figure 2. Workpackage and manpower bar-chart of the project.

Materials and methods

The workplan of the three-year project was divided in five sections (Workpackages, WP) containing the synthesis, purification and characterisation of the steroid metabolites (WP1-2), excretion studies and isolation of the excreted metabolites as well as comparison of the synthesised to the excreted urinary metabolites (WP3), development of LC–MS/MS methods (WP4) and finally inter-laboratory comparison (WP5) of the applicability of the developed LC–MS/MS method was tested in seven laboratories. Workpackages were divided further in tasks, which formed a uniform section from a laboratory work point of view. The project construction is shown as Workpackage and manpower bar-chart in Figure 2.

WP1: Synthesis and characterisation of phase-I metabolites

First phase metabolites (Table 1) were synthesised in Cologne laboratory by using classical chemical synthesis methods described in the literature [1,3]. After structural characterisation by GC/MS and ^{13}C-NMR these compounds were applied in the next stage of the project, i.e. starting material in synthesis of the AAS glucuronides.

Table 1. Chemically synthesised phase-I metabolites

17α-methyl-5α-androstan-3α,17β-diol	17α-methyl-5β-androstan-3α,17β-diol
17β-methyl-5β-androst-1-en-3α,17α-diol	1-methylen-5α-androstan-3α-ol-17-one
5α-estran-3α-ol-17-one	16,16,17-d_3-nandrolone
2,2,4,4-d_4-5α-estran-3α-ol-17-one	2,2,3,4,4-d_5-androstane-3α,17β-diol
16,16,17-d_3-testosterone	

WP2: Synthesis and characterisation of phase-II metabolites

Steroid glucuronidation was carried out by two parallel methods. The Cologne laboratory synthesised the conjugates in chemical syntheses by modified Koenigs-Knorr reactions [4,5], whereas the University of Helsinki performed the glucuronidation via enzyme-assisted pathway [6,7] using a pool of Arochlor-induced rat liver microsomes as the source of UGT-enzymes (Figure 3). Steroid glucuronide structures were characterised by ^{1}H-NMR and LC-MS/MS (positive and negative electrospray ionisation, ESI), and chemically synthesised AAS-glucuronides also by ^{13}C-NMR.

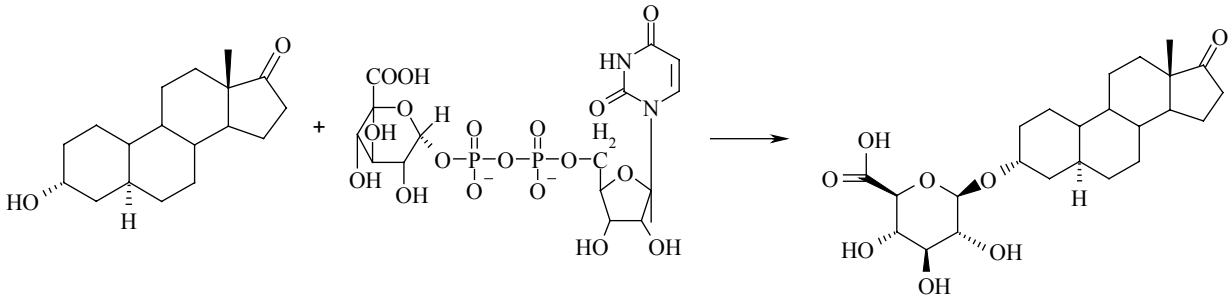

Figure 3. Enzyme-assisted glucuronidation of 5α-estran-3α-ol-17-one. In the reaction uridine-diphospho-glucuronic acid (UDPGA) serves as source of glucuronic acid moiety, and it is catalysed by rat microsomal uridine-diphospho-glucuronsyl transferases (UGTs).

WP3: Comparison of synthesised AAS glucuronide with authentic metabolites

In order to compare the synthesised metabolites to authentic ones, excretion studies were carried out in the doping control laboratories of Athens and Helsinki. The compounds administered, their doses and administration routes are shown in Table 2. Following the sample collection the metabolites were identified as steroid aglycones by using GC-MS procedures of routine doping control (enzymatic hydrolysis, liquid-liquid extraction and trimethylsilylation) and also as steroid glucuronides by using solid phase extraction (Isolute IST C18(EC) and Isolute IST PE-AX) and LC-MS fractionation (Zorbax 300SB-CN (4.6 x 150 mm, 5 µm), acetonitrile/ammonium acetate (pH 3.5) gradient, flow 1 ml/min) for the clean-up and isolation of AAS conjugates. The isolates were then compared to chemically synthesised references by LC-MS/MS.

Table 2. Compounds, their doses and administration routes within the excretion studies.

Compound	**Dose / Route of administration**
Nandrolone decanoate	50 mg / i.m.
Metandienone	20 mg / p.o.
Methyltestosterone	20 mg / p.o.
Metenolone acetate	25 mg / i.m.
Testosterone undecanoate	40 mg / p.o.

WP4: Development of LC-MS/MS methods for the analysis of AAS glucuronides in urine

Method development for the analysis of AAS glucuronides in urine consisted of the optimisation of sample preparation system, liquid chromatographic separation and mass spectrometric conditions (polarity and most representative MS/MS-transitions). The final method was validated by University of Helsinki and then applied also in the final stage of the

project – inter-laboratory comparison. Anabolic steroid glucuronides were spiked as a mixture into a blank urine, which was a pool of human urine spot samples. Detection limits, recovery (n=5), repeatability of injection (n=5), within-day repeatability (n=5) and between-day repeatability (n=5) were investigated and suggested LC-MS/MS procedure is presented in Table 3.

Table 3. Optimised LC-MS/MS procedure for AAS glucuronides is human urine.

Sample preparation	Oasis HBL (30 mg/ 1 ml) - 1st wash: 5% MeOH in H_2O - 2nd wash: 2% CH_3COOH in MeOH:H_2O (1:9, v/v) - elution: 2% NH_4OH in MeOH:H_2O (6:4, v/v)
Liquid chromatography	Phenomenex Luna CN (2 x 150 mm, 3 µm) - A: CH_3COONH_4, pH 3.5 (CH_3COOH) - B: ACN - 0.2 ml/min; runtime 25 min
Mass spectrometry	Applied Biosystems API3000 - ESI, positive ion mode, MRM; offset voltages 15-70 V - $[M+NH_4]^+$, $[M+H]^+$ $\rightarrow$ $[M+H-Glu]^+$, $[M+H-Glu-H_2O]^+$, $[M+H-Glu-2H_2O]^+$

WP5: Inter-laboratory comparison of LC-MS/MS method

Participants of the inter-laboratory comparison consisted of seven laboratories equipped with LC-MS/MS instrumentation (six triple quadrupole instruments, various models from three manufacturers and one ion trap instrument). The laboratories were provided with reference material (5α-NG, 5α-MEG, 5α-MTG, and 5β-MTG) for set-up of the method, six excretion urine samples (nandrolone, metenolone and methyltestosterone, each separately in high and low concentration), three blank urine samples, and three blank urine samples fortified with 0.4 nmol/ml of each reference compound. Sample preparation protocol, LC conditions as well as data acquisition sheet example were also suggested and the laboratories were requested 1) to optimise MS-conditions for each compound, 2) to prepare the samples in triplicates (single injection of each), 3) to measure peak areas and heights of each fragment ion and 4) to indicate each modification to suggested procedure. Relative abundances of employed ion transitions were determined, and results of all spiked urine samples and all excretion study

urine specimens were compared and evaluated according to WADA guidelines (WADA 2003).

Results and Discussion

WP1: Synthesis and characterisation of phase-I metabolites

All AAS phase-I metabolites synthesised within the project yielded 50-300 mg of the desired material with purities greater than 90% as determined by GC-MS. The degree of deuteration of the particular compounds (d_3-testosterone, d_4-norandrosterone and d_5-androstandiol) ranged from 94-96%. The only exception from the original project plan was the synthesis of 18-nor-metabolite of metandienone, which was not successful due to complicated synthesis and purification steps with low yields and the compound was excluded from further project.

WP2: Synthesis and characterisation of phase-II metabolites

Both chemical and enzymatic syntheses were carried out in order to compare the pathways in the production of AAS conjugates. The synthesis yields are shown in Table 4. The amounts of AAS glucuronides produced by chemical synthesis method were typically between 20 to 50 mg the yield being 15-40%. Enzymatic synthesis method produced significantly lower amounts (1-7 mg), the yields being 15-50%. However, chemical synthesis method is relatively laborious and small amounts of AAS glucuronides can be produced rapidly by using enzymatic synthesis if induced rat microsomes are available. Larger amounts of glucuronides could be produced by enzymatic manner by increasing synthesis volume. However, this would lead to increased use of expensive co-substrate (UDPGA) and increased expenses of enzymatic synthesis.

WP3: Comparison of synthesised AAS glucuronides with authentic metabolites

The final step in the structural analysis of the synthesised metabolites was to compare them with authentic metabolites found in urine after administration of the drug. The following conjugated metabolites were isolated both as aglycones and conjugates: 5α-NG, 5α-MTG, 5β-MTG, 5β-EPIMG, 5α-MEG and 5α-1-MEG. Two metabolites, (NG and MTG) could not be isolated in conjugated form due to their low concentration in urine. All measurements confirmed the synthesised AAS glucuronides identical with the authentic metabolites purified from urine samples.

Table 4. Synthesis yields of AAS glucuronides (see Figure 1 for abbreviations).

Compound	Chemical Synthesis	Enzymatic Synthesis	Compound	Chemical Synthesis	Enzymatic Synthesis
5α-NG	15 mg	1 mg	5β-MTG	20-40 mg	3 mg
d_4-5α-NG	25 mg	2 mg	5α-MEG	20-40 mg	3 mg
5β-EPIMG	10 mg	1 mg	d_5-AG	50 mg	2 mg
d_3-TG	20 mg	7 mg	5α-1-MEG	20-40 mg	2 mg
d_3-NG	20 mg	4 mg	NG	215 mg	4 mg
5α-MTG	20-40 mg	2 mg	MTG	10 mg	4 mg

WP4: Development of LC-MS/MS methods for the analysis of AAS glucuronides in urine

Recovery of the SPE extraction varied between 89-100 %. The repeatability of analyte peak area varied within day from 2 to 10 % (relative standard deviation) and between days from 8 to 32 %. The repeatability of analyte peak height varied within day from 3 to 12 % and between days from 8 to 28 %. The repeatability of retention time was below 0.1 % within day and below 1 % between days. Detection limits in urine were from l ng/ml to 40 ng/ml, which corresponds to 20-800 ng/ml in injected solution. Detection limits in buffer were from 0.1 to 15 ng/ml, which corresponds to 2-300 ng/ml in injected solution. The results obtained show that the developed LC/MS provides a reliable method for the detection of AAS glucuronides. However, the limits of detection measured with standards prepared in pure solvent were one-two orders of magnitude lower than those obtained with urine samples. This shows that the selectivity of the method is limited due to endogenic compounds in urine samples. This is true in spite of use of tandem mass spectrometry.

WP5: Inter-laboratory comparison of LC-MS/MS method

According to WADA guidelines for the confirmation of prohibited compounds, 68% of urine samples containing 5α-NG, 71% containing 5β-MTG, and 95% containing 5α-MEG tested positive for the respective metabolites. In addition, two laboratories succeeded to determine the metabolite 5α-MTG present in very low concentration in excretion study urine specimens. Problems were reported from two laboratories regarding the chromatographic separation of either signals interfering with the analyte of interest (here 5α-NG) or marginal resolution of the metabolites of methyltestosterone. Moreover, the limited stability of ammonium adduct precursor ions in ion trap mass spectrometers appeared to be a major issue for efficient

MS/MS experiments. For the metenolone metabolite 5α-MEG, the protonated precursor ion was generated yielding results comparable to triple quadrupole mass spectrometers, but with 5α-NG and 5β-MTG the ammonium adduct had to be isolated and fragmented by collision-induced dissociation resulting in inconclusive sets of data.

Conclusions

All the compounds within the project plan were possible to conjugate by chemical and enzymatic synthesis using rat liver microsomes as the source of UGT enzymes. The amounts of AAS glucuronides produced by chemical synthesis method were typically between 20 to 50 mg the yield being 15-40%. Enzymatic synthesis method produced significantly lower amounts (1-7 mg), the yields being 15-50%. However, chemical synthesis method is relatively laborious and small amounts of AAS glucuronides can be produced rapidly by using enzymatic synthesis if induced rat microsomes are available. Larger amounts could be produced by enzymatic manner by increasing synthesis volume. However, this would lead to increased use of expensive co-substrate (UDPGA) and, consequently, to increased expenses of enzymatic synthesis. As a conclusion chemical synthesis is better suited to produce AAS glucuronides than enzymatic synthesis. However, if chemical synthesis will fail, the enzymatic synthesis can be used instead. The experiments performed for characterization AAS glucuronides showed them to have aimed structures. Comparing the synthesised glucuronides with authentic metabolites obtained in excretion studies after administering the respective anabolic steroid further corroborated the correct structures.

The developed assay is transferable to other laboratories equipped with different LC-MS/MS instruments. An LC-MS/MS method adaptable to both screening and confirmation was developed. Careful sample purification is essential, for the purpose SPE proved most suitable. However, the limits of detection measured with standards prepared in pure solvent were one-two orders of magnitude lower than those obtained with urine samples. This shows that the selectivity of the method is limited due to endogenic compounds in urine samples in spite of use of tandem mass spectrometry. The inter-laboratory testing showed that the developed assay is transferable to other laboratories equipped with different LC-MS/MS instruments. Triple quadrupole mass spectrometers appear advantageous over ion trap analysers owing to the unstable nature of adduct ions formed during atmospheric pressure ionisation. Both

ionisation modes, i.e. ESI and APCI, can be employed for the detection of steroid glucuronide conjugates, and main reasons for inconclusive results are based on chromatographic issues.

Acknowledgments

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References

1. Schänzer W, Donike M. Metabolism of androgenic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Anal. Chim. Acta* 1993; **275**: 23.

2. Schänzer W. Metabolism of anabolic androgenic steroids. *Clin. Chem.* 1996; **42**: 1001.

3. Schänzer W, Donike M. Synthesis of deuterated steroids for GC/MS quantification of endogenous steroids. In Donike M (Ed.), *Proceedings of the 12th Cologne Workshop in Dope Analysis*, 1994, Sport und Buch Strauß, Köln, 1995: 93.

4. Thevis M, Opfermann G, Schmickler H, Schänzer W. 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. *J. Mass Spectrom.* 2001; **36**: 159.

5. Thevis M, Opfermann G, Schmickler H, Schänzer W. Mass spectrometry of steroid glucuronide conjugates. II. Electron impact fragmentation of 3-keto-4-en- and 3-keto-5α-steroid-17-*O*-β glucuronides and 5α-steroid-3α,17β-diol 3- and 17-glucuronides. *J. Mass Spectrom.* 2001; **36**: 998.

6. Kuuranne T, Aitio O, Vahermo M, Elovaara E, Kostiainen R. Enzyme-assisted synthesis and structure characterization of glucuronide conjugates of methyltestosterone (17α-methylandrost-4-en-17β-ol-3-one) and nandrolone (estr-4-en-17β-ol-3-one) metabolites. *Bioconjugate Chem.* 2002; **13**: 194.

7. Kuuranne T, Kurkela M, Thevis M, Schänzer W, Finel M, Kostiainen R. Glucuronidation of anabolic androgenic steroids by recombinant human UDP-glucuronosyltransferases. *Drug Metab. Dispos*. 2003; **31**: 1117.