

Applications of *in vivo* urinary steroid detection in uPA^{+/+}-SCID chimeric mice

¹⁾ DoCoLab, Department of Clinical Biology, Microbiology and Immunology, Ghent University, Belgium

²⁾ IMIM, Institut Municipal d'Investigació Mèdica-Hospital del Mar, Barcelona, Spain

³⁾ CEVAC, Center for Vaccinology, Department of Clinical Biology, Microbiology and Immunology, Ghent University and Hospital, Belgium

Abstract

Successful validation of the chimeric mouse model for the *in vivo* study of the human metabolism with 3 model steroid compounds, resulted in the use of the mouse model to investigate how anti-doping control laboratories can screen for prohormones or designer steroids. Therefore some selected "nutritional supplements", containing designer steroids or prohormones, were administered to the chimeric mice. The preliminary results of the excretion studies with Promagnon and Superdrol are discussed.

Introduction

Anabolic steroids are a popular class of doping substances due to their performance-enhancing properties. They are forbidden in sports and are mentioned on the prohibited list of the World Anti-Doping Agency (WADA) [1]. Screening of their misuse is usually performed in urine but like many other drugs, most of the steroids are transformed in the human body. In order to make their urinary detection possible, it is important to have knowledge about their metabolism, which is mainly performed in the human liver. However, due to ethical constraints correlated to the many side effects of steroid use, no human excretion studies can be performed with designer steroids. By using a chimeric mouse model, transplanted with human hepatocytes, an alternative is offered to perform *in vivo* metabolic studies. Previous studies have demonstrated that the steroids administered to these chimeric mice underwent approximately the same metabolic transformations as in human subjects [2]. In this way the *in vivo* human metabolism of illicit substances can be studied without involving human volunteers.

The aim of the current study was to use the chimeric mouse model to investigate the metabolism of prohormones and/or designer steroids, sold as "food supplements". These supplements are not marketed as pharmaceutical preparations and in many cases a proper labeling of the contents is missing. However, previous studies have indicated that the supplements can contain (designer) steroids or prohormones [3,4]. Prohormones are considered as steroid precursors which convert to the active compound in the body, while designer steroids are modified steroid compounds with similar or enhanced effects but are especially synthesized to circumvent doping analysis.

Promagnon was the first supplement administered to the chimeric mice to investigate its detection and metabolism. According to the label it contains as active compound 4-chloro-17 α -methyl-andro-4-ene-3,17 β -diol, a substance structurally related to clostebol (4-chlorotestosterone) and 17-methylclostebol (Figure 1). Numerous results of the metabolism of clostebol in human [5-7] and cattle [8,9] have been published. But concerning promagnon and methylclostebol, only very limited data are available about their metabolism in humans.

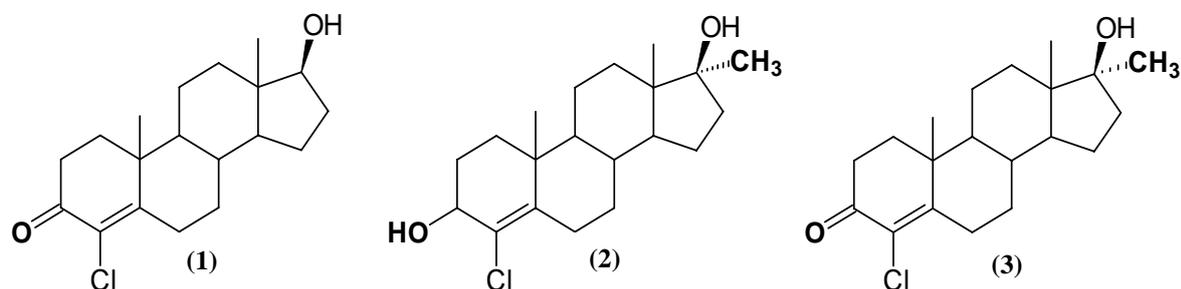


Figure 1: Chemical structures of Clostebol (1), Promagnon (2) and 17-Methylclostebol (3).

The second nutritional supplement tested in the chimeric mice was Superdrol. According to the label it contains 2 α ,17 α -dimethyl-5 α -androst-3-one (Figure 2). Superdrol, also known as methasterone or 17 α -methylhydrostanolone, is on the WADA prohibited list since 2006. In the mean time some serious health disorders have been reported after the use of Superdrol supplements [10]. One of the metabolites reported *in vivo* is the reduction of the keto-function in the C₃ position to form 2 α ,17 α -dimethyl-5 α -androstane-3 α ,17 β -diol (DM-AD) [11,12]. Via *in vitro* cultures the detection of some additional 16-hydroxylated compounds was also described [13].

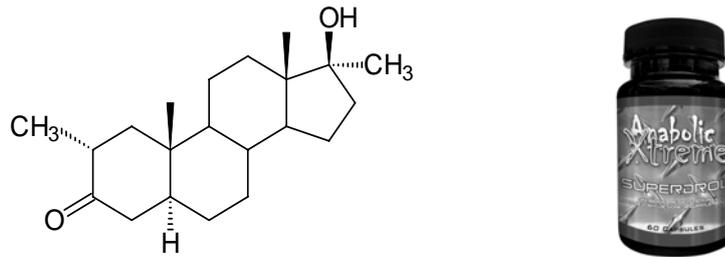


Figure 2: Chemical structure of the active compound in the "nutritional supplement" Superdrol.

Materials and Methods

References

The "supplements" were ordered via the internet. Promagnon was bought from Peak Performance Laboratories and Superdrol from Anabolic Xtreme/Designer Supplements. The reference standards of methylclostebol and superdrol were purchased from Toronto Research Chemicals (TRC, Canada). 6 β -Hydroxymethylclostebol, produced by *in vitro* cultures, was a kind gift from the Department of Chemistry, Wroclaw University (Wroclaw, Poland).

Study protocol

The project was approved by the Animal Ethics Committee of the Faculty of Medicine of the Ghent University (ECD 06/09). The chimeric uPA^{+/+}-SCID mice were produced and maintained at the Center for Vaccinology (CEVAC, Ghent) as described by Meuleman et al. [14].

The administration studies were performed in special metabolic cages from Tecniplast (Someren, The Netherlands). In this way the mice had free access to water and powdered food. Moreover, the design of the cages allows for an easy collection of the mouse urine, perfectly separated from the faeces, without disturbing the mice. The excretion studies were performed by oral gavage of a Phosphate Buffered Saline (PBS) suspension containing the supplement or the reference standard of the steroids. Prior to the administration, blank urine was collected. After a single dose administration, the urine was collected after 24 h, followed by storage at -20°C , awaiting analysis. Also non-transplanted mice were included in these administration studies as a control group for the interspecies differences.

Sample preparation

A general sample preparation method, used in routine doping control screening, was followed to extract the steroids from the mouse urine. The mice only produce a limited amount of urine a day (average of 1.5 mL/24 h) therefore the extraction method starts from only 100 μ L of mouse urine. As described previously, after hydrolysis of the mouse urine a liquid-liquid extraction with diethyl ether was performed [2].

Results and Discussion

Promagnon-Methylclostebol

Before administration of the supplement, the pills were analysed for their content with gas chromatography-mass spectrometry (GC-MS) and the presence of promagnon was confirmed. Compared to previous administration studies [2], a high dose (10 mg/mL) was needed to allow for the detection of metabolites when using full scan MS (Figure 3).

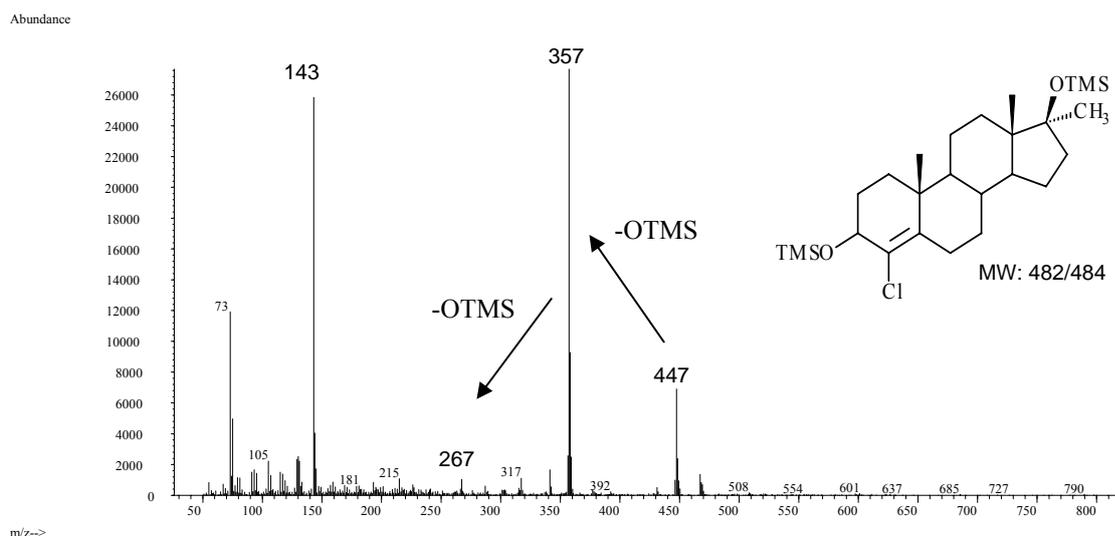


Figure 3: Mass spectrum of the derivatised Promagnon, detected in the chimeric mouse urine after its administration.

The spectrum of the trimethylsilyl (TMS)-derivatised Promagnon (MW 482/484) has mass to charge ratios (m/z) of 447 and 357 as most abundant and typical fragment ions (Figure 3), resulting from the loss of the chlorine group (loss of -35/37) and the OTMS group (loss of -90) respectively. These findings are in correlation with previously reported results [15]. However, during the quality control of the pills, it became apparent that the Promagnon supplement was contaminated with another chlorinated substance. This impurity was already reported in 2008 [16]. Identification with a reference standard revealed the presence of

methylclostebol (Figure 1). Regarding the metabolism of methylclostebol, only a few studies with *in vitro* cultures are found in literature [17,18]. Methylclostebol and promagnon are not explicitly mentioned on the WADA Prohibited List, while clostebol is [1]. In fact, all these compounds are 4-chlorinated substances introducing more anabolic activities and less androgenic aromatisation [8]. In humans, it seems that methylclostebol was already abused in Germany during the time period 1966-1974 as a substance named STS482 [19].

Because methylclostebol is available as a pure reference standard (Figure 4), a suspension of 10 mg/mL was also administered in a separated study to the chimeric and non-chimeric mice. The urine was collected on the day before the single administration and 24 h after the administration.

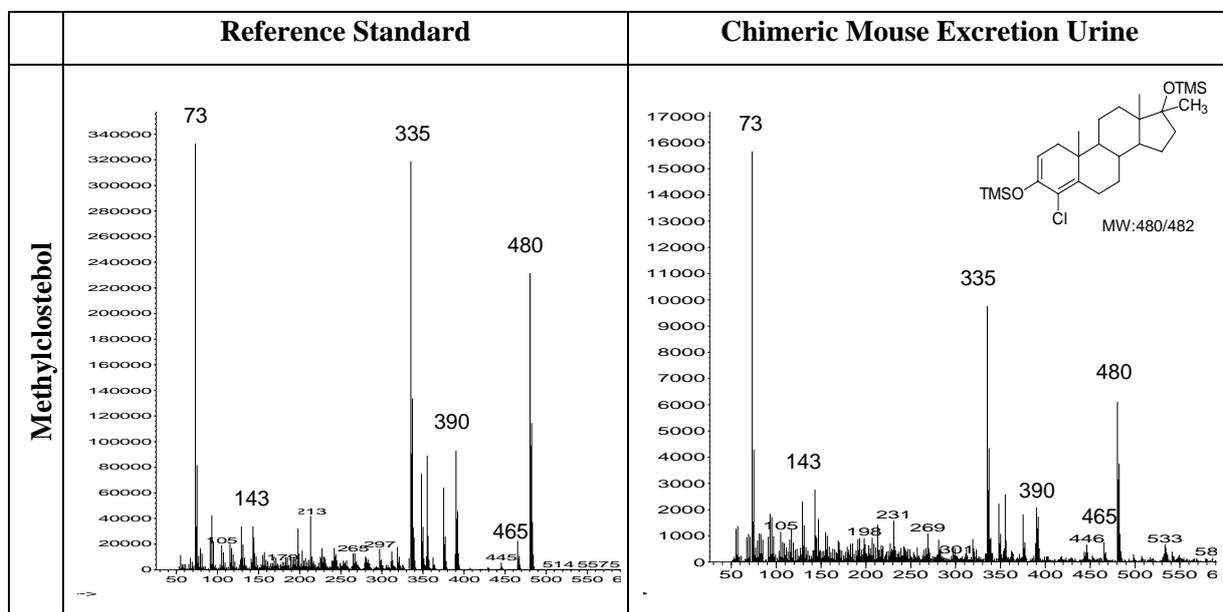


Figure 4: GC-derivatised-mass spectra of Methylclostebol, as a reference (left) and as the compound found in the 24 h excretion urine of a chimeric mouse (right).

Methylclostebol, itself, was detected in all mouse urine samples, as evidence of a successful administration with a sufficient dose. Besides the parent peak (2), the 3-hydroxylated metabolite (=promagnon) was found in all samples, indicated as peak (1) in Figure 5.

Several poly-hydroxylated compounds were detected in the post-administration chimeric and non-chimeric mouse urine (Figure 5.B). The fragment ions at m/z 218 and 231 indicated that these substances are 17-methyl-16,17-dihydroxylated steroid compounds (Figure 5.B, Table 1) [7]. Peak (3) with a molecular weight (MW) of m/z 570-572 (chlorine cluster) was correlated with a proposed structure of 16-hydroxylated promagnon.

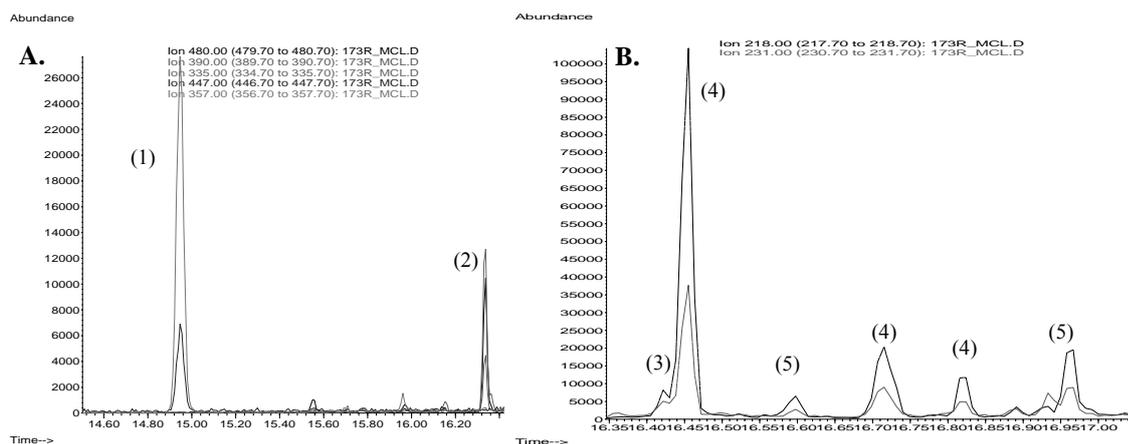


Figure 5: GC-MS chromatograms of a Methylclostebol post-administration chimeric mouse urine. **A.** Promagnon (1) and methylclostebol (2) **B.** Several hydroxylated metabolites, with extracted ions at m/z 218 and 231. Legends for peak identifications see Table 1*.

Based on mass spectra research, in comparison with the theoretical masses and typical losses for steroids, the following structure was proposed for peaks (4): x,16-dihydroxylated-promagnon (MW 658). Peaks (5) indicated a closely related spectrum (Table 1). Additionally, a peak was detected in the chimeric mouse urine with m/z 143 and 170 as only characteristic ions, which are typical for 12-hydroxylated steroids [7] (Table 1). This compound could not be detected in the non-chimeric mice after methylclostebol administration. However, synthesis of the reference standards of these compounds is necessary to unequivocally identify the structures of these compounds. Direct comparison with a reference standard, remarkably, showed the absence of 6 β -hydroxymethylclostebol in the mouse urine sample, while in literature this is one of the major metabolic pathways for 17-hydroxylated,17-methylated compounds [7,17].

Table 1: Overview of the detected metabolites after methylclostebol administration to the chimeric mice.

*Figure 5	Compound	Characteristic TMS-ions m/z	RT
(1)	Promagnon	447/357	14.89
(2)	Methylclostebol	480-482/390/335	16.35
	12-hydroxylated compound	143/170	16.20
(3)	16-hydroxylated-promagnon	570-572/480/218/231	16.44
(4)	x,16-dihydroxylated promagnon	658-660/568/218/231	16.46 16.72 16.80
(5)	x,16-dihydroxylated promagnon, with reduced double bond	660-662/218/231	16.60 16.99

Superdrol

GC-MS analysis of the superdrol pill confirmed the presence of superdrol in high amounts (Figure 6). An excretion study with the reference standard, even with a high dose (10 mg/mL), resulted in the recovery of only a very minor amount of the parent drug from the mouse urine. Other attempts were made to formulate the suspension differently e.g. with PEG400, as used in toxicity studies. The solubility with PEG was slightly better but no differences in detected compounds could be observed (data not shown).

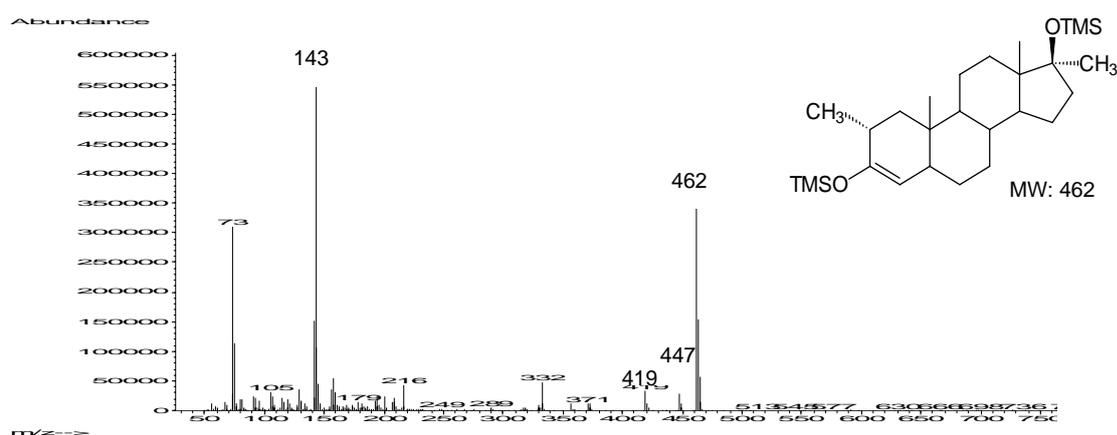


Figure 6: GC-MS spectrum of the TMS-enol-TMS-ether derivatised superdrol (MW=462).

As shown in Figure 7 several metabolites were detected by overlaying the post-administration with the pre-administration urines. Comparison of the mass spectra of these metabolites with previous reported results from *in vivo* human studies indicated that the chimeric mice revealed other metabolic pathways [11,12]. The only reported metabolite *in vivo*, namely the 3-keto-reduced metabolite of superdrol: 2 α ,17 α -dimethyl-androstanediol (DM-AD) was not detected in the mouse urine. However, one of the compounds mentioned in the *in vitro* cultures of Gauthier et al. could be detected in the chimeric mouse urine, namely peak (2) (Figure 7). This compound was described as a 2,3,16,17-tetrol metabolite [13], here indicated as 2,16-dihydroxy-DM-AD (Table 2).

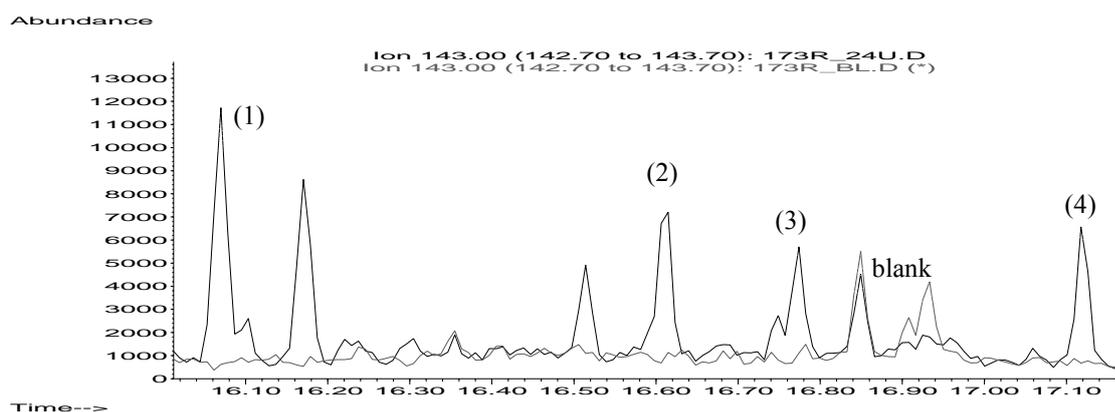


Figure 7: Overlay of chromatograms (m/z 143): a pre- and 24 h post-superdrol-administration urine of a chimeric mouse. Legends for peak identifications see Table 2**.

Nevertheless several other compounds were found, e.g. peak (1) with derivatised M^+ at m/z 550, which is closely related to the metabolites previously reported [13]. This is a hydroxylated compound, but not at the C_{16} position since ions m/z 218 and 231 are not present in the spectrum [7]. The proposed structures/metabolites in this paper are solely based on fragmentation patterns (Table 2). Most of the compounds showed fragment ions at m/z 218/231, e.g. peaks (2) and (3), which indicates these compounds as 16-hydroxylated metabolites. However, full structure elucidation is still necessary for the identification of the compounds.

Table 2: Overview of the detected metabolites after superdrol administration to the chimeric mice.

**Fig. 7	Compounds	Characteristic TMS-ions m/z	RT
	Superdrol	462/447/419	14.70
(1)	x-hydroxy-superdrol	550/460/143	16.08
(2)	2,16-dihydroxy-DM-AD	640/550/218/231	16.60
(3)	x,16-dihydroxy-superdrol	638/218/231	16.77
(4)	trihydroxy-superdrol	726/711/636/546	17.10

DM-AD= $2\alpha,17\alpha$ -dimethyl- 5α -androstane- $3\alpha,17\beta$ -diol, the 3-hydroxy-superdrol.

Evaluation and comparison with the excretion urine of the non-chimeric mice revealed that only peaks (1) and (4) were detected in the non-chimeric mice, while peaks (2) and (3) could not be detected in the non-chimeric mice. This indicates that these two latter compounds are exclusively produced in the chimeric mice and could therefore be considered as typical human metabolites.

Conclusion

This study involving the detection of chlorinated and 2,17-dimethylated substances in the framework of doping analysis, showed that the results of the previous reported *in vitro* cultures could not completely be confirmed via the chimeric mouse model. For superdrol one metabolite and several closely related compounds were found and for methylclostebol the 3-keto-reduced promagnon was detected together with some mono- and dihydroxylated compounds. Compared to *in vitro* cultures the 6 β -hydroxymethylclostebol was not detected. This *in vivo* chimeric mouse model, with transplanted human hepatocytes, revealed essential data on the metabolism of these steroids.

Acknowledgements

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