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Metabolism of "new" anabolic steroids: Development of *in vitro* methodology in metabolite production and analytical techniques

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Abstract

The aim of the project was to develop a flexible *in vitro* synthesis procedure, which can be applied to study and predict the metabolic patterns of new derivatives of anabolic androgenic steroids (AAS) with respect to most prominent target compounds for doping control purposes. Microsomal and S9 fraction of human liver preparations were used as source of metabolizing enzymes, and the co-substrates of the synthesis mixture were selected to favor phase-I metabolic reactions and glucuronidation as phase-II conjugation reactions.

The correlation between *in vitro* metabolism of human microsomes and *in vitro* excretion studies in human was compared with model compounds and subsequently, the applicability of the *in vitro* model for prediction of AAS metabolic pathways for new doping agents was evaluated. Analytical methods based on liquid chromatographic-mass spectrometric (LC/MS) instrumentation were developed, relying on the electrospray ionization (ESI). With respect to routine applications a comparison was performed between two instrument set-ups (both with triple quadrupole analyzers) to reveal those potential differences that should be taken into account when transferring the LC-MS/MS method from one instrument to another.

All the AAS examined within this study were successfully metabolized using an *in vitro* model, hydroxylation, reduction and glucuronide-conjugation being the most prominent reaction pathways. Due to an excessive amount of the steroid aglycone, combined fraction of

hepatic enzymes and relatively extensive reaction times the number of *in vitro* formed metabolites was typically higher than those observed from *in vivo* excretion urine samples of corresponding AAS. The need of parallel application of both gas chromatography-mass spectrometry (GC/MS) and LC/MS was observed, especially when analyzing non-conjugated phase-I AAS metabolites with completely saturated A-ring structure.

Introduction

Due to excessive metabolism that AA undergo in human body only a little or no unchanged steroid is excreted in the urine, which requires careful elucidation of metabolic pathways to ensure correctly targeted analysis in doping control as well as fast and flexible response to analytical challenges. *In vivo* excretion studies are one approach to the study of metabolic pathways, but require a heavy administrative and medical workload when applied to human subjects. Severe problems may also be encountered with designer steroids or a pipeline product, when there is neither legal pharmaceutical reference product nor sales permission available. The aim of this study was to develop a flexible *in vitro* procedure to screen the structure-metabolic patterns of relatively new AAS with respect to most prominent target compounds for doping control purposes.

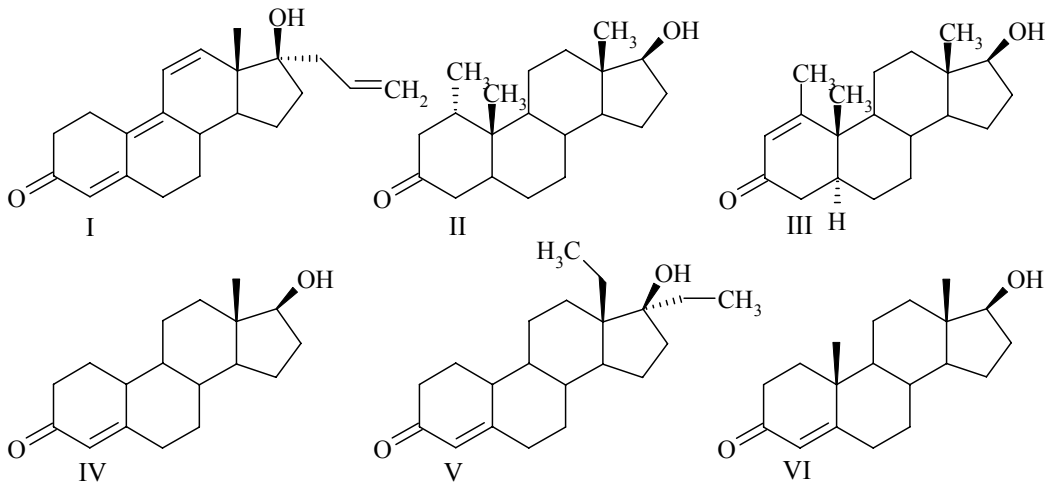

Figure 1. Structures of the steroids within the study: I allyltrenbolone, II mesterolone, III methenolone, IV nandrolone, V norbolethone, and VI testosterone.

Materials and Methods

Compounds within the study are presented in Figure 1. Metabolic reactions were activated by using commercially available human microsomal and S9 fractions of enzymes to promote subsequently phase-I and glucuronidation in a single experiment. Incubated samples and urine samples of excretion studies were purified by solid phase extraction and analyzed by LC-MS(/MS) methods applying positive ion mode ESI. This study is the second part of our recent WADA-funded project, and the details of enzyme assays, sample clean-up and analytical parameters are described elsewhere (Kuuranne et al. 2006 and Kuuranne et al. 2008). The analytical GC/MS procedure for the detection of steroid metabolites was based on the routine screening of anabolic steroids.

Excretion study samples were collected from male volunteers according to following dosing and collection times. The studies were approved by the local ethical committee.

Compound	**Preparation**	**Dose**	**Urine fraction**
Methenolone	Primobolan®	1 x 25 mg; p.o.	13-54 hrs
Mesterolone	Proviron®	1 x 25 mg; p.o.	0-42 hrs
Nandrolone	Deca-Durabolin®	50 mg; i.m.	7-15 hrs

Two different LC-MS/MS set-ups with triple quadrupole analyzers were applied to the instrument comparison part of the study.

Set-up	**LC; manufacturer**	**MS; manufacturer**
A	Surveyor; Thermo Finnigan	TSQ Quantum; Thermo Finnigan
B	1100 Series LC; Agilent	API3000; Applied Biosystems

Results and Discussion

In vitro assays

The aim of the project was to combine both phase-I and phase-II metabolic reactions in one assay, using the intact steroid as the substrate. The incubation conditions of the assay (NADPH and UDPGA solely added as a co-substrates, but not PAPS) were selected to allow only phase-I reactions and glucuronidation, which is the main conjugation pathway for steroids in human body. Although the set of model compounds applied to this study represented structurally variable group of steroids they were all metabolized using the same *in vitro* model.

Reduction and hydroxylation were the main phase-I reaction pathways for all the substrates (Table 1), but also oxidized metabolites were detected from methenolone, nandrolone and testosterone. Incubations with allyltrenbolone and mesterolone resulted also in the formation of dihydroxylated metabolites (M+2+2*16 and M+2*16). Substrate concentration of *in vitro* assay was relatively high (50 μM) to allow the formation of detectable amounts of steroid metabolites. The relative abundance of free phase-I metabolites was low, the non-reacted steroid substrate being typically the most abundant analyte detected. Norbolethone was an exception, as one hydroxylated metabolite (M+16) was most abundant analyte of the *in vitro* assay. Theoretically, hydroxylation should enhance the glucuronidation activity of the analyte by exposing new and potentially stereochemically better available sites for *O*-glucuronidation. In most cases these hydroxylated metabolites were indeed further conjugated, proving the applicability of this combined *in vitro* synthesis set-up.

Conjugation was detected also with hydrogenated metabolites, but in the case of oxidation the number of potential attachment sites also decreases and in our assay we did not observe any conjugated oxidized metabolite. Glucuronidation was the only supported reaction for the phase-II metabolism and it was also detected to occur directly for all the substrates. This indicates that the steric hindrance of various types of 17α-alkylation, at least in *in vitro* conditions, is not too high and 17β-hydroxyl group is amenable for UGT enzymes and allows the *O*-glucuronidation. This is also in good agreement with our earlier results with methyltestosterone (Kuuranne et al. 2003a), which was glucuronidated by rat liver microsomal UGTs.

In vitro assay consisted of combined microsomal and S9 fraction of human liver enzymes. The enzymatic activity of drug and steroid metabolizing CYP450 isoforms 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A of the commercial human microsomal and S9 preparations were controlled and determined by the provider, but in addition to those the pool of tissue preparations also contains several other unidentified enzymes which participate in the metabolic reactions (e.g. UGT isoenzymes which are responsible of the glucuronidation). In our procedure the reactions were standardized with respect to total protein concentration of the enzymatic preparations. β-glucuronidase inhibitor, *D*-saccharic acid-1,4-lactone, was added to the reaction mixture to protect the glucuronide conjugates potentially formed in incubation, but the effect of other catalysts or inhibitors was not tested

within this project. Steroids, as extremely non-polar analytes, represent probably the most difficult type of compounds for the *in vitro* assay, as the enzymatic protein does not tolerate high concentration of organic solvents. The applied amount (10 %) of DMSO is a compromise between insufficient solubility of the substrate and precipitation of the protein. For more polar analytes the synthesis yields may be higher, although the enzyme-assay should be optimized to exclude both substrate and product inhibition, especially if the aim of the process is to synthesize metabolites of a certain steroid in higher amounts than in these small-scale studies. Other parameters to optimize then are also incubation time of phase-I and phase-II steps, co-substrate concentration and protein concentration as well.

In vitro-in vivo correlation

All the metabolites observed in LC-MS/MS and GC/MS studies of *in vivo* methenolone sample were also detected from the *in vitro* sample, which included also several additional metabolites. The most remarkable deviation was the *in vitro* formation of several hydroxylated metabolites (M+16) and their glucuronide-conjugates, although the relative abundance of these metabolites was less than 8 %. In the analysis of methenolone metabolites it was also obvious that only the combination of LC-MS/MS- and GC/MS-based methods may result into relevant data on the complete metabolic behavior, the former providing the data on intact glucuronide conjugates, the latter of those phase-I metabolites for which proton affinity is not sufficient for ESI.

Mesterolone was more extensively metabolized than methenolone, and *in vivo* study and LC-MS/MS analysis of mesterolone excretion study resulted in the detection of six glucuronide-conjugated metabolites (Table 1). These all were detected also from *in vitro* samples, which resulted in the formation of three additional glucuronide-conjugated metabolites with different combinations of hydrogenation and hydroxylation as phase-I reactions. In the free fraction of *in vivo* study samples three metabolites were observed, from which the hydrogenated and oxidized metabolite (M+2-2) was not detected in enzyme-assisted synthesis. Two additional hydrogenated (M+2) and one dihydroxylated (M+2*16) mesterolone metabolites were formed in *in vitro* assay in comparison to *in vitro* results.

From *in vivo* excretion study sample of nandrolone two phase-I metabolites and two glucuronide-conjugated metabolites were detected. Despite of the other phase-I metabolite,

these all were also present in the *in vitro* assay. With the corresponding study of methyltestosterone during the first project year (Kuuranne et al 2006), these isomeric 5α/5β-metabolites were produced also *in vitro*, which gives evidence of the activity of 5α- and 5β-reductase enzymes in the tissue preparation and of the capability of simulating also these metabolic reactions, which are typical of the steroids with 3-one-4-ene conjugated double-bond structures. *In vitro* formed metabolites of nandrolone (Table 1) were numerous in comparison to *in vivo* study, the formation of dihydrogenated metabolites (also as glucuronide conjugates) and hydroxylated metabolites being the most significant deviation between these two assays.

As a general conclusion from the *in vitro–in vivo* –correlation it can be drawn that *in vitro* assay yields higher number of metabolites than seem to form in the human body. However, the main metabolites of this *in vivo* experiment were among those metabolites synthesized in the enzyme-driven process. Excretion studies were now performed as single administration to one individual, and thus also inter-individual variation in metabolism and time of sample collection (short-term vs. long-term metabolites) are factors influencing the correlation.

High concentration of steroid substrate is not totally consumed in the overall process, and the presence of the non-polar starting material causes also the most obvious difference between *in vitro* and *in vivo* samples. Being extremely non-polar in nature, steroid substrates are extensively metabolized and typically the parent compound is not detected in excretion studies. Also assay-dependent factors include the lack of enterohepatic circulation, which involves also extra-hepatic enzymes e.g. intestinal UGT-enzymes in the overall metabolic processes.

Combining the sulfonation as one part of the total *in vitro* assay could enable the epimerization reactions of 17α-methyl-17β-hydroxyl structured steroids and could thus also enhance the *in vitro-in vivo* correlation. In theory, our *in vitro* assay contains all the elements for sulfonation, except the co-substrate PAPS, as S9 fraction of enzymes includes the cytosolic SULTs. From this family of enzymes SULT2A1 subfamily has been reported to display substrate specificity toward an array of e.g. hydroxysteroids.

Method transfer

As suggested, based on an extensive double-bond conjugation and thus high proton affinity, e.g. allyltrenbolone, its six phase-I metabolites and two phase-II metabolites were all detected almost completely as protonated molecule $[M+H]^+$, and the results from two different instruments were also closely similar.

ESI behavior of methenolone metabolites gave a strong indication of the stability of 1-ene-3-one structure towards metabolic enzyme reactions, as all but one (hydroxylated metabolite) of the detected metabolites were present mainly as protonated molecule $[M+H]^+$. However, compared to allyltrenbolone, there was now a more clear difference between two different instrument set-ups with respect to the distribution of ionized species, as with instrument set-up A methenolone metabolites were detected mainly as $[M+H]^+$, whereas with set-up B more abundant $[M+NH_4]^+$ and especially $[M+Na]^+$ were formed (Table 2).

Table 2. Distribution of ESI ion species with different LC-MS instruments.

Compound	**$[M+H]^+$**	**$[M+NH_4]^+$**	**$[M+Na]^+$**
Methenolone	*m/z* 303	*m/z* 320	*m/z* 325
Set-up A	100 %	7 %	n.d.
Set-up B	100 %	68 %	20 %
Hydroxylated metabolite	*m/z* 319	*m/z* 336	*m/z* 341
Set-up A	100 %	21 %	n.d.
Set-up B	100 %	61 %	19 %
Glucuronide-conjugate	*m/z* 479	*m/z* 496	*m/z* 501
Set-up A	100 %	14 %	5 %
Set-up B	99 %	100 %	29 %

The main focus of the analytical part of the project was to examine the applicability of LC-MS/MS method in the detection of phase-I and phase-II metabolites of AAS. One LC gradient was applied to the detection of *in vitro* synthesized AAS metabolites. Despite of the specificity of MS/MS detection, the screening of unknown metabolites requires also reliable chromatography to avoid co-eluting peaks. In our study the resolving power of C_{18}-based column was observed limited to distinguish between methenolone and its metabolite, 3α-hydroxy-1-methylen-5α-androstan-17-one, which share the same precursor ion in LC-MS/MS and as a further step more selective phase material, e.g. cyano-substituted material, could be tested.

Despite of the initial plan to carry out all the analysis by LC/MS-based methods it was mandatory to apply both GC/MS and LC-MS/MS to ensure the detection of wide range of AAS metabolites. This should be especially taken into account in the case of potential formation of phase-I AAS metabolites with completely saturated A-ring structure. Proton affinity of those metabolites is too low to yield $[M+H]^+$ and the analyte is not detectable in LC-MS/MS analysis. However, conjugation with glucuronic acid enhances the ionization efficiency and phase-II metabolites of those same compounds are detected. The situation is completely opposite for the AAS metabolites with extensive conjugated double bond systems among A-, B- and C-rings (e.g. allyltrenbolone and its phase-I metabolites), which are problematic or impossible to monitor in GC/MS analysis. Due to charge delocalization and high proton affinity, LC-MS/MS is a method of choice for analysis of those AAS metabolites. Soft ionization technique, ESI, combined with LC separation also allows the direct detection of intact glucuronide-conjugated AAS metabolites.

Inter-laboratory comparison of the LC-MS-based method was carried out with two instrument set-ups, both applying ESI and triple quadrupole analyzer to examine the ease and critical points of method transfer. In general, the distribution of ion species was well in accordance with earlier results (Kuuranne et al. 2000, Kuuranne et al. 2003b) and depends on the proton affinity (i.e. saturation level or conjugated double bond systems) of the steroid metabolite. Systematic difference in the adduct formation and distribution of different adducts was observed between ion sources, which phenomenon should be kept in mind when screening the *in vitro* metabolic assay samples of new compounds, in order to allow the monitoring of not only $[M+H]^+$ ions but also the potential adduct ions of metabolites.

Based on the data obtained from the studies with two different instruments, it may be concluded that there indeed are differences between overall ionization processes, most obviously caused by ion source construction (e.g. orientation of gas flow and applied heat) or the ion transfer system of MS-instrument. Here the differences were in the orientation of the ion spray with respect the collection of the ions inside the MS instrument (set-up A: 90° off-axis orthogonal structure, set-up B: direct on-axis structure). Both source constructions had two separate and heated gas flows to assist the spraying and drying of the LC-eluent. From these two, set-up B allowed more extensive formation of NH_4^+ and Na^+ adducts. Despite the similar initial ESI process behind these hardware-attached features should be taken into

account and also clarified with test compounds when transferring the method to a new instrument.

This instrument comparison was aimed at the monitoring of the distribution of ionized species and thus run only in MS-mode, but when an MS/MS method is applied also the collision offset voltage level should be evaluated individually for each instrument, as the differences may occur between the different collision gases (e.g. argon and nitrogen). Adduct formation may be a critical issue also when considering the application of different MS-analyzers in the detection of adducts. Based on our earlier experience, triple quadrupole analyzer is superior in comparison to ion trap instrument, in which the kinetic MS^n isolation process may be already too high to maintain the adduct, which leads into a dramatic decrease of the sensitivity.

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Table 1. Number of each type of *in vitro* formed metabolites (*number of corresponding in vivo formed metabolites in parenthesis*).

Phase-I metabolism	**Reaction**	**Allyltrenbolone**	**Mesterolone**	**Methenolone**	**Nandrolone**	**Norbolethone**	**Testosterone**
Hydroxylation	M+16	3	1 *(1)*	4	2	3	3
Dihydroxylation	M+2*16		1				
Hydrogenation	M+2		3 *(1)*	1 *(1)*	1 *(2)*		
Dihydrogenation	M+2*2				1		
Oxidation	M-2			1 *(1)*	1		1
Hydrogenation and hydroxylation	M+2+16				1		
Hydrogenation and dihydroxylation	M+2+2*16	2					
Hydrogenation and oxidation	M+2-2	1	*(1)*				
Phase-II metabolism							
Glucuronidation	M+176	2	2 *(2)*	1	1	2	1
Hydroxylation + glucuronidation	M+16+176		1 *(1)*	2		2	
Dihydroxylation + glucuronidation	M+2*16+176		1 *(1)*				
Hydrogenation + glucuronidation	M+2+176		3 *(2)*		2 *(2)*		1
Dihydrogenation + glucuronidation	M+2*2+176				2		1
Hydrogenation, hydroxylation + glucuronidation	M+2+16+176		1				
Hydrogenation, dihydroxylation + glucuronidation	M+2+2*16+176		1				