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Alternative detection strategies for anabolic steroid abuse by NMR metabonomics and endocrine feedback loop analyses

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Abstract

So far, NMR metabonomics showed its potential for a range of applications in medicine, biochemistry, drug development, nutrition and related disciplines. During a pilot study NMR metabonomics was used for the discrimination between pre- and post-administration urines of a single dose administration trial with testosterone undecanoate (Andriol®) in humans. Discrimination was possible for almost 48 hours applying non-targeted principal components analysis while it was possible even for two weeks using a refined data analysis.

Another indirect detection technique for anabolic androgenic steroid abuse appears to be the analyses of pituitary profiles. The exogenous administration of anabolic steroids, including testosterone, is known to affect the production of endogenous hormones via feedback mechanisms on the hypothalamic pituitary gland axis. Within our investigation we have analyzed the effect of anabolics administration on different hormonal serum concentrations. Multiple doses were monitored and resulted in decreased LH and inhibin concentrations in rats. These findings were confirmed by samples collected from bodybuilders abusing steroids. Additionally the members of the "abuser" group were found to have significantly higher serum prolactin levels compared to normal males.

Introduction

In human sports doping control the (mis-)use of anabolic steroids or other anabolic substances is generally detected using mass spectrometry (MS) based assays such as GC-MS(/MS) and LC-MS(/MS). However, the examples of designer steroids have demonstrated that even substances with a chemical structure typical for this class of substances may sometimes be challenging if their exact chemical structure and metabolism is unknown. Therefore, orthogonal detection techniques may be very helpful to supplement the traditional mass spectrometric detection of anabolic substances.

In recent years, a new approach in metabonomics, namely the use of nuclear magnetic resonance (NMR) spectroscopy, can be found in an increasing number of applications [1,2]. As major advantage, NMR metabonomics does not require any extraction or derivatization of samples and, after setup of an initial profile, can lead straightforward to results in a fraction of time.

In a another approach hormones of the hypothalamic pituitary gonadal axis are targeted as they are known to be influenced by administration of androgens via feedback mechanisms (FIGURE1). Thus, the administration of anabolic steroids in sports might be detectable by monitoring endogenous indicators. For this study cortisol, TSH, FSH, LH, inhibin, prolactin, estradiol, thyroxin (T4), testosterone, IGF-1, activin receptor II beta (ActR-IIB), follistatin and myostatin were selected as candidates. Cortisol, TSH, FSH, LH, inhibin, prolactin and IGF-1 were monitored before and after administration of metandienone or estradienedione in rats for 21 days. Bodybuilders abusing steroids were included in the study as well to allow a proof of concept of the approach.
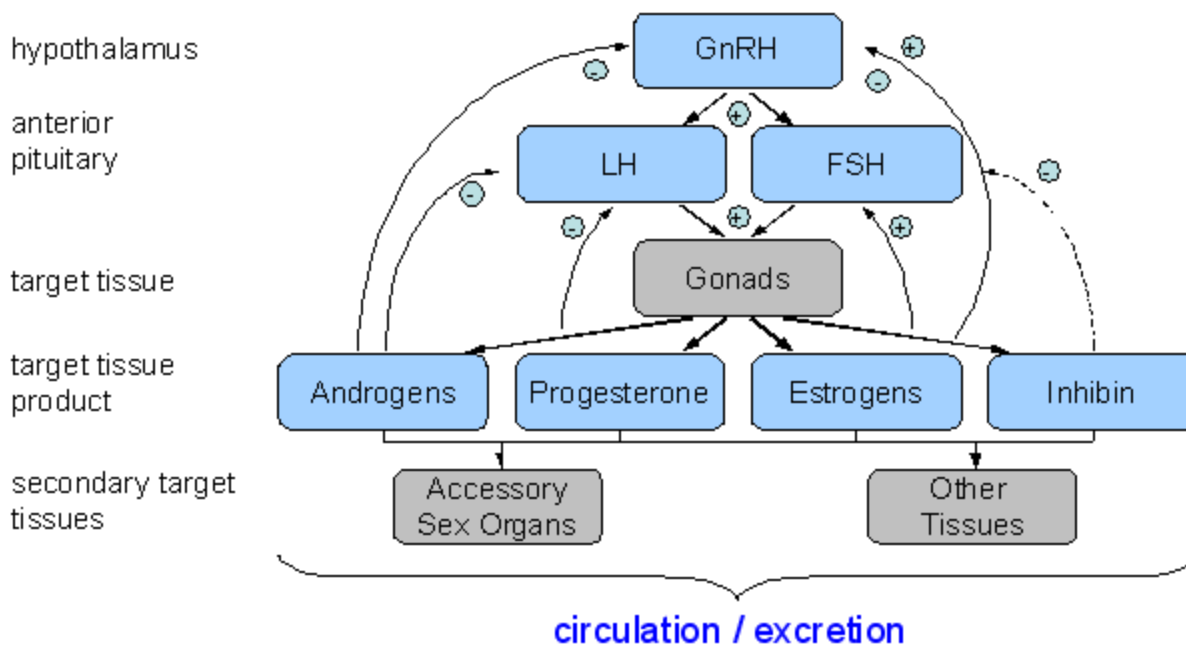
FIGURE1: Regulation of Steroid Secretion - Hypothalamic-Pituitary-Gonadal Axis, adapted from [3]

Experimental

Administration studies

An administration study was conducted in a human volunteer, who received a single dose of 40mg testosterone-undecanoate (one capsule of Andriol®). Urine samples were collected for two weeks before and two weeks after the administration and stored at –20°C until analyses.

In a second trial intact male wistar rats (n=8) were treated with metandienone or estra-4-9-diene-3,17-dione (s.c., 0.5, 1.5 mg/kg BW for 21 days). Urine and serum samples were collected after sacrifice.

Human serum samples were collected from ten recreational bodybuilders. Based on a questionnaire and urinary GC-MS(/MS) analysis (blind testing) five of them (age 24.20 ± 6.3 years, body weight 102.00 ± 13.77 kg, height 1.84 ± 0.07 m) were identified as anabolic steroid abusers, while the others (n=5, 34.4 ± 14.57 years, 94.50 ± 10.47 kg, 1.82 ± 0.07 m) were found negative for anabolic substances. Male laboratory staff members (n=6; 29.7 ± 9.35years; 81.92 ± 4.18kg; height 1.83 ± 0.05m) acted as control group. The study was approved by local ethical and animal welfare committees.

NMR analyses

The urine samples of the testosterone administration study were analyzed on a Bruker Avance II$^+$ NMR instrument at 600MHz (1D NOESY-presaturation, 300 K) and spectra were calibrated on 3-trimethylsilyl propionate (TSP=0ppm) and normalized on creatinin-CH_3-peak. Data were processed using AMIX software package (Bruker, Rheinstetten, Germany). Sample were handled as described in the standard protocol [4], i.e. diluted (9:1, v:v) with buffer (K_2HPO_4, NaN_3, TSP in D_2O/ H_2O) prior to analysis.

Serum hormone levels

Serum levels of TSH, FSH, LH, inhibin, and IGF-1 were determined by specific ELISAs, and cortisol by RIA.

Urinary anabolic steroid detection by GC-MS(/MS)

For comparison urinary analysis was performed using the routinely used anabolic steroid screening as described elsewhere [5,6].

Results and Discussion

NMR analyses of the urine samples of the testosterone administration study resulted in spectra as displayed in FIGURE2.
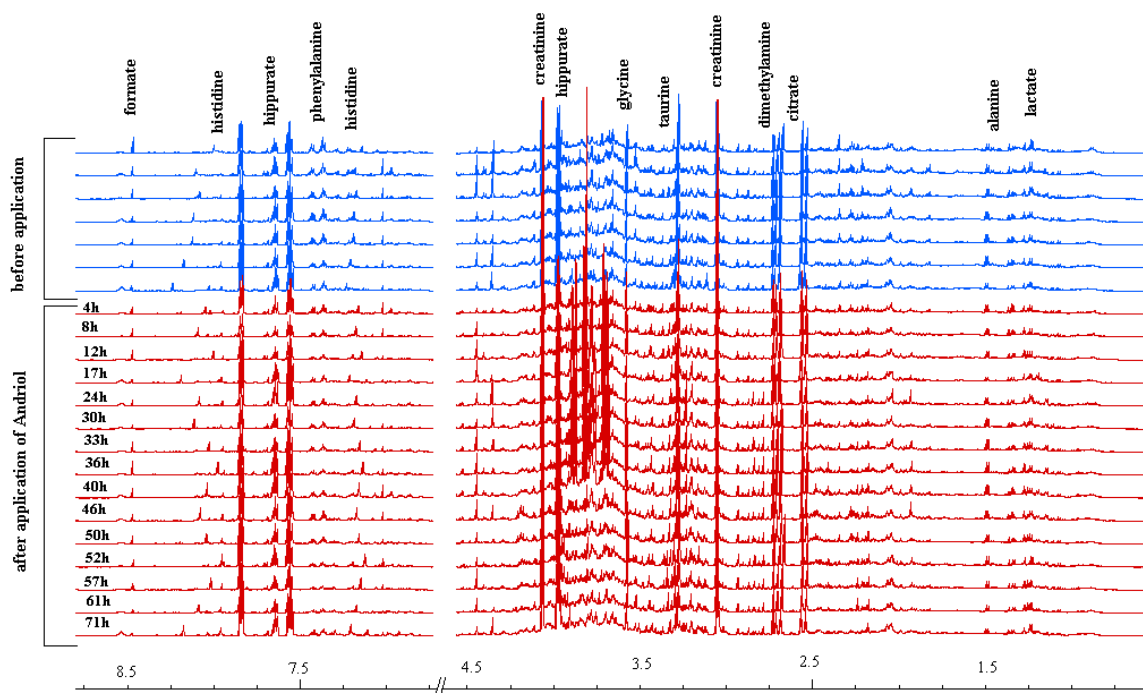

FIGURE2: 1H NMR spectra series of human urine before (blue) and after application of Andriol® (red, phosphate buffer in D2O, pH 7.4, 600 MHz, 300 K, ns 64, NOESYpresat sequence)

Already with a non-refined, standardized protocol for sample preparation and experiments and by non-targeted principal components analysis (PCA) of the experimental data, changes in the urine profile were detectable after administration of a single oral dose of 40mg of testosterone undecanoate and could be traced for almost 48 hours after administration (FIGURE3). With a more refined data analysis protocol implementing Monte-Carlo simulation, preliminary results indicate that a change in the profile is observable even in the last sample collected 15 days after administration. Alterations in the urinary steroid profile indicative for a testosterone administration were only detected within the first day after the administration when routine GC-MS methods of doping control were used for determination.

In the administration study in rats significant alterations of serum hormone levels could be detected. MD and EDD administration resulted in significantly decreased serum LH (FIGURE4). Additionally, inhibin and cortisol concentrations were decreased not significantly.

Five out of ten bodybuilders did not show any suspicious finding for anabolic steroids either in GC-MS or GC-MS/MS analyses. Five bodybuilders had adverse analytical findings for testosterone, nortestosterone and/or metandienone. These results were in accordance with the declarations on the questionnaire.

Bodybuilders identified to be positive for anabolic steroids by GC-MS(/MS) showed significantly increased serum levels for prolactin in comparison to the control group. In contrast serum levels of inhibin, LH and TSH were significantly decreased (FIGURE5). Cortisol levels remained unaffected. Comparison of the data with bodybuilders not abusing steroids showed that observed effects on TSH seemed to be a result of training whereas the altered LH, inhibin, and prolactin concentrations seemed to be the result of the administered substances. In summary the data of this study indicate that measurements of LH and inhibin could be a promising strategy to screen for an abuse of anabolic substances. Further hormones of the hypothalamic pituitary gonadal axis will be included in future investigations.
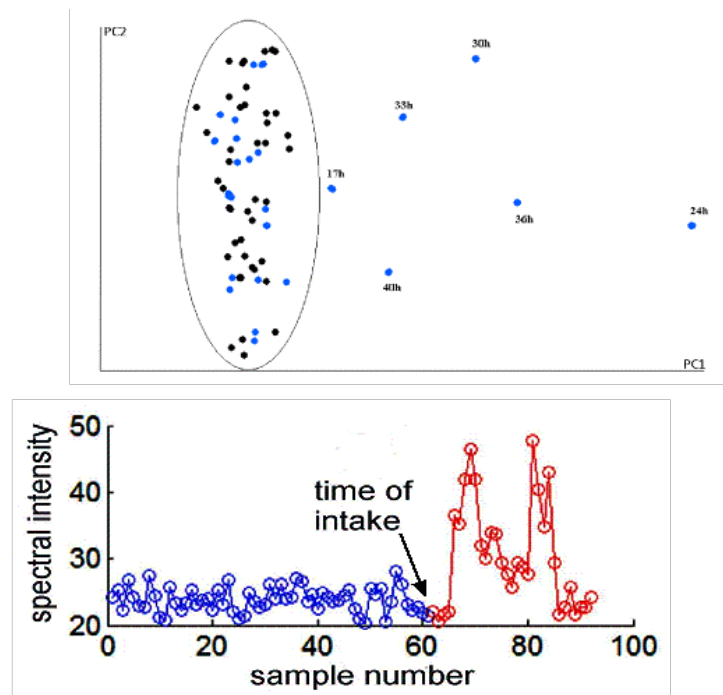
FIGURE3: Principle component analysis (PCA) of total NMR spectra (upper) and NMR based kinetics on basis of signals at 2.75-2.95 ppm (lower)
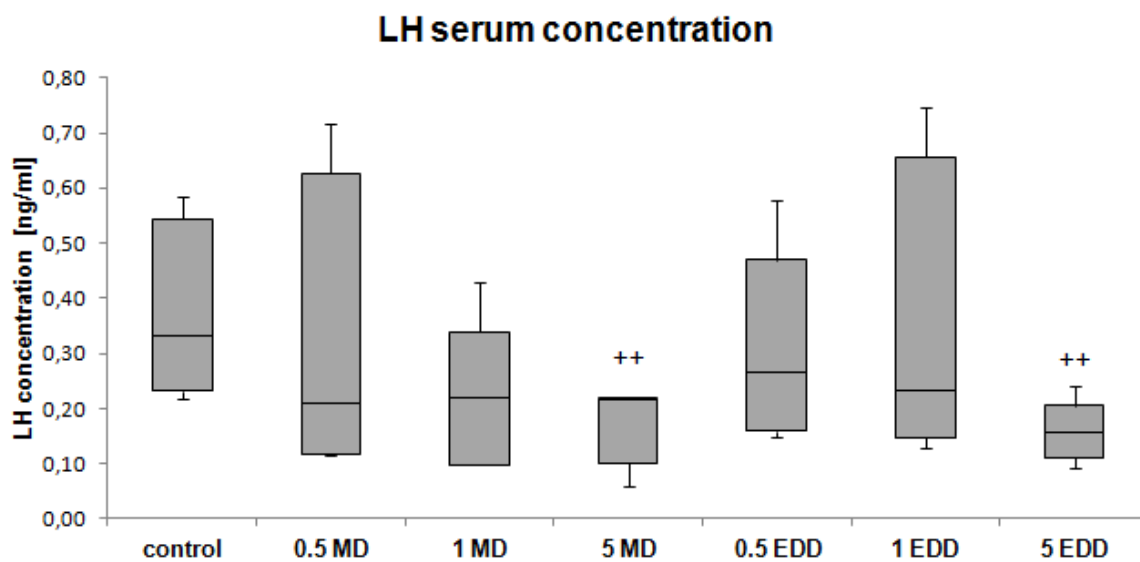

FIGURE4: Down-regulation of serum LH after s.c. administration of metandienone (MD) or estra-4,9-diene-3,17-dione (EDD) in rats administration of respective steroids for 21 days to intact male wistar rats (MD 0.5: 0.5 mg/kg BW/d, MD 1: 1 mg/kg BW/d, MD 5: 5 mg/kg BW/d, EDD 0.5: 0.5 mg/kg BW/d, EDD 1: 1 mg/kg BW/d, EDD 5: 5 mg/kg BW/d) dissolved in 20% peanut oil and 80% ethanol. ** means significant against control ($p<0.01$, Wilcoxon-Mann-Whitney test)
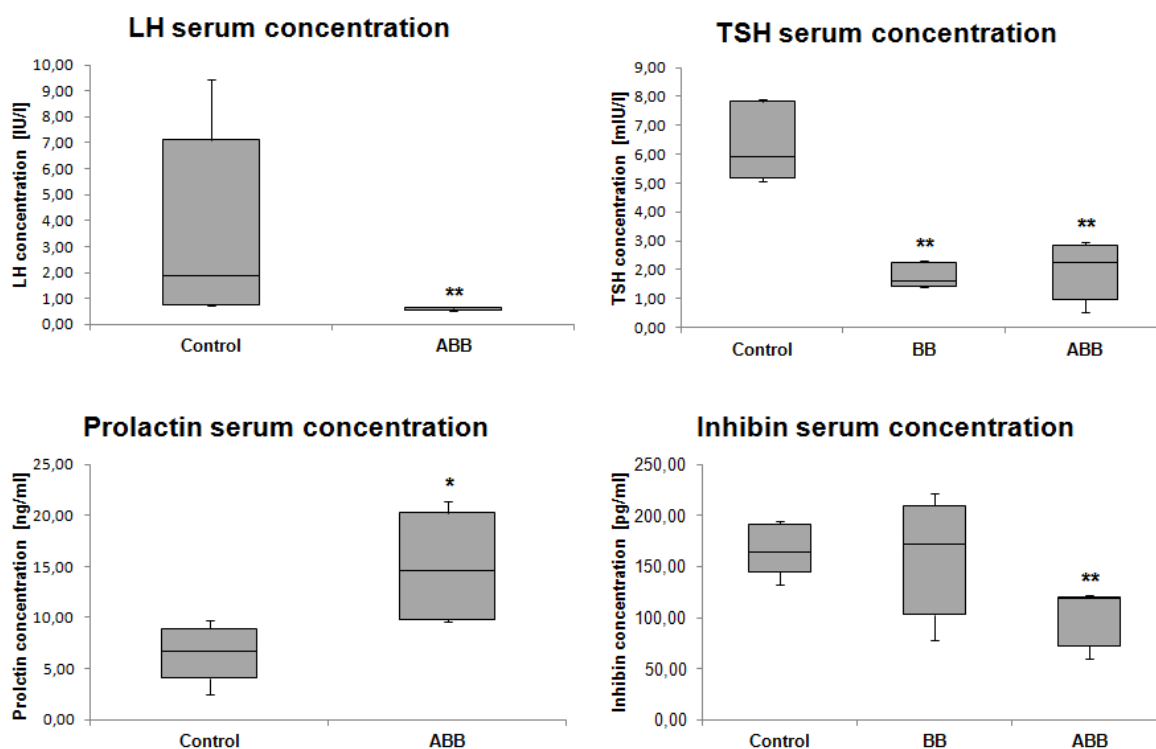
FIGURE5: Significantly altered serum parameters in bodybuilders abusing anabolic steroids (ABB) and non abusing bodybuilders (BB). Non-steroid using laboratory staff members served as control. ** means significant against control (p<=0.01, Wilcoxon-Mann-Whitney test). * means significant against control (p<=0.05, Wilcoxon-Mann-Whitney test).

Conclusions

Orthogonal detection techniques are useful to supplement conventional MS to screen for anabolics.

The analyses of pituitary profiles resulted in decreased serum LH and inhibin and increased prolactin following anabolic steroid administration. Another promising approach is based on NMR metabonomics, a fast technique manageable with almost no sample preparation. It resulted in detectable alterations in profile after one single oral dose of testosterone in non-targeted PCA for 48h after single dose and for 2 weeks using a refined approach for data analyses.

Future research is needed on inter-individual profile differences, identification of altered compounds recognized by NMR and on the influence of different preparations and administration routes as well as to better understand the metabolic feedback.

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