Quantitative Determination of Metabolic Products of 19-Norandrostenediol in Human Plasma using Gas Chromatography / Mass Spectrometry

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Abstract

Prohormones such as 19-norandrostenediol (estr-4-ene-3β,17β-diol) have been added to the list of prohibited substances of the World Anti-Doping Agency as they are metabolized to the common nandrolone metabolites norandrosterone and noretiocholanolone. So far, no studies on the metabolism and *in vivo* conversion of 19-norandrostenediol after oral or sublingual administration have been reported, nor have quantified data on resulting plasma nandrolone levels. In the present study, an open label crossover trial with eight healthy male volunteers was conducted. After application of capsules or sublingual tablets of 19-norandrostenediol plasma concentrations of 19-norandrostenediol, nandrolone, as well as major metabolites (19-norandrosterone and 19-noretiocholanolone) were determined using a validated assay based on gas chromatography/mass spectrometry (GC/MS). The administration of 100 mg capsules of 19-norandrostenediol yielded maximum plasma total concentrations (i.e. conjugated plus unconjugated compounds) of 1.1 ng/mL (± 0.7) for 19-norandrostenediol, 4.0 ng/mL (± 2.6) for nandrolone, 154.8 ng/mL (± 130.8) for 19-norandrosterone and 37.7 ng/mL (± 6.9) for 19-noretiocholanolone. The use of 25 mg sublingual tables resulted in 3.3 ng/mL (± 1.0) for 19-norandrostenediol, 11.0 ng/mL (± 6.4) for nandrolone, 106.3 ng/mL (± 40.1) for 19-norandrosterone and 28.5 ng/mL (± 20.8) for 19-noretiocholanolone. Most interestingly, the pharmacologically active unconjugated nandrolone was determined after administration of sublingual tablets (up to 5.7 ng/mL) in contrast to capsule applications. These results demonstrate the importance of prohibiting prohormones such as 19-norandrostenediol, in particular as plasma concentrations of nandrolone between 0.3-1.2 ng/mL have been reported to influence endocrinological parameters.
Introduction

Nandrolone (17β-hydroxy-estr-4-en-3-one, NL, Fig. 1A) is an anabolic steroid usually administered as the decanoate ester in the form of oily intramuscular injections. Nandrolone has been used as an anabolic agent after debilitating illness, for the treatment of postmenopausal osteoporosis, postmenopausal metastatic breast carcinoma, anaemia due to chronic renal failure, and aplastic anaemia. In addition to these therapeutic applications, NL gained public recognition due to its abuse by athletes for the purpose of increasing muscle mass and muscular strength (Yesalis and Bahrke, 1995). As a result, among others, NL was banned in 1974 by the International Olympic Committee (IOC). To provide evidence of NL doping the main urinary metabolites 19-norandrosterone (3α-hydroxy-5α-estran-17-one, NA, Fig. 1B) and 19-noretiocholanolone (3α-hydroxy-5β-estran-17-one, NE, Fig. 1C) have been used as target analytes (Schänzer and Donike, 1993; Schänzer, 1996; Saugy et al., 2000; Kicman and Gower, 2003). As a reference, NA has to exceed a threshold level of 2 ng per mL of urine in order to produce a positive doping test result. In its 2004 Adverse Analytical Findings Report the World Anti-Doping Agency (WADA) listed 339 positive specimens related to nandrolone abuse, which represented a proportion of 10% of the overall doping offences detected in the WADA-accredited laboratories. As recently published, not only deliberate appliance of nandrolone products have led to verifiable concentrations of NA in urine but also the degradation of endogenously produced androsterone to NA in so called “active” urine specimens (Grosse et al., 2005). Moreover, the administration of contaminated nutritional supplements (Catlin D. H. et al., 2000; Geyer et al., 2004) and possible endogenous production of nandrolone (Le Bizec et al., 1999; Kohler and Lambert, 2002) are associated with the detection of NA in urine samples. In particular the ingestion of nutritional supplements containing 19-norandrostenedione (estr-4-ene-3,17-dione, NDione, Fig. 1D) and 19-norandrostenediol (estr-4-ene-3β,17β-diol, NDiol, Fig. 1E) resulted in positive drug tests
owing to the presence of NA (Uralets and Gillette, 1999; Colker et al., 2001; Tseng et al., 2005). Supplement companies introduced both so-called prohormones as legal alternatives to illegal androgens in 1998, and anabolic properties were advertised due to an assumed conversion to NL. Since 1999 NDione and NDiol were classified as anabolic agents as defined by the IOC Anti-Doping Code and, as such, were banned in most sports. Under disguise of The Dietary Supplement Health and Education Act (DSHEA) these prohormones were available as nutritional supplements for over-the-counter (OTC) sales in the United States until the commencement of the Anabolic Steroid Control Act of 2004. Several studies on nandrolone prohormones dealing with the analysis of urinary metabolites or effects on athletic performance have recently been conducted (Uralets and Gillette, 1999; Colker et al., 2001; Van Gammeren et al., 2001; Van Gammeren et al., 2002; Tseng et al., 2005), but except for results of a pilot study (Machnik, 2001) no scientific data were published dealing with the in vivo conversion of these hormone precursors to their active form, i.e. NL. To investigate the in vivo biotransformation of NDiol, an evaluation of plasma levels of 19-norsteroids, in particular of unconjugated nandrolone (NL_{unc}), was performed within a clinical trial. To gain information if the route of administration affects the metabolic rate of the compound, NDiol was tested formulated as capsules as well as sublingual tablets.
Materials and methods

Chemicals

Potassium hydroxide (p.a.), sodium hydroxide (p.a.), and sodium dihydrogen phosphate monohydrate (p.a.) were purchased from Merck (Darmstadt, Germany). β-Glucuronidase from Escherichia coli was supplied by Roche Diagnostics (Mannheim, Germany). Methanol and tert.-butyl methyl ether were obtained from Kraemer & Martin (St. Augustin, Germany) and distilled before use. n-Pentane (≥ 99%) was bought from Merck Schuchard (Hohenbrunn, Germany). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was supplied by Chem. Fabrik Karl Bucher (Waldstetten, Germany) and distilled before use. Ammonium iodide and ethanethiol (≥ 97%) were purchased from Sigma-Aldrich (Steinheim, Germany).

Steroids

Nandrolone (NL, 17β-hydroxy-estr-4-en-3-one, >99%) was bought from Sigma-Aldrich (Steinheim, Germany), and 3,4,5-2H3-3α-hydroxy-5β-estran-17-one (d3-NE; 98%) was purchased from LGC Promochem (Wesel, Germany). The steroids 19-norandrostenediol (NDiol, estr-4-ene-3β,17β-diol, 95%), 19-norandrosterone (NA, 3α-hydroxy-5α-estran-17-one, >97%), 19-noretiocholanolone (NE, 3α-hydroxy-5β-estran-17-one, >97%), 16,16,17-2H3-estr-4-ene-3β,17β-diol (d3-NDiol; 3β/3α-isomers 3:1, w:w), and 16,16,17-2H3-17β-hydroxy-estr-4-en-3-one (d3-NL; 96%) were synthesized in our laboratory according to established procedures (Schänzer and Donike, 1993; Schänzer and Donike, 1995).

Subjects

Eight healthy men (age 32.8 ± 7.5 y; weight 81.6 ± 10.7 kg) volunteered to participate in this study. Prior to the study, subjects underwent physical examinations in order to check up on entrance and exclusion criteria (e.g. medical history, physical examinations, hematological
tests, and blood and urine evaluations). Each participant read and signed a health history and informed consent that detailed the outline of the study.

Regulatory and ethical aspects

According to the German Drug Act, nutritional supplements containing prohormones such as NDiol have never been considered as food supplements but as drug products without regulatory approval. Because of this status, our present investigation was categorized as a phase I-clinical trial. Ethical committee application, study protocol and informed consent form were prepared in accordance with the Declaration of Helsinki and approved by the Cologne University Human Subjects Committee. Registration at the local health authority and the Federal Institute for Drugs and Medical Advices (BfArM, Bonn) was applied.

Supplementation

Two nutritional supplements containing NDiol were purchased via the Internet. Norandrodiol (Kaizen, Los Angeles, CA) was consumed in 100 mg capsules. Cyclo-Nordiol (Kaizen, Los Angeles, CA) was provided as sublingual tablets containing a hydroxypropyl-ß-cyclodextrin-norandrostenediol complex (25 mg of NDiol per tablet). Supplements were analyzed for purity and authenticity in our laboratory. The study was designed as an open crossover trial. Administration of each of the respective products was performed once for each individual. Supplement administrations were separated by a period of at least two weeks. NDiol capsules were swallowed with water. Sublingual NDiol was placed under the tongue and allowed to dissolve for at least 10 minutes before swallowing tablet residues. Subjects were instructed to fast before and for 2 hours after administration of NDiol supplements.

Blood sample collection

On the day of administration an indwelling catheter was inserted into a forearm vein. Blood samples (10 mL) were collected immediately before supplementation and at 10, 20, 30, 45,
60, 90 minutes, 2 h, 3 h, 4 h, 6 h, and 8 h from \( t = 0 \). An additional sample was obtained by venipuncture 24 h after administration.

Blood samples were collected in potassium-EDTA tubes, centrifuged immediately, and the separated plasma was stored at \(-20 \, ^\circ\mathrm{C}\) until analysis.

**Plasma sample preparation**

For each analysis a volume of 0.5 mL of plasma was used. Each specimen was fortified with 20 µL of an internal standard mixture (0.1 µg/mL of \( \text{d}_3\)-NDiol, 0.1 µg/mL of \( \text{d}_3\)-NL and 1.0 µg/mL of \( \text{d}_3\)-NE, Fig. 2A-C).

In case of determination of total plasma steroid concentrations (unconjugated plus conjugated steroids; \( \text{NDiol}_{\text{tot}}, \text{NL}_{\text{tot}}, \text{NA}_{\text{tot}}, \text{NE}_{\text{tot}} \) ) 0.5 mL of phosphate buffer (0.2 M, pH 7) was added and an enzymatic hydrolysis was performed utilizing 50 µL of \( \beta \)-glucuronidase from *E. coli* at 50 °C for 60 min. After adjusting to pH 12 with 0.5 mL of aqueous KOH (0.5 M) a first liquid-liquid extraction (LLE) was performed by adding 6 mL of tert.-butyl methyl ether and shaking for 15 min. After centrifugation for 10 min at 1200 g the organic layer was transferred into a fresh tube and evaporated to dryness. The residue was resuspended in 1.0 mL of sodium hydroxide (0.01 M). A second LLE was carried out using 5 mL of n-pentane and shaking for 15 min. After centrifugation for 10 min at 1200 g the organic phase was collected and reextracted by shaking with 2 mL of a mixture of methanol/water (95/5, v/v) for 15 min. Subsequent centrifugation for 10 min at 1200 g was followed by removal of n-pentane and evaporation of the aqueous-methanolic layer. Finally, analytes were derivatized with 50 µL of a mixture of MSTFA/NH₄I/ethanethiol (1000/2/3, v/w/v) by incubation for 15 min at 60 °C. For the determination of \( \text{NL}_{\text{unc}} \), plasma sample preparation started directly with the first basic LLE skipping the enzymatic hydrolysis. If concentrations determined in plasma samples obtained after NDiol supplementation exceeded the defined working ranges, 0.25 mL...
of respective specimens was diluted using blank plasma (1:1, v/v) prior to reapplied sample preparation.

**Analysis by GC/MS**

Gas chromatography/mass spectrometry (GC/MS) analysis was performed with a quadrupole mass selective detector HP 5973 coupled to an HP 6890 gas chromatograph (Agilent Technologies, Waldbronn, Germany). A HP 5 crosslinked methyl silicone capillary column (length: 17 m; i.d.: 0.25 mm; film thickness 0.25 µm) was employed with helium as carrier gas at a constant flow-rate of 1.0 mL/min. A 2 µL aliquot of sample was injected onto the GC column in the splitless mode. The temperature program started at 100 °C, increased by 40 °C/min up to 190 °C, 5 °C/min up to 240 °C and finally 40 °C/min up to 320 °C and rest for 3 min. Injection port and transfer line were heated to 300 °C. Data acquisition was performed using electron impact ionisation at 70 eV, and selected ion monitoring (SIM) mode with dwell times of 20 ms for each ion was employed. Two fragment ions of each target analyte were selected for qualitative and quantitative determination (Table 1). To facilitate and optimize quantitation results, three stably deuterated compounds were employed as internal standards (ISTDs): d3-NDiol, d3-NL, and d3-NE (Fig. 2, A-C) were used for isotope-dilution mass spectrometry of NDiol, NL, NA, and NE, respectively. In Table 1, characteristic fragment ions of target analytes and ISTDs are listed, the underlined mass-to-charge values of which represent ions used for quantification purposes. Peak areas of deuterated ISTDs were corrected by subtracting the isotope signals of corresponding unlabeled analytes. As depicted in Fig. 3, informative fragment ions are influenced by deuterium labeling, e.g. the molecular ion M* at m/z 420 and (M*−15) at m/z 405 are incremented by 3 u to m/z 423 and 408, respectively. Moreover, fragment ions resulting from cleavages of the steroid nucleus such as m/z 129 and 142 (Table 1) demonstrate the diagnostic character of EI mass spectra used for qualitative and quantitative determination of target analytes. While the ion at m/z 129 was
generated from the steroidal D-ring under hydrogen rearrangement as substantiated by the presence of its deuterated counterpart at \( m/z \) 131 in case of \( \text{d}_3\)-NDiol, the fragment at \( m/z \) 142 resulted from the A-ring as reported in the literature earlier (Thevis and Schänzer, 2005).

**Validation of the analytical method**

*Calibration curves*

A calibration curve was generated for the quantification of each analyte. Therefore, male control blank plasma was fortified with respective analytes before passing sample preparation. Samples were run in duplicates at six different non-zero concentrations for all standard curves to verify linearity for all analytes within the defined working ranges (Table 2).

*Accuracy and precision*

Accuracy and precision were determined by replicate analysis of samples containing known amounts of the analytes. Depending on the extent of the working ranges accuracy and precision were measured using five determinations each of up to three different concentrations levels (Table 2).

*Lower limit of quantification*

The lower limits of quantification (LLOQ) were established using five samples for each analyte. At these levels analyte peaks should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120% (U.S. Food and Drug Administration, 2001).

*Recovery*

Recovery of all analytes were determined as follows: Six blank plasma samples were spiked with defined amounts of NDiol, ND, NA, and NE (Table 2) before sample preparation, and another six blank plasma samples were extracted according to the described protocol. Before the last drying and subsequent derivatization step, non-spiked blank plasma samples were
fortified with respective norsteroids, and all samples were enriched with 20 µL of the ISTD mixture. Recoveries were calculated by comparison of mean peak area ratios of analytes and ISTD of samples fortified prior to and after sample preparation. Linearity, LLOQ, accuracy, precision, and recovery were determined for total plasma steroid concentrations (unconjugated plus conjugated steroids) of NDiol, NL, NA, and NE as well as for NLunc.

**Pharmacokinetic and statistical evaluation**

The pharmacokinetic parameters $t_{\text{max}}$ (time of peak plasma concentration) and $C_{\text{max}}$ (peak plasma drug concentration) were calculated for NDiol$_{\text{tot}}$, NL$_{\text{tot}}$, NA$_{\text{tot}}$, NE$_{\text{tot}}$, and NL$_{\text{unc}}$ for each subject. Individual values for the area under the plasma level-time curve (AUC) were determined by the trapezoidal rule method. Calculations resulted directly from the measured plasma concentration data without curve fitting. Variables for $t_{\text{max}}$ were analyzed by nonparametric Wilcoxon signed rank test. Data for $C_{\text{max}}$ and AUC were tested for normal distribution (Kolmogorov-Smirnov test) and were compared between both treatment groups (capsule and sublingual tablet) by means of paired t-test. All parameters were tested for significant differences at the 5% level and p-values were adjusted by Bonferroni correction. The programs BIAS. for Windows (8.2), and R (R Development Core Team, 2005) were used for the statistical analysis.

**Results**

GC-MS analyses of plasma samples enabled the determination of all target analytes as depicted exemplarily in Figure 4. The specimen was collected 1 h after administration of a sublingual tablet (25 mg of NDiol), and NDiol (Fig. 4A) as well as the metabolites NL (NL$_{\text{tot}}$ Fig. 4B, NL$_{\text{unc}}$ Fig. 4D), NA and NE (Fig. 4C) were detected using the established approach at concentration levels ranging from 1.8 – 109.2 ng/mL.
Validation of the analytical method

Validation results are summarized in Table 2. Linearity was proven for all analytes within the defined working ranges (ANOVA). The lowest levels of the working ranges represent the respective lower limits of quantification (LLOQ). Results for calibration curves, LLOQ, accuracy and precision met requirements of the Guidance for Industry for Bioanalytical Method Validation (U.S. Food and Drug Administration, 2001).

Plasma concentrations of norsteroids after administration of a 100 mg NDiol capsule

Plasma total concentrations (unconjugated and conjugated steroids)

After oral administration of a 100 mg capsule, NDiol\textsuperscript{tot} was quantified in low amounts up to 2.3 ng/mL (Fig. 5A). Concentrations of NDiol\textsuperscript{tot} were detected during the entire monitoring period (8 h), peaked out between 1 and 8 h after application and returned to baseline levels within 24 h. Only plasma samples of one volunteer did not contain NDiol\textsuperscript{tot}. NDiol\textsuperscript{tot} concentrations of another volunteer, referred to as V8 in the following, remained at baseline about 4 hours before increasing during a subsequent 4-hours period. After 24 h plasma level concentrations of V8 and yet another volunteers still remained above LLOQ, determined at 0.6 ng/mL and 1.0 ng/mL, respectively. NL\textsuperscript{tot} was detected in plasma samples of the volunteers up to 8 h after administration. Plasma samples of the particular volunteer, which already showed no NDiol\textsuperscript{tot}, did also not contain NL\textsuperscript{tot}. Maximum values of the remaining volunteers varied between 1.6 and 8.8 ng/mL (Fig. 5B). The plasma concentration course of V8 remained at baseline about 4 h before increasing after NDiol administration. Oral ingestion of a 100 mg capsule of NDiol resulted in maximum total plasma concentrations of NA\textsuperscript{tot} and NE\textsuperscript{tot} ranging from 53.4 to 461.7 ng/mL (Fig. 5C) and 22.0 to 44.9 ng/mL (Fig. 5D), respectively. For about 4 hours plasma level concentrations of these metabolites remained at baseline values for V8 increasing rapidly during the subsequent 4-hours period, especially for NA\textsuperscript{tot}. Plasma levels of NA\textsuperscript{tot} and NE\textsuperscript{tot} decreased slowly, and after 24 hours
following the administration considerable amounts up to approximately 30 ng/mL for both compounds were still detected.

**Plasma concentrations of unconjugated nandrolone**

After ingestion of one capsule *Norandrodiol* containing 100 mg NDiol only very low amounts of NL$_{unc}$ were detected in plasma samples of the volunteers. In fact, values of only one volunteer exceeded the limit of quantification of 0.5 ng/mL as determined at 0.7 ng/mL and 0.6 ng/mL 45 and 60 minutes after administration, respectively.

**Plasma concentrations of norsteroids after administration of a 25 mg NDiol sublingual tablet**

**Plasma total concentrations (unconjugated and conjugated steroids)**

Administration of NDiol by the sublingual formulation resulted in fast absorption of the parent compound, which was detected in plasma samples of all volunteers. Maximum concentrations were determined in a range from 1.6 ng/mL to 4.3 ng/mL for each volunteer (Fig. 6A). Concentrations peaked out within 45 minutes and declined to baseline levels not later than 4 hours after administration. Already ten minutes after application of *Cyclo-Nordiol* the formation of NL was detected in plasma samples of all volunteers. Maximum plasma total concentrations of NL$_{tot}$ varied between 4.4 ng/mL and 20.2 ng/mL and were reached within one hour after application (Fig. 6B). Also, concentrations of the main metabolites NA and NE increased at an early stage. Maximum values for NA$_{tot}$ varied between 41.8 and 157.5 ng/mL (Fig. 6C) and for NE$_{tot}$ between 12.0 and 72.2 ng/mL (Fig. 6D). Trace amounts of NA$_{tot}$ were still detected 24 hours after administration but remained below LLOQ.

**Plasma concentrations of unconjugated nandrolone**

Ten minutes after sublingual administration of 25 mg NDiol sublingual tablets considerable amounts of NL$_{unc}$ in plasma samples of all volunteers were quantified. Plasma concentrations
of each volunteer peaked within 30 minutes after application and were determined between 3.2 and 5.7 ng/mL (Fig. 7). Even though concentrations fell below the lower limit of quantification of 0.5 ng/mL, NL_{unc} was still detected in all specimens three hours after application.

**Statistical and pharmacokinetic evaluation**

Mean values (± SE) of $C_{max}$, $t_{max}$ and AUC were calculated for NDiol_{tot}, NL_{tot}, NA_{tot}, NE_{tot}, and NL_{unc} as obtained from eight volunteers after administration of one 100 mg NDiol capsule and one 25 mg NDiol sublingual tablet, respectively (Table 3).

**Discussion**

In the current investigation, we compared metabolite profiles in plasma resulting from administrations of two differently formulated NDiol products. In contrast to urine analysis, blood analysis has enabled the detection of the applied prohormone and the intermediately formed NL in addition to the main metabolites NA and NE.

After consumption of a 100 mg NDiol capsule, NDiol_{tot} as well as NL_{tot} were determined after enzymatic hydrolysis in plasma samples of seven out of eight volunteers in low amounts (0.8 – 2.3 ng/mL and 1.6 – 8.8 ng/mL, respectively, Fig. 5). In plasma samples of all volunteers NA and NE were identified as major metabolites, while NL_{unc} (i.e. without hydrolysis) was quantified in low amounts near the LLOQ in one case only (0.7 ng/mL, data not shown). The deviant course of the concentration curves of all quantified norsteroids in plasma samples of V8 might result from a delayed dissolution of the capsule.

Concentration curves after administration of 25 mg NDiol sublingual tablets are characterized by a fast absorption of the prohormone as well as a fast increase of its metabolites in samples of all volunteers. Furthermore, NL_{unc} was detected and quantified in considerable quantities in plasma samples of all subjects (3.2 – 5.7 ng/mL).
Considering the fact that the administration of sublingual tablets applies only one fourth of the dose administered when using NDiol capsules, this drug formulation leads to significantly higher mean peak concentrations for NDiol_{tot} and NL_{tot}. Even though only low amounts of the parent compound were determined in plasma samples of both test groups (capsule and sublingual tablet) demonstrating no significant difference regarding mean AUC ($p = 0.1977$), the mean $C_{\text{max}}$ value for NDiol_{tot} is significantly higher after administration of the 25 mg NDiol sublingual tablet ($p = 0.0001$). In addition, plasma NL_{tot} concentrations reached a mean value of 11.0 ng/mL (± 6.4), which accounts for nearly the threefold amount registered after ingestion of the 100 mg NDiol capsule ($p = 0.0083$), but the mean AUC for NL_{tot} is two times higher in the 100 mg capsule test group ($p = 0.0324$). No significant differences for $C_{\text{max}}$ of the main metabolites NA and NE have been observed ($p = 0.3333$ and $p = 0.3692$, respectively). Regarding mean AUCs, values for both, NA_{tot} and NE_{tot}, are significantly higher after administration of NDiol capsules compared to the sublingual formulation ($p = 0.048$ and $p = 0.0001$, respectively). While detecting only low concentrations of NL_{unc} in plasma samples of one volunteer after capsule administration, sublingually administered NDiol was effective in raising plasma NL_{unc} levels, determined at 4.4 ng/mL (± 1.0). Furthermore, sublingual administration of NDiol produced uniform hormonal responses and reduced mean time to peak concentrations ($t_{\text{max}}$) as well as fast decrease in plasma concentrations for all analytes compared to oral administration (Fig. 5-7). The $t_{\text{max}}$ values for NDiol_{tot}, NL_{tot}, NA_{tot} and NE_{tot} ($p = 0.009$, 0.0533, 0.0624 and 0.0083, respectively) were significantly different only for NDiol_{tot} and NE_{tot}.

These findings result from different absorption kinetics due to the different drug formulations. Initially, prohormones such as NDiol have widely been used in form of capsules or pills. Manufacturers introduced sublingual formulations of prohormones to the supplement market intending to improve effectiveness of their products. This pharmaceutical form should prevent
prohormones from assumed hepatic catabolism if consumed orally. Sublingual tablets are
designed for drug release under the tongue and are used for compounds, which are able to
penetrate mucosal membranes. In sublingual tablets (or “lozenges” as labeled by the
manufacturer), hydroxypropyl-ß-cyclodextrin forms an inclusion complex with NDiol to
forward dissolution. Compounds absorbed through the oral mucosa shall be distributed within
the body prior to the first hepatic metabolism. As a consequence, sublingual application may
provide better bioavailability and a shorter onset time over oral administration for drugs with
noticeable first-pass effect.

To evaluate if concentrations of NL_{unc} generated from prohormones are pharmacologically
relevant, pharmacokinetic investigations regarding NL-containing therapeutics have been
considered. These drugs have generally been marketed as depot injections and not for oral
administration. The single \textit{i.m.} application of 100 mg of NL decanoate to healthy male
volunteers resulted in mean peak plasma concentrations of NL 4.3 ng/mL (2.9-6.9 ng/mL)
(Bagchus et al., 2005), and weekly injections of 200 mg of NL-3-(p-hexoxyphenyl)-
propionate for 10 weeks yielded NL plasma levels up to 5.5 ng/mL (Belkien et al., 1985).
Here, plasma testosterone suppression was considered as an important indicator for the
pharmacological activity of NL, and testosterone levels did not recover from suppressive
effects until NL_{unc} concentrations declined to 0.3-1.2 ng/mL (Belkien et al., 1985; Knuth et
al., 1985; Minto et al., 1997). Hence, this concentration range of NL_{unc} can be regarded as the
minimum effective concentration of the drug that will result in a pharmacological effect.

Sublingual uptake of 25 mg NDiol resulted in maximum plasma levels of NL_{unc} between
3.7 and 5.7 ng/mL, i.e. all volunteers have been exposed to pharmacologically relevant
plasma concentrations of NL_{unc} for at least two hours if compared to acute therapeutic
nandrolone application. Although these concentration levels remained for 2-3 hours only,
pharmacological effects cannot be ruled out, especially considering a multiple intake of the respective supplement.

To the best of our knowledge, this study is the first reporting on the in vivo conversion of nandrolone prohormones into nandrolone. Related studies examined the acute effect of orally administered testosterone prohormones such as androstenedione and androstenediol, which did not result in uniform findings concerning the elevation of plasma testosterone concentrations of young healthy men (King et al., 1999; Brown et al., 2000; Earnest et al., 2000, Leder et al., 2000). In accordance to our results, an increase of plasma testosterone concentrations after sublingual androstenediol intake was reported (Brown et al., 2002) demonstrating the effectiveness of this route of drug administration.

**Conclusion**

The observed plasma NL unc concentrations after application of NL prohormones as determined in the present study are alarming in particular as NDiol was marketed as nutritional supplement and may have been (mis)used in amateur and professional sport. Our findings suggest that NDiol supplementation may have exposed the users to unpredictable health risks as its in vivo conversion to the active compound NL was demonstrated. Moreover, the rationale for prohibiting prohormones such as NDiol in sports was substantiated as the prohibited compound NL was generated in addition to the common metabolites NA and NE.

**Acknowledgments**

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References


Footnotes

This project has been carried out with support from the World Anti-Doping Agency (WADA).

* only one volunteer generated quantifiable amounts; mean values were not calculated
Legends for figures

Figure 1: Chemical structures of A) nandrolone (NL, mol wt = 274), B) 19-norandrosterone (NA, mol wt = 276), C) 19-noretiocholanolone (NE, mol wt = 276), D) 19-norandrostenedione (ND, mol wt = 272), and E) 19-norandrostenediol (NDiol, mol wt = 276).

Figure 2: Chemical structure of A) \(\text{16,16,17-2H}_3\text{-estr-4-ene-3\beta,17\beta-diol}\) (d\(\text{3-NDiol}\), mol wt = 279), B) \(\text{16,16,17-2H}_3\text{-17\beta-hydroxy-estr-4-en-3-one}\) (d\(\text{3-NL}\), mol wt = 277), and C) \(\text{3,4,5-2H}_3\text{-3\alpha-hydroxy-5\beta-estran-17-one}\) (d\(\text{3-NE}\), mol wt = 279).

Figure 3: EI mass spectra of the target analyte NDiol bis-TMS (A, mol wt = 420) and its deuterated analogue d\(\text{3-NDiol}\) bis-TMS (B, mol wt 423). The molecular ion as well as characteristic fragment ions such as \(m/z\) 330 and 240 differ by 3 u due to deuterium labeling, while \(m/z\) 129 resulting from the steroidal D-ring is shifted to \(m/z\) 131 by two deuterium atoms only. The ion at \(m/z\) 142 remains constant as it is suggested to originate from the unlabeled A-ring.

Figure 4: GC-MS analysis of a plasma sample collected 1 h after administration of 25 mg of NDiol (sublingual tablet). The extracted ion chromatograms contain target analytes (solid lines) and respective deuterated internal standards (dotted lines): (A) 1.8 ng/mL of NDiol\(_{\text{tot}}\), (B) 11.9 ng/mL of NL\(_{\text{tot}}\), (C) 109.2 ng/mL of NA\(_{\text{tot}}\) and 20.7 ng/mL of NE\(_{\text{tot}}\), and (D) 2.2 ng/mL of NL\(_{\text{unc}}\).
Figure 5: Plasma total concentrations of NDiol\textsubscript{tot} (A), NL\textsubscript{tot} (B), NA\textsubscript{tot} (C), and NE\textsubscript{tot} (D) after administration of a capsule containing 100 mg of NDiol (eight volunteers). For clarity purposes, V8 is shown using a dotted line.

Figure 6: Plasma total concentrations of NDiol\textsubscript{tot} (A), NL\textsubscript{tot} (B), NA\textsubscript{tot} (C), and NE\textsubscript{tot} (D) after administration of a sublingual tablet containing 25 mg of NDiol (eight volunteers).

Figure 7: Plasma total concentrations of NL\textsubscript{unc} after administration of a sublingual tablet containing 25 mg of NDiol (eight volunteers).
Tables

Table 1: Characteristic ions of steroids used for qualitative and quantitative determination. All steroids were analyzed as bis-TMS-derivatives.

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<td>142</td>
<td>129</td>
</tr>
<tr>
<td>d3-NDiol</td>
<td>423</td>
<td>408</td>
<td>142</td>
<td>131</td>
</tr>
<tr>
<td>NL</td>
<td>418</td>
<td>403</td>
<td>194</td>
<td>182</td>
</tr>
<tr>
<td>d3-NL</td>
<td>421</td>
<td>406</td>
<td>194</td>
<td>182</td>
</tr>
<tr>
<td>NA</td>
<td>420</td>
<td>405</td>
<td>225</td>
<td>169</td>
</tr>
<tr>
<td>NE</td>
<td>420</td>
<td>405</td>
<td>225</td>
<td>169</td>
</tr>
<tr>
<td>d3-NE</td>
<td>423</td>
<td>408</td>
<td>228</td>
<td>169</td>
</tr>
</tbody>
</table>
Table 2: Validation parameters and results for plasma total concentrations (conjugated and unconjugated) of NDIol, NL, NA, NE and unconjugated NL (NLunc)

<table>
<thead>
<tr>
<th></th>
<th>Working range</th>
<th>Correl. coeff. ($R^2$)</th>
<th>Accuracy (%)</th>
<th>Precision (CV, %)</th>
<th>Mean recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDIol tot</td>
<td>0.5 – 10 ng/mL</td>
<td>0.998</td>
<td>11.4 (0.5 ng/ml)</td>
<td>6.5 (0.5 ng/ml)</td>
<td>55.3 (5 ng/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.4 (10 ng/ml)</td>
<td>2.2 (10 ng/ml)</td>
<td></td>
</tr>
<tr>
<td>NL tot</td>
<td>0.5 – 80 ng/mL</td>
<td>0.999</td>
<td>5.5 (0.5 ng/ml)</td>
<td>2.5 (0.5 ng/ml)</td>
<td>66.1 (5 ng/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.6 (25 ng/ml)</td>
<td>2.2 (25 ng/ml)</td>
<td>67.8 (50 ng/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.7 (80 ng/ml)</td>
<td>1.6 (80 ng/ml)</td>
<td></td>
</tr>
<tr>
<td>NA tot</td>
<td>5 – 2000 ng/mL</td>
<td>0.998</td>
<td>12.2 (5 ng/ml)</td>
<td>3.1 (5 ng/ml)</td>
<td>74.8 (10 ng/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.1 (400 ng/ml)</td>
<td>1.1 (400 ng/ml)</td>
<td>81.1 (500 ng/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.3 (2000 ng/ml)</td>
<td>0.6 (2000 ng/ml)</td>
<td></td>
</tr>
<tr>
<td>NE tot</td>
<td>5 – 2000 ng/mL</td>
<td>0.998</td>
<td>10.4 (5 ng/ml)</td>
<td>2.1 (5 ng/ml)</td>
<td>74.3 (10 ng/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.0 (400 ng/ml)</td>
<td>2.3 (400 ng/ml)</td>
<td>75.6 (500 ng/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.9 (2000 ng/ml)</td>
<td>1.1 (2000 ng/ml)</td>
<td></td>
</tr>
<tr>
<td>NL unc</td>
<td>0.5 – 5 ng/mL</td>
<td>0.999</td>
<td>9.9 (0.5 ng/ml)</td>
<td>7.4 (0.5 ng/ml)</td>
<td>61.8 (2.5 ng/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.8 (5 ng/ml)</td>
<td>1.7 (5 ng/ml)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Summary of pharmacokinetic evaluation

<table>
<thead>
<tr>
<th>dosage form (dose/unit)</th>
<th>$C_{\text{max}}$ (ng/mL)</th>
<th>$t_{\text{max}}$ (h)</th>
<th>AUC 0-24 (h*ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>capsule (100 mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDioltot</td>
<td>1.1 (± 0.7)</td>
<td>4.3 (± 2.7)</td>
<td>11.4 (± 13.6)</td>
</tr>
<tr>
<td>NLtot</td>
<td>4.0 (± 2.6)</td>
<td>2.6 (± 2.6)</td>
<td>33.5 (± 24.2)</td>
</tr>
<tr>
<td>NAtot</td>
<td>154.8 (± 130.8)</td>
<td>2.8 (± 2.3)</td>
<td>1371.9 (± 1365.0)</td>
</tr>
<tr>
<td>NEtot</td>
<td>37.7 (± 6.9)</td>
<td>3.8 (± 2.6)</td>
<td>529.1 (± 185.7)</td>
</tr>
<tr>
<td>NLunc $^a$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NLtot</td>
<td>11.0 (± 6.4)</td>
<td>0.6 (± 0.3)</td>
<td>15.3 (± 7.6)</td>
</tr>
<tr>
<td>NAtot</td>
<td>106.3 (± 40.1)</td>
<td>0.9 (± 0.3)</td>
<td>277.9 (± 122.9)</td>
</tr>
<tr>
<td>NEtot</td>
<td>28.5 (± 20.8)</td>
<td>0.8 (± 0.3)</td>
<td>49.5 (± 31.0)</td>
</tr>
<tr>
<td>NLunc</td>
<td>4.4 (± 1.0)</td>
<td>0.4 (± 0.1)</td>
<td>5.0 (± 1.2)</td>
</tr>
</tbody>
</table>

$^a$ only one volunteer generated quantifiable amounts; mean values were not calculated.
Figure 1

A

B

C

D

E
Figure 2

A

B

C
Figure 3
Figure 4
Figure 5
Figure 6

A

B

C

D

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Figure 7