Results of several (small) research projects at DoCoLab in 2007

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1. Introduction
The paper is a summary of two research projects that were initiated in 2007 at DoCoLab and which deal with the analysis of new prohormones and the detection of diethylstilbestrol as a contaminant in a nutritional supplement.

2. Prohormones
Similar as last year supplements containing “new” prohormones have been purchased through a WADA funded project and these supplements have been analyzed for the presence of prohibited or new steroids.

2.1. 1,4,6-Androstatriene-3,17-dione and 3-hydroxy-4-androstene-6,17-dione
1,4,6-Androstatriene-3,17-dione is sold under several commercial names, including novedex and recently 4AD. “Attitude” is a preparation combining 1,4,6-androstatriene-3,17-dione and 3-hydroxy-4-androstene-6,17-dione. Last year data on the elimination and detection of 1,4,6-androstatriene-3,17-dione was published, which show that it is largely excreted unchanged, yet also difficult to detect via GC-MS due to derivatisation problems [1, 2]. Therefore the supplements (4AD and Attitude) were analyzed by LC/MS. In the LC-MS/MS analysis, a peak was obtained corresponding with the expected m/z for the [M+H]^+ of 1,4,6-androstatriene-3,17-dione. No abundant adducts were observed (abundances of [M+MeOH+H]^+ and [M+MeOH+Na]^+ were lower than 10%). Therefore, the use of LC-MS/MS using [M+H]^+ as precursor ion is advised for the detection of this analyte.
In case of Attitude, several abundant adducts were detected with water, ammonia, sodium, methanol or combinations for 3-hydroxy-4-androstene-6,17-dione.

It is also clear from this analysis that the Attitude capsules contain relatively high amounts of 3-hydroxy-4-androstene-6,17-dione compared (2.5E8 abundance) to 1,4,6-androstatriene-3,17-dione (1.3E7 abundance). Indeed, 1,4,6-androstatriene-3,17-dione has a conjugated oxo function in the 3 position which normally results in better ionization and hence better sensitivity for LC-MS. Nevertheless, the abundance for this compound is far smaller than for 3-hydroxy-4-androstene-6,17-dione.
In contrast to 1,4,6-androstatriene-3,17-dione, 3-hydroxy-4-androstene-6,17-dione is easily analyzed by GC-MS after derivatisation to an enol-TMS-ether-TMS-derivate. The GC-MS mass spectrum of this substance is shown in Fig. 2.

**Fig. 1.** Ion chromatograms and LC/MS mass spectra of 3-hydroxy-4-androstene-6,17-dione and 1,4,6-androstatriene-3,17-dione in the supplement “Attitude”

**Fig. 2.** Mass spectrum of 3-hydroxy-4-androstene-6,17-dione, tris-TMS
2.2. 2α,3α-epithio-17α-methyl-5α-androstan-17β-ol

Epistane is a 17alpha-methylated analogue of epitiostanol, the main metabolite of mepitiostane. The metabolism and excretion of mepitiostane were previously investigated and described [3]. Mepitiostane is marketed/used as a hematopoetic agent, while epistane is also marketed for its claimed myotrophic properties.

GC/MS analysis of the trimethylsilylated residue of a solution of a capsule in methanol resulted in the detection of two peaks. The GC-MS spectrum of these peaks is shown in Fig.3.

![Fig. 3. Total ion chromatogram and mass spectra of madol and the oxidized analogue after analysis of the supplement “Hemaguno”](image)

Both spectra show an intense m/z 143 indicating a 17-alkylated steroid. The obtained spectrum of the first peak shows an M⁺ of m/z 360 which is corresponding to 17α-methyl-17β-hydroxy-androstan-2-ene. The spectrum of the second peak shows an M⁺ of m/z 362 corresponding to 17α-methyl-17β-OH-androstan.
Based upon these results and comparison with an authentic reference standard it was concluded that madol (2-ene analogue only) and an reduced analogue were detected. No data was obtained by LC-MS/MS using a water:methanol gradient (both 1mM NH₄OAc) as mobile phase and electrospray as ionization source. This result was expected due to the absence of any keto moiety in the molecule.

Using both GC-MS and LC-MS no epistane was detected in the analyzed preparations. Feedback by Dr. Ray Kazlauskas (Sydney laboratory) however indicated that after clean-up and analysis of an analogous supplement, called hemapolin, using NMR, the thioepoxide was detected. Hence, the presence of the thioepoxide can not be excluded, but under routine circumstances in doping control laboratories madol will be detected.

3. **Diethylstilbestrol as a contaminant**

Besides the analysis of prohormones, last year DoCoLab was also involved in the analysis of nutritional supplements as an element in the protection of the health of the athletes and to minimise the risks of testing positive for a doping substance after the intake of a non-prohibited supplement.

Since January 2008, the WADA code prohibits WADA-accredited laboratories analyzing supplements, but late 2007 a supplement was received from a company requesting an analysis for androgens and estrogens. Initially this seemed strange since we had never received a request for an analysis for estrogens..., but not much attention was paid to this fact, since it was believed to be a mistake of the customer. The supplement was supposed to contain several extracts of natural products, but in fact nothing that would raise suspicion: Phytosterol 95%, Resveravine (=8% resveratrol extract), Quercitine, Sabal extract 4 :1, Pygeum africanum cotrex pulvis, Panax ginseng extract 15%, Ganoderma chinensis conc. (Reishi), Calcium hydrogen phosphate, Magnesium stearate, Colloidal silicium dioxide, Microcristallin cellulose, Adeps solidus, Sodium starch glyconate.

Analysis by GC-MS in full scan and SIM mode did not reveal the presence of any steroid traditionally screened for [6, 7], but showed two abundant peaks with the mass spectra given in Fig. 4. Based upon these mass spectra, it was concluded that the substances detected were most probably isomers.
From the mass spectrum it was clear that the substance is trimethylsilylated, but nothing in the mass spectrum has any resemblance with routinely detected steroids. Nevertheless, due to their abundance, the nature of these substances was investigated.

The mass spectral data obtained in these spectra is quite limited. Indeed, besides the evidence that the substance is trimethylsilylated there is only evidence for the loss of an ethyl group, so it was decided to analyze the supplement without a derivatisation step.

Again, the two isomers were detected, but little additional information was obtained, except that the molecular mass was 268 and hence that based upon, the mass difference between this molecular mass and the mass of the TMS-derivatised substance, it was clear that the substance contained two hydroxy groups.

Fortunately, standard pre-installed libraries on mass spectrometers contain a high number of mass spectra of underivatised substances and a quick run on the Wiley and NIST library indicated that the detected substances were cis- and trans-diethylstilbestrol (DES).

DES is an estrogen and is one of the substances included in the screening methods of the laboratories in the European Union which are involved in the control of food stuffs for prohibited substances and veterinary residues.
DES is an orally active synthetic nonsteroidal estrogen that was first synthesized in 1938. In 1971 it was found to be a teratogen when given to pregnant women and its use can result in malformations of the foetus.

Based upon these results the company manufacturing the supplement as well as the public health authorities were warned. The public authorities have started a full investigation of this case. Moreover some questions remain upon the knowledge of the company prior to the analysis, since they initially requested an analysis for androgens and estrogens.

However, very importantly, this example also clearly illustrates the need for analysis of supplements prior to their introduction on the nutritional market and the role our laboratories played in the prevention of health problems and health control prior to the prohibition of WADA to analyze supplements.

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5. References