PROJECT REVIEW

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

T. Kuuranne (United Laboratories Ltd; Doping Control Laboratory; Helsinki, Finland); **R. Kostiainen, L. Hintikka** (Division of Pharmaceutical Chemistry University of Helsinki); **M. Thevis** (Institute of Biochemistry German Sport University, Cologne, Germany)

Anabolic-androgenic steroids (AAS) are synthetic testosterone derivatives, which are designed to maintain the anabolic (beneficial effects) and to minimize the androgenic (side effects) activities of the endogenous prototype. More than 600 testosterone analogues have been synthesized since 1940s and in addition to conventional and bona fide pharmaceutical manufacturers, anabolic steroid products are provided in the market by a wide variety of illicit laboratories.

As recently experienced, designer steroids have become a new threat for doping control as the compounds are "tailor made" to avoid the detection in the current analytical procedures. In man, AAS undergo extensive metabolism, in which they are converted into more polar compounds and are better excreted in urine. Therefore, besides new structure modifications, also the metabolism of the "new" AAS is generally not known, which leads to the lack of target compounds in doping control. To ensure the fast response to analytical challenges, the development and set-up of new analytical methods or implementation of new compounds in present procedures should be fast.

Despite of its use to some extent, *in vivo* study of drug metabolism is difficult and time- and resource-consuming because of legislation aspects. Furthermore, designer drugs which are not registered as clinical preparations are not normally allowed to be used in human metabolic studies. An alternative pathway for the examination of metabolism are *in vitro* applications, which may utilise e.g. mammalian liver slices, cytosolic or microsomal preparations as source of metabolising enzymes. This approach is already well-adapted for *in vitro* studies in the field of pharmaceutical industry, especially in drug discovery. Utilisation of *in vitro* preparations is moderately straightforward, fast and amenable for experienced staff, and therefore a method of choice in metabolic studies of new AAS.

The aim of the present project is to develop flexible *in vitro* procedures which can be applied in order 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 microsomes and *in vitro* excretion studies in human will be compared with model compounds and subsequently, the applicability of the *in vitro* model for prediction of AAS metabolic pathways for new doping agents will be evaluated.

Parallel to the synthesis work an analytical aspect is also closely attached to the project. Liquid chromatographic-tandem mass spectrometric (LC-MS/MS) methods will be developed for the detection of new AAS metabolites to support the prevailing instrument methodology. Analytical experience gained in metabolic studies will be finally exploited in implementation of potential LC-MS/MS methods in routine doping control.

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Results and Conclusions

All the anabolic androgenic steroids (AAS) applied within this study were metabolized using an in vitro model consisting of combined microsomal and S9 fraction of human enzymes. Due to an excessive amount (50 □M) of the steroid aglycone, combined fraction of hepatic enzymes and relatively extensive reaction times (4-5 hrs and 16-17 hrs for phase-I and phase-II reactions, respectively), the number of in vitro formed metabolites was typically higher than those observed from in vivo excretion urine samples of corresponding AAS.

The main focus of the analytical part of the project was to examine the applicability of liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method in the detection of phase-I and phase-II metabolites of AAS. As a conclusion it should be drawn the advantage of parallel application of both GC/MS and LC-MS/MS to cover the wide range of AAS metabolites. This is especially taken into account in the case of potential formation of phase-I AAS metabolites with completely saturated A-ring structure (e.g. 5□-estran-3□-ol-17-one, the main metabolite of nandolone). 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 extremely problematic in GC/MS analysis. Due to chage delocalization and high proton affinity, LC-MS/MS is a method of choise for analysis of those AAS metabolites. Soft ionization technique, ESI, combined with LC separation also allow the direct detection of intact glucuronide-conjugated AAS metabolites.