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ASDTL Supplements Project 2009

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Introduction

Steroids described as "designer" drugs continue to be sold in preparations described as "supplements". There appears to be a deliberate attempt to put substances with anabolic and/or androgenic activity into these materials knowing that by advertising these as performance enhancing, bodybuilders and others will readily purchase them. All these substances have no reasonable clinical trials in humans and at most have had some testing in animals prior to publication. Some may also have been synthesised purely on the basis of similarity to known biologically active compounds. This is often done by substituting a methyl group at the C17-position of the steroid. Further manipulations of stereochemistry at the C5 position as well as functional groups at C3, C6 and C7 positions may provide a host of compounds that can be unleashed onto an unwary public.

Experimental

A general extraction process has be developed which can be routinely used with only minor modifications to accommodate the variety of compounds that may be encountered. The process includes extraction from the capsule or preparation, screening by GC/MS or other techniques, then chromatography on silica gel with further HPLC clean up as needed.

Extraction of steroids: These can rapidly be extracted by packing the capsule contents (or finely ground tablets) into a Varian BondElut Reservoir #12131017 (10 cm x 2 cm) or #12131018 (15 cm x 3 cm) with a frit secured in the base. The powder is packed by tapping the column while under vacuum in an SPE manifold as well as tamping down with a flat edged tube. When the material is tightly packed, components can be eluted with organic solvents. Hexane will remove most lipids and some steroids, with most material being removed in two to four column volumes. Dichloromethane (DCM) removes most other compounds, again in approximately two to four column volumes. Analysis of these extracts allows a decision to be made on whether they may be combined or treated separately. Evaporation of the solvent provides material that is suitable for chromatography.

Chromatography: Rapid separation of components can be achieved by chromatography using short columns filled with TLC grade silica. The silica is tightly packed dry by tapping while under vacuum on an SPE reservoir. When the column is packed the top is tightly pressed down using a suitable flat object to ensure it is even. A non-polar solvent such as hexane (20 mL) is passed through the column but at no time should the column be allowed to

run dry. If it does then the top must be pressed down with the flat object before solvent is added to prevent channelling down the sides of the column. The mixture to be separated is added in a minimum volume of a non-polar solvent such as toluene (may be added while still hot from heating needed to help the material to dissolve) and this is allowed to run onto the column under vacuum. A small amount of the solvent is used to rinse the sides of the column. Elution follows a set path – using volumes of approximately 15 mL each collected into separate tubes using an initial gradient starting from hexane, followed by 50% hexane/(DCM), then finally DCM. A second gradient elution is performed starting with 1% ethyl acetate/DCM, followed by 2%, 5%, 10%, 20%, 40% and 80% ethyl acetate/DCM, before finishing with 100% ethyl acetate. An additional wash with 5% methanol in ethyl acetate may be needed for more polar compounds such as trihydroxy steroids. The fractions are verified by thin layer chromatography with visualisation using a vanillin/sulfuric acid spray and heating or under UV light. The fractions may also be checked either underivatised or derivatised using MSTFA/TMSI/ethanethiol reagent by GC/MS. Appropriate fractions are combined and if sufficiently pure, recrystallised or if needed further purified by a repeat column or by HPLC.

General identification process: This is performed in several steps. One tablet/capsule in methanol (10 mL) is shaken/sonicated for 20 mins, an aliquot (100 μL) is diluted with ethyl acetate (1 mL) and the underivatised and derivatised GC/MS data is obtained. Often by comparing with a library of compounds it is possible to identify the steroid and this is verified against a standard to match spectrum and retention time, according to WADA criteria.[1] If no reference material is available then it is necessary to purify and identify the compounds present by structural elucidation. A common process is to extract 20 tablets/capsules by grinding to a powder then packing onto an empty SPE column as above and eluting the capsules with DCM. Usually after 3 washes the amount extracting is minimal. The solvent is evaporated then made up in an appropriate solvent for chromatography and the components separated as described above. Purified compounds are analysed using GC/MS, ^{1}H NMR and ^{13}C NMR. Once identified they may be synthesised to obtain authentic reference materials.

Supplements studied

Alri restore: The label states that the capsules contain 6-bromodione and MbAET. The capsules were extracted with hexane then DCM and the DCM extract was chromatographed to separate the components. These were identified as 6β-bromoandrostenedione and 17β-acetoxy-17α-methylandrost-5-ene-3β,7β-diol. The 6-bromo-compound decomposes in the injection port of the GCMS when analysed without derivatisation to give androst-4,6-dien-3,17-dione and small amount of the bromo-compound which places the bromine in the 6-position of androstenedione. The ^{1}H NMR spectrum for the bromo compound in this supplement is shown in Figure 1. It has as a finely coupled doublet of doublets for the proton attached to C6 indicating an equatorial configuration with the axial β-bromo stereochemistry. The second compound isolated from this supplement has an acetyl function and the large downfield shift of a methyl group attached to a quaternary carbon indicates this acetate to be at C17. The remainder of the ^{1}H NMR is similar to 7β-hydroxyDHEA thus helping define the

positions and stereochemistry. B-AET appears to translate to β-androstenetriol on internet blogs so apparently MbAET is methyl-β-androstenetriol as the acetyl derivative (Figure 2).

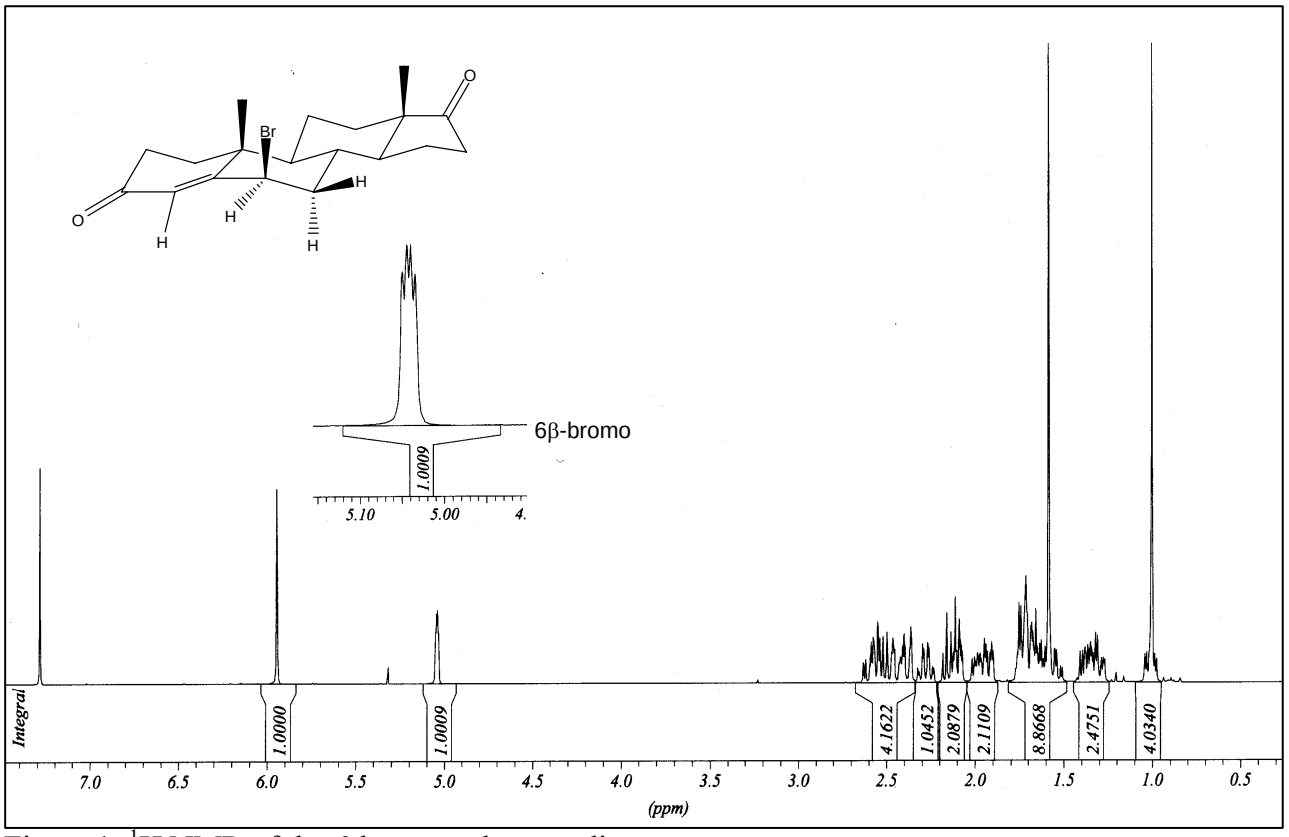

Figure 1: ^{1}H NMR of the 6-bromoandrostenedione.

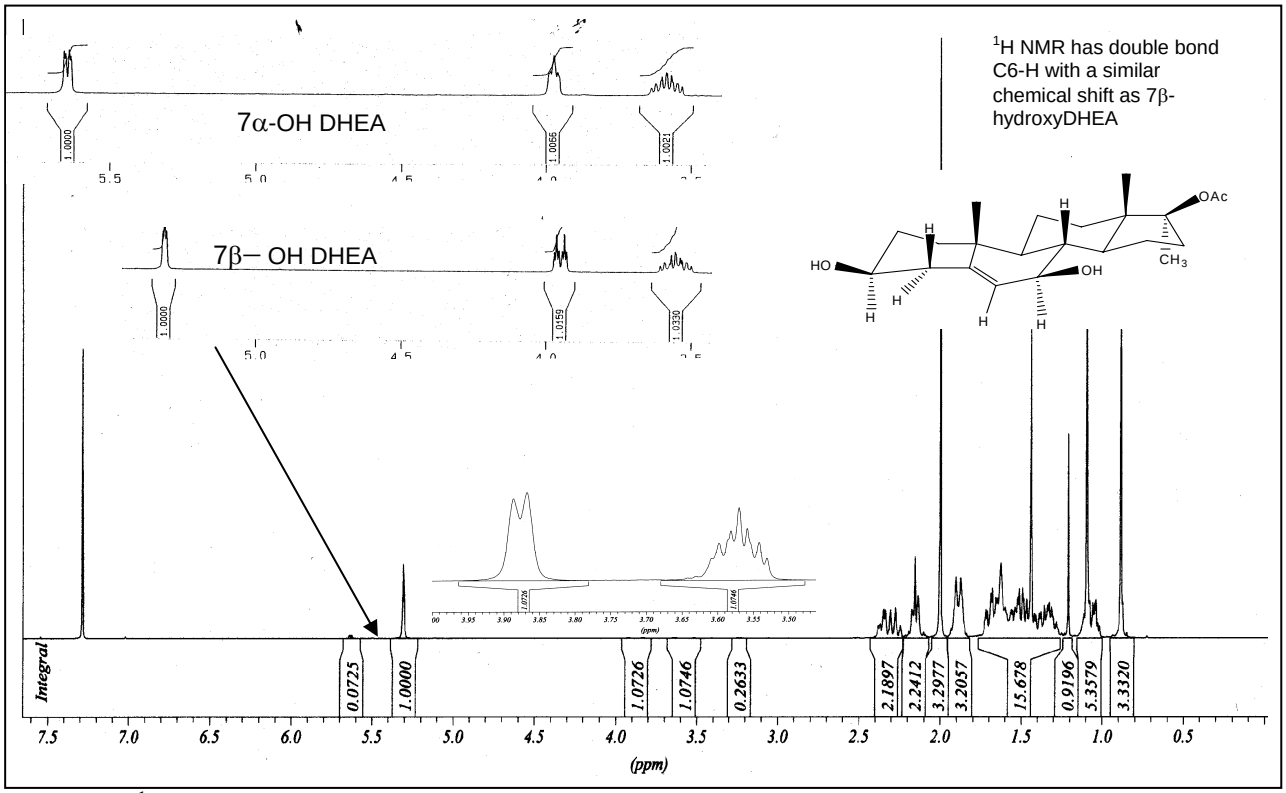

Figure 2: ^{1}H NMR of the 17β-acetoxy-17α-methylandrost-5-ene-3β,7β-diol showing the similarity of chemical shifts with 7β-hydroxyDHEA.

Analysis of the DCM extract by ^{1}H NMR prior to purification provided a relatively clean spectrum that allowed identification of the two components as shown in Figure 3. This process can be useful to identify supplements that may have mixtures of stereoisomers. In the spectrum a small doublet of doublets (highlighted) at δ5.54 ppm may in fact be due to the 7α-hydroxy isomer as this corresponds to the similar signal in 7α-hydroxyDHEA.

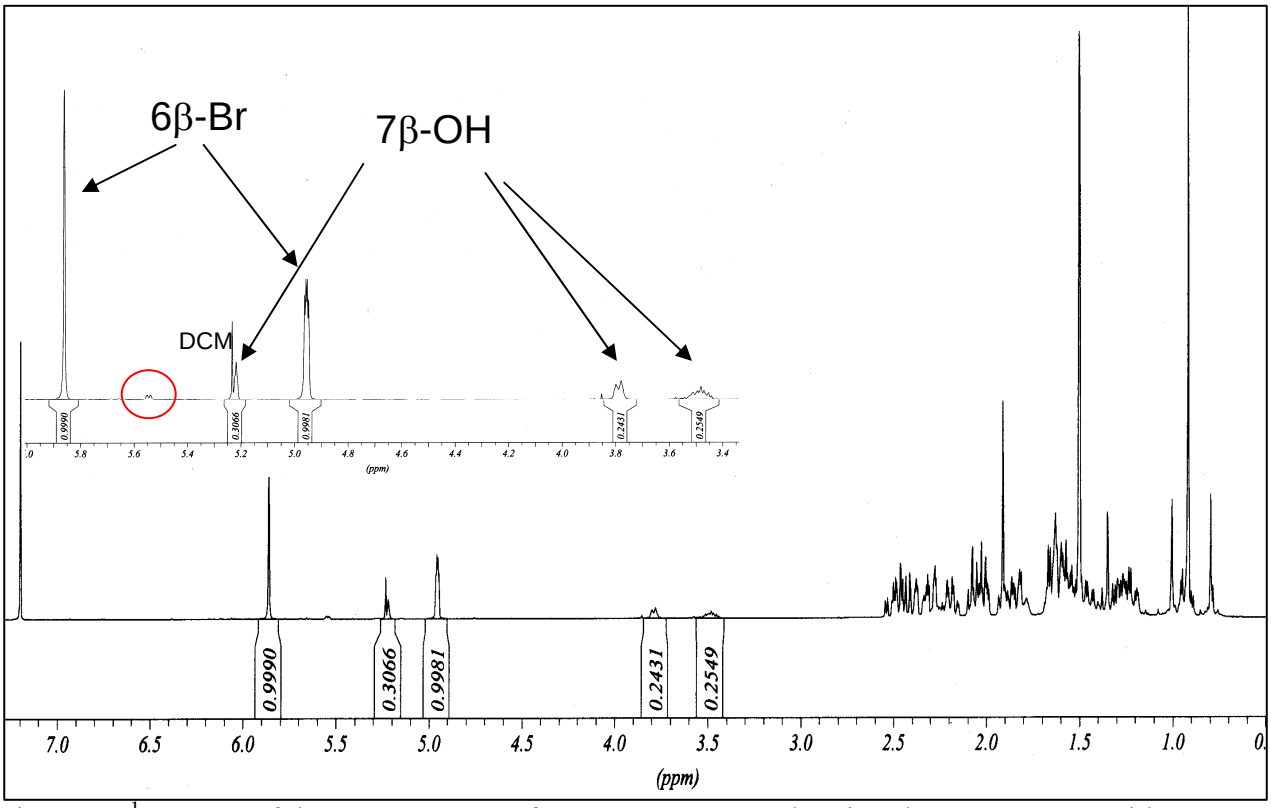

Figure 3: ^{1}H NMR of the DCM extract of ALRI RESTORE showing the components with very little background. This also allows a good estimation of the proportions of each compound.

X2-Hyperdrol: Similar analysis of the DCM extract of X2-Hyperdrol was found to contain 6-bromoandrostenedione and the presence of both the 6α and 6β-bromo isomers (Figure 4). 6α-Bromoandrostenedione shows the large couplings for the axial proton with those protons at C7. The preparation also contains osthol which contains aromatic and olefinic double bond protons.

Androst-4,9-diene-3,17-dione: This substance was found to occur in a large number of supplements purchased. The ingredient is usually correctly named on the label, with GC/MS and ^{1}H NMR confirming the stated structure. The amount of material in the supplements does vary considerably (Table 1). Approximately half of the preparations contained the amount stated on the label. For the majority of others the amount is much less showing poor quality control. In individual capsules the variation is small except for MD1T which has a low amount compared to the label as well as a large variation between capsules.

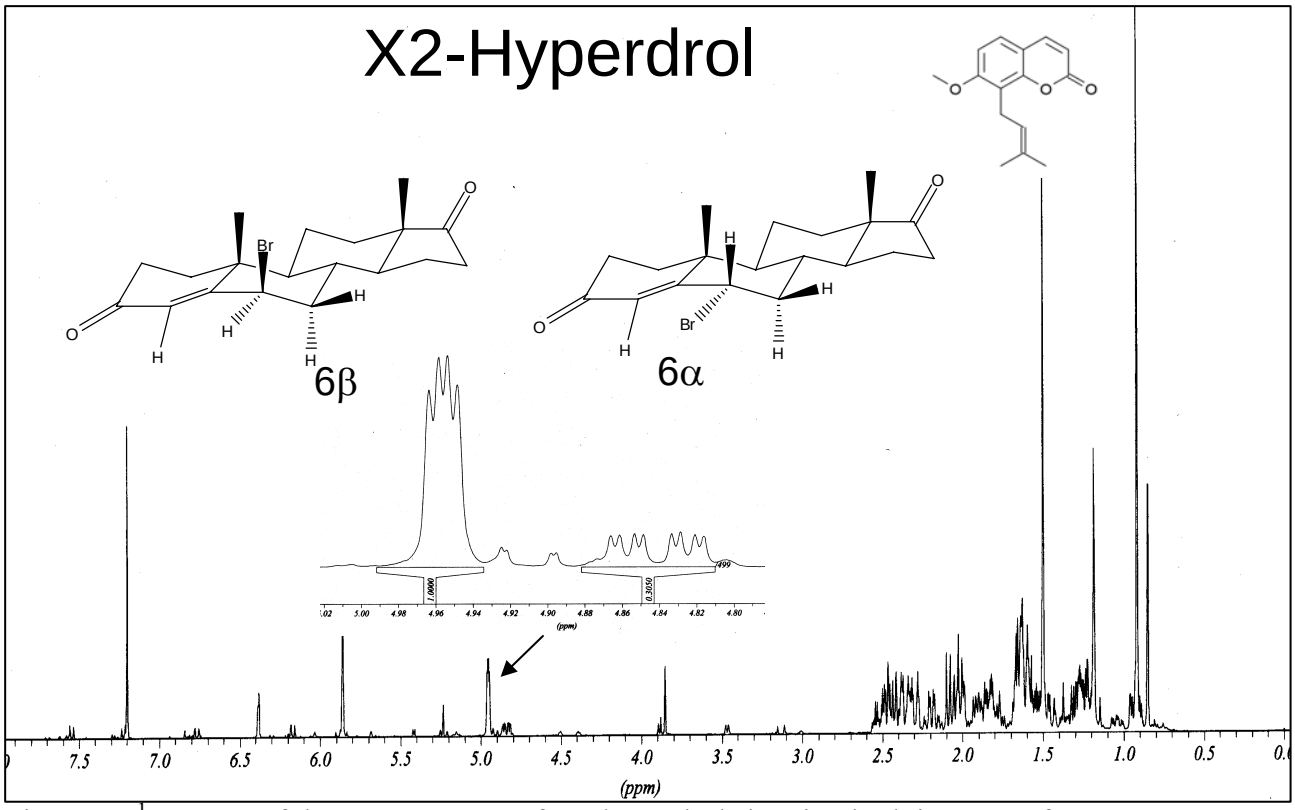

Figure 4: ^{1}H NMR of the DCM extract of X2-hyperdrol showing both isomers of bromoandrostenedione.

Table 1: Variation between the labelled value and that in the preparation for one capsule (top) and the variation between 5 capsules (bottom).

Supplement	Mean n=2 (mg)	Value on label (mg)
Trena	9	10
Tren-250	1	25
Super tren	17	25
Trenavol-V	32	30
Liquidrone	25	12.5
Epi-Tren	30	30
X-Tren	28	30
1T-Tren	22	81
Orafinadrol	45	50
Finaflex	30	25
Tren 19 nor	15	40
Dienedrone	50	50
MD1T	15	50
D-Drol	34	-

Supplement	Label value (mg)	N	Mean (mg)	RSD %
Trenavol V	30	5	24	4
Epi-Tren	30	5	27	6
Orafinadrol	50	5	60	10
MD1T	50	5	8	19
D-Drol	N/A	5	26	4

In vitro metabolism studies of steroids from supplements

Phase-I metabolites of new substrates were analysed using the *in vitro* metabolism assay based on the method by Kuuranne *et al.*[2] The total volume of the incubation reaction mixture was 500 µL using 50 mM phosphate buffer pH 7.4 containing 5 mM $MgCl_2$ as the incubation buffer. The concentration of the substrate was 10 µM with 5 mM NADPH (Roche Diagnostics) as the co-factor. Total concentration of the enzymatic protein was 2.0 mg/mL of the S9 fraction of human liver enzymes (BD Gentest™, MA, USA). The reaction mixture was incubated at 37°C for three and/or 24 hours (with additional NADPH). After incubation, d_3-testosterone (4 µg/mL, 50 µL) was added as surrogate. Steroids were extracted from the reaction mixture using 3M Empore C18® solid phase extraction cartridges (Varian, CA, USA) and supplemented with 17-methyltestosterone (4 µg/mL, 50 µL) as internal standard. The steroid extracts were dried, derivatised with MSTFA/TMSI/Ethanethiol (55 µL) at 60ºC for 15 minutes. The TMS steroid derivatives were analysed by full scan GC/MS. The steroids that were analysed by *in vitro* metabolism are summarised in Table 2.

Table 1. Summary of *in vitro* metabolism studies of steroids from supplements

Parent substrate	Incubation time (h)	Metabolite/s observed	Comments
Estra-4,9(10)-diene-3,17-dione	3	17β-hydroxyestra-4, 9(10)-diene-3-one (45%)	Did not observe 3α/3β metabolites
Androst-1,4,6-triene-3,17-dione (ATD)	3	No metabolite observed	Parent compound observed
6α–Methyl-androst-4-ene-3,17-dione (TMS M+ 444)	3	446 ion – 38% 448 ion – 3.5%	41% parent ion remaining
	24	446 ion – 31.7% 448 ion – 13.2%	43.4% parent remaining
2α, 17α-Dimethyl DHT (TMS M+ 462)	3	2α, 17α-Dimethyl-5α-androstan-3α, 17β-diol (464 ion) – 4.5%	34% parent 462 remaining
	24	464 ion - 3.2%	33.9% parent remaining
3α-Hydroxy-4-androstene-6,17-dione (TMS M+ 518)	3	(both 522 ion) 6β-Hydroxyandrosterone - 2.1% 6β-Hydroxyetiocholanolone – 1.9%	4% parent remaining 18% of a compound with 518 M+ detected, possibly 17β-hydroxy-4-androstene-3,6-dione)
17α-Methyl-2α,3α-thioepoxy-androstane-17β-ol	3	No metabolite observed	Loss of thio group detected
6ζ-Bromoandrostenedione (TMS M^+ = 510)	3	6α-Hydroxytestosterone (520 ion) – 33.5% (against 516 ion) 6α-Hydroxyandrostenedione – level increased from 940 ng/mL to 2040 ng/mL Androstenedione – level increased from 875 ng/mL to 915 ng/mL 6-Oxo-androstenedione – levels decreased from 3413 ng/mL to 459 ng/mL No detectable 510 ion	**Starting material contains:** 6-Oxo-androstenedione (major) – 516 ion 6α-Hydroxyandrostenedione (minor) – 518 ion Androstenedione (minor) – 430 ion

Initial experiments were performed using two liver enzymes; human liver microsomes (HLM) and S9 liver fraction. However, *in vitro* assays using the HLM fraction did not pass quality

control using testosterone and nandrolone as control substrates. Hence, all additional *in vitro* assays were performed using only the S9 liver fraction.

The majority of metabolites were detected after 3 hours incubation with the S9 liver fraction. If a high percentage of starting material remained, steroids were incubated with the enzyme for up to 24 hours to provide a satisfactory product yield. In cases, such as 2α, 17α-dimethyl DHT and 3α-hydroxy-4-androstene-6,17-dione, longer incubation times did not increase the concentration of metabolites.

References

1. WADA Technical Document – TD2010IDCR. Identification criteria for qualitative assays incorporating column chromatography and mass spectrometry. World Anti-Doping Agency, Montreal, 2010.
2. Kuuranne, T.; Kurkela, M.; Thevis, M.; Schänzer, W.; Finel M.; Kostiainen, R. *Drug Metbolism and Disposition* **31** (2003) 1117-1124.

Acknowledgements

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