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

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Introduction

Anabolic-androgenic steroids (AAS) are synthetic testosterone derivatives, which are designed to maintain the anabolic (beneficial effects) and to minimise the androgenic (side effects) activities of the endogenous prototype. In addition to conventional and *bona fide* pharmaceutical manufacturers, AAS products are provided in the market by a wide variety of illicit laboratories, and as recently experienced, "tailor made" designer steroids are produced to avoid the detection of routine doping control procedures. Due to excessive metabolism that AAS undergo in human body the prediction of metabolic reactions is a key issue to ensure fast and flexible response to analytical challenges.

The aim of this on-going WADA-funded project is to develop flexible *in vitro* procedure to study and predict the metabolic patterns of new AAS with respect to most prominent target compounds for doping control purposes. The correlation between *in vitro* metabolism of human hepatic enzymes and human *in vivo* excretion studies is examined with model compounds (e.g. methyltestosterone and methandienone) whose metabolism is already published [1,2]. Then the applicability of *in vitro* model for the prediction of AAS metabolic pathways is evaluated for "new" anabolic steroids. The steroids within the project scope are described in Figure 1. This project was planned for two years and this paper is a summary report after the research work of the first year.

Experimental

For the *in vitro* experiments enzyme assays for the combined and subsequent phase-I and phase-II metabolic reactions were incubated in a total volume of 100 µl and the components of the matrix are given in Table 1.

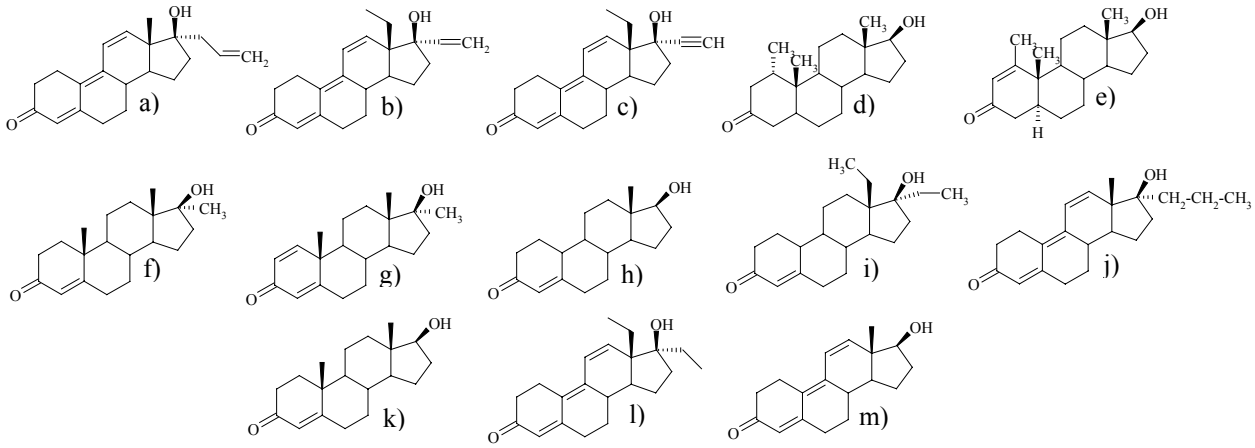


Figure 1. Structures of the steroids within the project: a) allyltrenbolone, b) dihydrogestrinone, c) gestrinone, d) mesterolone, e) methenolone, f) methyltestosterone, g) methandienone, h) nandrolone, i) norbolethone, j) propyltrenbolone, k) testosterone, l) tetrahydrogestrinone, and m) trenbolone.

Enzymatic protein was combined microsomal and S9 fractions of commercially available human liver homogenates, pool of five individuals (Gentest, Woburn, MA). Specific enzyme activities of preparations were not measured, but protein concentration was used to standardize the amount of enzymes in the syntheses. Phase-I reaction was initiated with NAPHD addition, carried out for 4-5 hours in a dry bath of 37°C. Then UDPGA was added to initiate glucuronidation reaction, the incubation period of which was performed for 16-17 hours. The overall reaction was terminated by protein precipitation (4 M perchloric acid, 10 µl), and transferring the tubes into ice bath. The tubes were then centrifuged (16 100 x g, 10 min) and supernatant was purified by SPE (OASIS®, 30 mg, Waters, USA) according to the procedure described in Figure 2 in detail. To examine *in vitro-in vivo* correlation, excretion study samples collected after intake of gestrinone, methandienone, or methyltestosterone (Table 2) were purified and analysed similarly to *in vitro* incubation samples.

Table 1. Composition of *in vitro* incubation matrix.

Compound	**Concentration**	**Compound**	**Concentration**
Phosphate buffer pH 7.4	50 mM, 5 mM $MgCl_2$	Substrate (steroid)	50 µM
Saccharic acid lactone	5 mM	NADPH	5 mM
Enzymatic protein	0.5 mg/ml	UDPGA	5 mM

Table 2. Excretion study samples.

Gestrinone	Dimetriose®	1 x 2.5 mg, p.o.	10-72 hrs, 8 fractions
Methandienone	Dianabol®	3 x 10 mg, p.o.	18-48 hrs, one fraction
Methyltestosterone	Neo-Hombreol®	3 x 25 mg, p.o.	18-48 hrs, one fraction

Mass spectrometer was TSQ Quantum triple quadrupole (Thermo Finnigan) applying positive ion ESI. The samples were first screened in SCAN mode method (*m/z* 200-650), and then precursor ion scan of ionized glucuronide moiety (*m/z* 177) as well as neutral loss of *m/z* 176 were also carried out. After the preliminary screening step the potential peaks were also analysed by LC-MS/MS. Details of LC-MS/MS parameters are listed in Table 3.

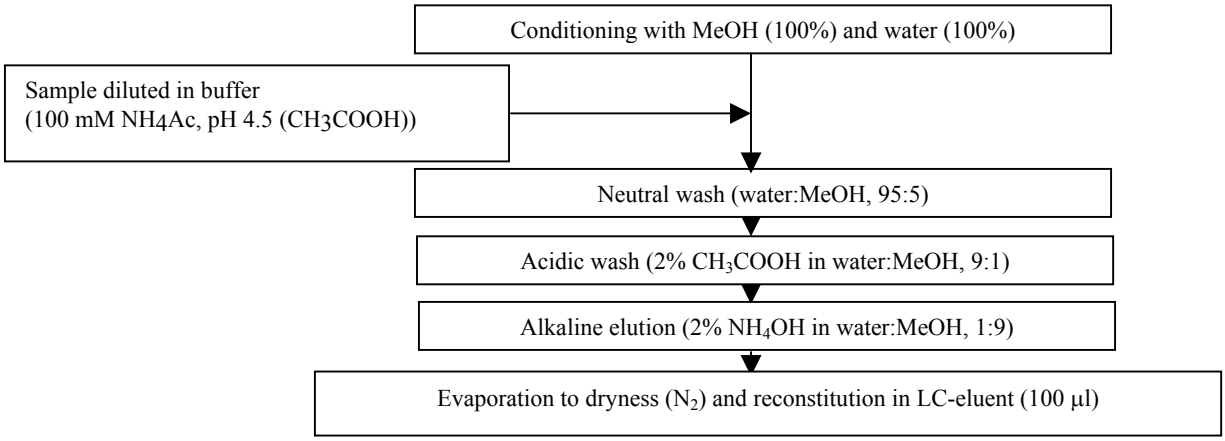


Figure 2. SPE clean-up procedure.

Table 3. LC-MS/MS parameters.

Column	Agilent Zorbax Eclipse, XDB-C18, 2.1x50 mm, 3.5 µm	**Eluent A**	2.5 mM ammonium acetate, 0.1 % acetic acid
Pre-column	Agilent Eclipse, XDB-C8, 2.1x12.5 mm, 5 µm	**Eluent B**	Methanol
Flow rate	200 µl/min	**Gradient program**	A: 75 %, 10 % /min ⇨ 5 % (3 min) ⇨ 75 % (3 min)
Injection	10 µl	**Ionization**	Positive ion electrospray 4500 V

Results and Discussion

Methyltestosterone

Methyltestosterone (MT) and methandienone (MDN) were pilot compounds in the project. With MT formation of two main metabolites indicated in literature (1,2), 17α-methyl-5α-androstan-3α,17β-diol (5α-MT) and 17α-methyl-5β-androstan-3α,17β-diol (5β-MT), was detected in enzymatically driven *in vitro* synthesis. In the experiments reactions using either microsomal enzymes or the enzymes of S9 fraction a clear difference was detected in relative formation of 5α- and 5β-isomers – S9 fraction favouring 5β-MT, whereas the use of only microsomal enzymes resulted in the higher abundance of 5α-MT isomer. In order to

maximize the formation of various metabolites, the combined fraction of S9 and microsomal enzymes was included in the routine metabolic *in vitro* metabolic process.

With MT correlation to *in vivo* sample was good, as both main metabolites were detected as glucuronide conjugates.

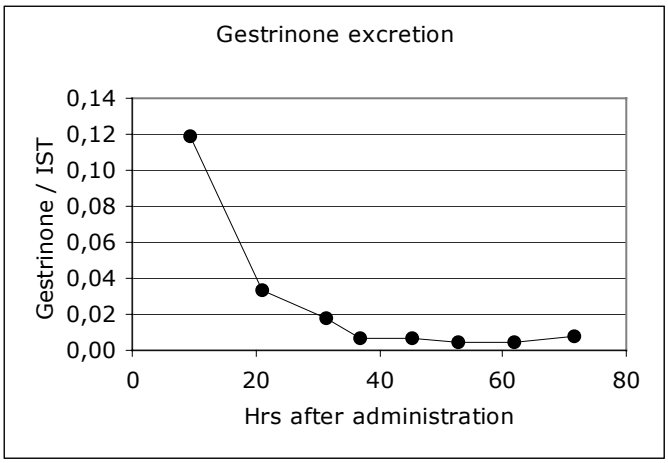
Methandienone

In vitro metabolism of MDN resulted in formation of only 6β-hydroxy-metandienone (6β-MDN) as phase-I metabolite, whereas formation of other reported metabolites, e.g. 17α-methyl-5β-androstane-3α,17β-diol (5β-MT), 17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol, or epimeric metabolites 17-epimetandienone, 17β-methyl-5β-androst-1-ene-3α,17α-diol was not detected in non-conjugated form in the mixture. For the absence of epimeric metabolites the most obvious reason is the lack of co-substrates involved in sulphate conjugation, which has been reported as transition state in the epimerisation process (2). From conjugated fraction glucuronide conjugate of 5β-MT was the only one detected. However, this is well in accordance with the earlier data, because e.g. 6β-MDN that was detected as phase-I metabolite has been reported to excrete in non-conjugated form in urine.

In vivo metabolism of MDN was examined from an excretion study sample, where the detected phase-I metabolites were 17β-methyl-5β-androst-1-ene-3α,17α-diol, 5β-MT and 6β-MDN, from which 5β-MT was also found in trace amounts as glucuronide conjugate.

Gestrinone

In vitro experiments of gestrinone (GES) four closely eluting but well-separated compounds were detected in ion trace *m/z* 325 corresponding to hydroxylated phase-I metabolite $[M+16+H]^+$, whereas phase-II metabolites consisted of one glucuronide conjugate of parent compound $[M+176+H]^+$, and two metabolites which were both hydroxylated and glucuronidated $[M+16+176+H]^+$(Table 4). This data is well in agreement with earlier data by Lévesque et al. (3). Excretion study of GES is depicted in Figure 3. A single dose of 2.5 mg resulted in the formation of glucuronide-conjugated gestrinone (up to 52 hrs) and two short-term hydroxylated and glucuronidated metabolites *in vivo* (up to 10 hrs post administration).



Fraction	Hrs from administration	Volume (ml)	pH	Spec.grav.
1	10	440	5,50	1,027
2	21	720	6,50	1,028
3	32	610	5,60	1,026
4	37	790	6,10	1,014
5	46	1020	7,00	1,016
6	52	890	7,00	1,015
7	62	630	6,10	1,024
8	72	520	6,10	1,028

Figure 3. Details of gestrinone excretion study and the LC-MS/MS detectability of unchanged gestrinone.

Dihydrogestrinone and tetrahydrogestrinone

From dihydrogestrinone (DHG) only a hydrogenated and hydroxylated phase-I metabolite was detected in trace amounts, and only detected in MS/MS mode. The direct conjugate of DHG was the only glucuronide-conjugated metabolite detected in *in vitro* experiments (Table 5).

Two hydroxylated metabolites $[M+16+H]^+$ were detected from tetrahydrogestrinone (THG), which again was in good agreement with earlier studies (3). Hydroxylation is proposed to be located in C-16 and C-18, but not yet confirmed by NMR from these incubated samples. As phase-II metabolites, direct glucuronidation of THG was detected $[M+176+H]^+$, as well as two conjugated hydroxylated metabolites $[M+16+176+H]^+$ (Table 5).

Trenbolone and propyltrenbolone

From trenbolone (TR) one hydroxylated metabolite was detected $[M+16+H]^+$, and the phase-II metabolic reactions resulted both in the direct conjugation of trenbolone $[M+176]^+$ and its hydroxylated metabolite $[M+16+176]^+$ (Table 6).

In vitro phase-I reactions of propyltrenbolone (PTR) resulted in formation of three hydroxylated metabolites $[M+16+H]^+$, which all were also glucuronide conjugated $[M+16+176+H]^+$. Direct glucuronidation of parent propyltrenbolone was also detected as phase-II metabolites (Table 6).

Table 4. *In vitro* results of gestrinone.

***In vitro* metabolite**	**Reaction**	**t_r (min)**
Gestrinone		7.27
Hydroxylated metabolite 1	M+16	5.28
Hydroxylated metabolite 2	M+16	5.87
Hydroxylated metabolite 3	M+16	6.26
Hydroxylated metabolite 4	M+16	6.50
Glucuronide-conjugated	M+176	5.84
Hydroxylated and glucuronide-conjugated metabolite 1	M+16+176	4.30
Hydroxylated and glucuronide-conjugated metabolite 2	M+16+176	5.00

Table 5. *In vitro* results of dihydrogestrinone and tetrahydrogestrinone.

***In vitro* metabolite**	**Reaction**	**t_r (min)**
Dihydrogestrinone		7.69
Hydroxylated and hydrogenated metabolite	M+2+16	6.90
Glucuronide-conjugated	M+176	6.15
Tetrahydrogestrinone		7.76
Hydroxylated metabolite 1	M+16	6.89
Hydroxylated metabolite 2	M+16	7.17
Glucuronide-conjugated	M+176	6.30
Hydroxylated and glucuronide-conjugated metabolite 1	M+16+176	5.56
Hydroxylated and glucuronide-conjugated metabolite 2	M+16+176	6.29

Table 6. *In vitro* results of trenbolone and propyltrenbolone.

***In vitro* metabolite**	**Reaction**	**t_r (min)**
Trenbolone		6.82
Hydroxylated metabolite	M+16	5.11
Glucuronide-conjugated	M+176	5.46
Propyltrenbolone		8.22
Hydroxylated metabolite 1		6.75
Hydroxylated metabolite 2	M+16	7.17
Hydroxylated metabolite 3	M+16	7.31
Glucuronide-conjugated	M+176	7.06
Hydroxylated and glucuronide-conjugated metabolite 1	M+16+176	6.36
Hydroxylated and glucuronide-conjugated metabolite 2	M+16+176	6.50
Hydroxylated and glucuronide-conjugated metabolite 3	M+16+176	6.71

Conclusion

In vitro synthesis model was applied to a group of selected compounds, in order to examine its applicability to serve as a model to predict metabolic pathways of "new" AAS. In the pilot experiments with methyltestosterone it became evident that the use of combined fraction (both microsomes and S9) is advantageous in the reaction mixture to ensure a maximal enzyme activity. Furthermore, in the experiments with methandienone it could be concluded that the drawback of the method is the lack of co-substrates (PAPS) responsible for the sulphatation process, which is apparently the limiting step in the formation of epimeric metabolites that are formed via sulphate-conjugated transition state.

Until now the developed *in vitro* metabolic reaction model has been applied for gestrinone, dihydrogestrinone, tetrahydrogestrinone, trenbolone and propyltrenbolone. The main observed metabolic pathway was hydroxylation, which all the selected substrates underwent, gestrinone and propyltrenbolone resulting in the formation of 4 and 3 metabolites, respectively. Phase-I reaction of dihydrogestrinone was only scarce, but exceptional, as the only generated metabolite was hydroxylation of hydrogenated compound. In general, this model has proven applicable for the purpose, as every compound tested has undergone phase-I and phase-II reactions at least to some extent. The results have been coherent also to those reported earlier in the literature. In our earlier studies (4) glucuronidation has been successful using the chemically synthesised phase-I metabolites as starting material, but now these both stages of metabolic reactions are combined in one experiment.

Acknowledgements

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