Nandrolone, or 19-nortestosterone, is an anabolic steroid initially introduced for the treatment of anemia, osteoporosis, and breast carcinoma (1, 2). Nandrolone is available in several pharmaceutical formulations as a 17β-hydroxy ester in an oily matrix or as a nandrolone salt (decanoate or sodium sulfate) in an aqueous solution. The pharmaceutical formulation most widely used is Deca-Durabolin® (3).

Because of its anabolic properties, nandrolone is used among athletes as a doping agent to accelerate muscle growth; to increase lean body mass, strength, and aggressiveness; and to allow faster recovery between athletic performances (4–6). Long-term use of anabolic steroids can lead to cardiovascular problems, neurologic disorders, and liver diseases such as peliosis hepatis, cholestasis, or hepatic tumors. Fatalities have been reported in young steroid abusers (7, 8).

Analyses for nandrolone according to the protocols set down by the International Olympic Committee are based on the identification of its two principal urinary metabolites, which in humans are glucuronides of 19-norandrosterone and 19-noretiocholanolone. For the main metabolite, 19-norandosterone, a limit of 2 μg/L for males and 5 μg/L for females has been set by the Olympic Movement Anti-Doping Code (9–11).

Because of the many positive cases involving professional athletes, various investigations and hypotheses have been made to exclude voluntary intake of nandrolone. The endogenous origin of nandrolone remains a controversial topic, although the existence of small amounts of naturally occurring nandrolone metabolites in urine under physiologic conditions or after physical effort

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has been demonstrated (12–21). Nevertheless, in almost all cases, the measured concentrations generally remain far below the threshold limits.

Other hypotheses are that some nutritional supplements contain traces of the steroids or prohormones, such as other norsteroids, not indicated on the label. Indeed, various studies have demonstrated that the consumption of some noncertified nutritional supplements may give rise to nandrolone metabolites in urine samples at concentrations higher than the respective cutoff values (23–28).

In this context, determination of the origin of nandrolone carbon by gas chromatography (GC)–combustion–isotope ratio mass spectrometry (MS) seems to be an interesting challenge, but investigations in this field of application are still in progress (28–36).

The aims of this study were to determine the interindividual variability in nandrolone excretion patterns and kinetics after the administration of [13C]nandrolone to healthy volunteers and to determine the influence of physical effort on the urinary excretion of labeled nandrolone metabolites (manuscript in preparation).

Material and Methods

CHEMICALS AND REAGENTS
All chemicals were analytical grade. n-Pentane was purchased from Fluka Chemie. Na₂CO₃ was obtained from Merck and NaHCO₃ from Acros; N-methyl-N-(trimethylsilyl)-trifluoroacetamide was provided by Macherey-Nagel; trimethylsilyl-dithioerythritol was from Sigma; and dithioerythritol was from Acros. Escherichia coli β-glucuronidase was purchased from Roche Diagnostics. Norandrosterone (NA) and noretiocholanolone (NE) were obtained from Sigma. [3,4,13C₂]Nandrolone standard (98% purity) came from the National Analytical Reference Laboratory (NARL Pymble, Australia). The pills for the study containing labeled nandrolone were prepared by the Pharmacy of the University Hospital (CHUV, Lausanne, Switzerland).

INSTRUMENTATION

Qualitative and quantitative analyses were performed on a Hewlett-Packard 6890 gas chromatograph (HP Analytical Division) equipped with a HP 7673 autosampler and coupled with a HP 5973 mass-selective detector. Instrument control and data processing were performed with a HP Vectra XA Computer and ChemStation software. GC separation was achieved on a ZB-5 (5% phenyl–95% dimethyl-polysiloxane; Phenomenex) column [30 m × 0.25 mm (i.d.), 0.25-μm film thickness] operated with a helium inlet pressure of 103 kPa and the following temperature programming: 100 °C for 1 min, increased at 16 °C/min to 220 °C, increased at 3.80 °C/min to 270 °C, and held for 3 min. Injections of 1-μL samples were made at 270 °C in the splitless mode (1 min) into a split-splitless injection port with an inner silanized glass liner, and the transfer line was heated at 280 °C. The ion source was operated in the electron impact mode at 70 eV.

The analyses were performed in single-ion monitoring mode with ions m/z 420, 405, and 315 for [12C]nandrolone; m/z 422, 407, and 317 for [13C]nandrolone; and m/z 446 and 301 for methyltestosterone (internal standard). In all cases, no interference from the urinary matrix was observed. The ions chosen for quantification were m/z 405 and 407 for [12C]nandrolone and [13C]nandrolone, respectively. In this study, regression curves (1–50 μg/L) were used for the quantification of low concentrations of labeled nandrolone metabolites, whereas high concentrations (>50 μg/L) were estimated by mean of two calibration points at 100 and 500 μg/L, introduced in every extracted batch. In the case of very high concentrations, urine samples were diluted with distilled water.

SELECTION OF VOLUNTEERS

The study was conducted at the Swiss Antidoping Laboratory in Lausanne, Switzerland, after approval of the protocol by the ethics committee from the Faculty of Medicine of the University of Lausanne. Participants were healthy male student volunteers of Caucasian origin, 19–27 years of age, from the Institute of Sports Sciences and Physical Education (University of Lausanne). A total of 34 volunteers provided informed consent for participation in the study. These students were not physically dependent on drugs and were instructed not to take any nutritional supplements or steroids that could affect their metabolism and steroid profile for a sufficient period before and during the study. In addition, all volunteers denied any participation in weightlifting or bodybuilding.

Volunteers who were prone to hormonal dysfunction, cardiovascular disease, or had used controlled substances or any medication known to affect steroid metabolism were excluded from the study. Participants were evaluated medically and psychologically at the beginning of the study, before the administration of nandrolone, and at the end of the study to monitor their health. In particular, several physiologic indices were measured (summarized in Table 1; for simplicity, only the minimum and maximum values, as well as the means and SD are given).

STUDY PROTOCOL

Two 25-mg oral doses of [3,4,13C₂]nandrolone were administered to 22 healthy male volunteers at 24-h intervals; 12 participants received placebo. The volunteers receiving labeled nandrolone or placebo were designed randomly. The pills containing [13C]nandrolone were administered between 0730 and 0800 in the morning. The volunteers were asked to collect ~100-mL aliquots from each of their spot urines for 5 days [excretion study (ES) period]. During that period of time, urines were kept by the

4 Nonstandard abbreviations: GC-MS, gas chromatography–mass spectrometry; NA, norandrosterone; NE, noretiocholanolone; and ES, excretion study.
volunteers at 4 °C until their delivery to the laboratory. After that, each urine sample was divided into 20-mL flasks and stored at −20 °C until extraction and analysis at the laboratory.

**ISOLATION OF NA AND NE FROM URINE**

To 1.5 mL of urine we added 0.75 mL of 0.8 mol/L phosphate buffer (pH 7.0) and 20 µg/L of methyltestosterone (used as internal standard); we then hydrolyzed the urine for 90 min at 50 °C with 75 µL of β-glucuronidase from *E. coli* (specific activity, 200 kU/L). After the addition of ~200 mg of solid carbonate buffer (Na₂CO₃–NaHCO₃, 1:10 by weight), the sample was extracted with 5 mL of *n*-pentane with shaking for 10 min. After centrifugation, (2500g for 5 min), the organic phase was collected and dried with Na₂SO₄ and the residue was derivatized with 50 µL of a solution containing 1 mL/L N-methyl-N-(trimethylsilyl)-trifluoroacetamide, 5 µL/L trimethylsилosilane, and 5 µg/L dithioerythritol for 30 min at 60 °C.

**Results**

The goal of this study was to investigate nandrolone metabolism in volunteers after administration of [13C]nandrolone (Fig. 1). The relevance of [13C]nandrolone administration was to focus on the analyses and interpretation on labeled metabolites only. The [13C] in positions 3 and 4 of the molecule was substituted by 13C. By this substitution we could clearly differentiate analyt-
ically between eventual endogenous and administered nandrolone, particularly at the end of the urine collections, when the excretion rates were lower. Nandrolone metabolism is not affected by these chemical changes, and the two main urinary nandrolone metabolites remain NA and NE. The corresponding mass spectra (Fig. 2), with fragment ions, allowed clear differentiation between nonlabeled and labeled metabolites (molecular ions and base peaks are $m/z$ 420 and 405 and 422 and 407, respectively). Because the presence in urine of very low concentrations of nandrolone metabolites could be attributable, in some cases, to physical effort or to contaminated supplements, it was easier to investigate and evaluate results obtained on labeled metabolites (single-ion monitoring mode with ion $m/z$ 407), than it would have been after administration of nonlabeled nandrolone. Moreover, $^{13}$C-nandrolone was chosen rather than the deuterated form because of its better stability during metabolism (data not shown).

Urine samples were extracted according to a published procedure (37–42) and analyzed by GC-MS in single-ion monitoring mode with ion $m/z$ 407. In both placebo- and steroid-treated volunteers, no significant amounts of endogenous nandrolone metabolites were detected [$<2 \mu g/L$ (International Olympic Committee cutoff value); data not shown]. Furthermore, the labeled nandrolone standard (98% purity) provided by the National Analytical Reference Laboratory was analyzed in the laboratory, and no unlabeled nandrolone was found.

![Mass spectra of $^{12}$C-NA (A) and $^{13}$C-NA (B) bis-trimethylsilyl derivatives.](image)
Numerous spot-urine samples were evaluated to determine the correlation between urinary creatinine and specific gravity. Urinary creatinine concentrations and specific gravity were determined in 750 spot samples (665 from steroid-treated volunteers and 85 from placebo-treated volunteers). Spot-urine samples were collected throughout the ES period, then for 5 days after the first intake of \([13C\]nandrolone. In spot samples, creatinine concentrations ranged between 0.051 and 3.790 g/L and specific gravity between 1.003 and 1.032, and 78% of creatinine values and 81% of specific gravity values fell within the reference intervals of 0.500–3.000 g/L and 1.010–1.030 for creatinine and specific gravity, respectively. No significant difference was observed between treated and nontreated volunteers, and we observed a high correlation \([y = 0.0068\ln(x) + 1.0172 \quad \left(r^2 = 0.773\right)\) and \([y = 0.0052\ln(x) + 1.0178 \quad \left(r^2 = 0.8142\right)\), respectively] between creatinine and specific gravity for both groups (Fig. 3).

\section*{Excretion Kinetics and Patterns}

Nandrolone is excreted in human urine as two main metabolites, NA and NE, mainly conjugated as glucuronides and in small amounts as sulfate esters (15, 43). In this study, only the free and conjugated fractions were extracted.

During the ES period, all nandrolone-treated volunteers showed a similar excretion pattern with a peak between 5 and 7 h after each intake of labeled nandrolone. The major elimination of labeled nandrolone via its two main metabolites is very fast, within 15–20 h, but it is interesting to note that small amounts were found over several days for some volunteers (Figs. 4 and 5). This observation has already been published in the literature (15, 43–45). It should be pointed out that for two volunteers, peak excretion for labeled NA and NE was at 12 h after the first intake. It is noteworthy that this difference in excretion is not as clear at the time of the second excretion peak.

For logistic reasons, it was not possible to collect all urine volumes but only an aliquot from each spot urine. For this reason, it was not possible to determine the exact amount of labeled nandrolone metabolites excreted at each time. Nevertheless, in a comparison between volunteers, the concentrations measured in urine during the ES period were very disparate (Fig. 4). In a doping control situation, two individuals previously treated with the same dosage at the same time could show very different concentrations of NA and NE (from 1180 to 34 043 µg/L and from 576 to 12 328 µg/L, respectively; see Table 2).
Even if the second nandrolone dose was close to the first one, no cumulative phenomenon was observed; the second excretion pattern was geometrically similar to the first one, as can be seen in Fig. 4 (exact values not shown). At the end of the labeled nandrolone excretion period (between 50 and 100 h after the first intake), the kinetic of excretion was different from one volunteer to another. Indeed, when we compared two volunteers (Fig. 5), the differences in the concentrations labeled nandrolone metabolites at that time of excretion was significant. For example, in some samples no nandrolone metabolites were detected, whereas in other cases, the concentrations were >100 μg/L.

**VARIABILITY IN NE:NA RATIOS**

The NE:NA ratio was never constant and varied during the entire excretion study. For all volunteers, the amount of labeled NA excreted was greater than that of labeled NE at the beginning of the excretion study as well as during the major elimination step (Fig. 6). This difference in labeled nandrolone metabolite production has already been pointed out in the literature, and the greater excretion of NA compared with NE is in agreement with previous studies (15, 43). Nevertheless, at the end of excretion period, the NE:NA ratio remained <1 for five volunteers and >1 for all the other volunteers (see Fig. 6). To our knowledge, this observation has never been clearly assessed in the literature. Consequently, it is possible to obtain a chromatogram in which the labeled NE peak is clearly higher than the NA peak.

**Discussion**

Nandrolone is an anabolic steroid widely used in some sports in which muscle mass and then strength are important factors. This substance is used mainly in bodybuilding but not exclusively for this purpose. Indeed, today various sport federations are concerned by positive cases of nandrolone use, and numerous publications and case reports have been published in the literature on this type of doping (4–6). The metabolism of nandrolone in terms of patterns and kinetics, the presence of its metabolites at low concentrations in some urines, and the possible natural excretion of these substances, which could increase with exhaustive exercise, are much debated. One aim of this study was to focus on the excretion kinetics and patterns of NA and NE in urine after administration of [13C]nandrolone to 22 volunteers. The administration of labeled nandrolone allowed a high selectivity and specificity for qualitative and quantitative analyses of nandrolone metabolites by GC-MS. We focused in this report on the elimination of labeled metabolites without interference from the urine matrix or possible natural excretion of these compounds, which could lead to erroneous interpretations. For simplicity, not all of the raw data are presented here.

In the case of spot-urine analyses, the urinary volume

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**Table 2. Labeled NA and NE concentrations at excretion peaks.**

<table>
<thead>
<tr>
<th></th>
<th>First excretion peak</th>
<th>Second excretion peak</th>
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<tbody>
<tr>
<td></td>
<td>NA, μg/L</td>
<td>NE, μg/L</td>
</tr>
<tr>
<td>Mean (n = 22)</td>
<td>12 225</td>
<td>5100</td>
</tr>
<tr>
<td>Range</td>
<td>1180–34 043</td>
<td>576–12 328</td>
</tr>
<tr>
<td></td>
<td>14 222</td>
<td>6181</td>
</tr>
<tr>
<td></td>
<td>4722–38 661</td>
<td>2249–11 640</td>
</tr>
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</table>
is influenced by various factors, e.g., glomerular filtration, tubular secretion and reabsorption, alimentary regimen, intake of liquids, and perspiration, which give rise to a differing extent of urinary dilution and, consequently, to changes in the concentrations of excreted substances that can lead to under- or overestimation of the final result. In particular, specific gravity depends on the degree of hydration and urinary solutes. Therefore, in the antidoping field, as in occupational medicine, adjustment for specific gravity and/or urinary creatinine concentration is currently performed (46). Consequently, for calculation of the urinary concentrations of the nandrolone metabolites, the following formula is applied by the majority of antidoping laboratories to correct values for samples for which the specific gravity is >1.020 (47):

\[
c_{\text{corr}} = f \times c_{\text{m}}
\]

where \(f = 0.02/(d - 1)\), in which \(f\) is the correction factor; \(c_{\text{corr}}\) is the corrected concentration; \(c_{\text{m}}\) is the calculated concentration (peak-area ratio); and \(d\) is the specific gravity of the urine sample.

In this study, the correlation between creatinine concentration and specific gravity was established through the analysis of 750 urine samples. We observed good correlation between these two indices for both the treated and placebo groups [\(y = 0.0068\ln(x) + 1.0172\) \(r^2 = 0.773\) and \(y = 0.0052\ln(x) + 1.0178\) \(r^2 = 0.81142\), respectively]. Consequently, in this study, concentrations of labeled nandrolone metabolites were systematically

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**Fig. 6. NE:NA ratios (top) for two volunteers after the oral administration of two doses of \[^{13}\text{C}\]\text{nandrolone and corresponding chromatograms (bottom) obtained by GC-MS from spot urines collected at 50.5 h (A) and 54 h (B) after the first nandrolone dose (t0). The arrow indicates the spot urine analyzed by GC-MS to obtain the corresponding chromatogram. IS, internal standard.}
corrected with the previous formula when specific gravity was >1.020.

All volunteers were evaluated for various physiologic and morphologic indices before and after the administration of nandrolone, before and after exhaustive exercise. The results obtained show that neither physical effort nor administration of labeled nandrolone had an influence on the measured values for both the treated and placebo groups (Table 1). This observation is important in relation to the interpretation of our results.

To evaluate the kinetics and patterns of nandrolone excretion, the volunteers were asked to collect their urines for 5 days after intake of two 25-mg doses of $^{13}$C-nandrolone (ES period). The volunteers and quantitative analyses of all these spot urines confirmed the interindividual variability in labeled nandrolone metabolite excretion. Urine aliquots were preferentially collected vs total volumes for two reasons: (a) difficulties in shipping and storage of all urines from 34 volunteers; and (b) a spot urine is more realistic in antidoping control and corresponds to a real sample received at the laboratory after collection.

Although the 22 excretion curves had roughly the same profiles, close inspection revealed interesting differences. The urinary concentrations of labeled NA and NE at the excretion peak were variable from one volunteer to another. In fact, large disparities were observed: for example, labeled NA concentrations reached 40,000 μg/L for one volunteer but <5000 μg/L for other cases (Fig. 4). Similarly, the excretion peak generally occurred around 6–7 h after intake, but for two volunteers, it took place after 10 h. The concentration of labeled NA excreted was generally greater than that of labeled NE (generally threefold higher), but this difference in excretion was, in some cases, not so obvious. Another observation was that although most of the nandrolone metabolites were rapidly eliminated, i.e., within 20 h, small quantities could be found for several days. For example, some urine samples contained labeled nandrolone metabolites at concentrations >100 μg/L 100 h after intake.

Another interesting point is that the elimination kinetics were different among volunteers. Indeed, the slopes of the excretion curves were very different at the end of labeled nandrolone elimination (Fig. 5). For example, at 77 h after the first intake, the labeled NA concentration could be below the cutoff of 2 μg/L in some urines and near 100 μg/L in other samples, although the same nandrolone dose was administered at the same time to the volunteers. All of these considerations suggest that the nandrolone metabolite elimination rate in urine is linked to the hydration of the volunteers at each time and that this can explain the observed differences in excretion patterns. Moreover, the differences obtained at the end of nandrolone elimination can also probably be explained by a difference in metabolism among volunteers.

These findings led us to consider the positive cases obtained in the laboratory. Indeed, regarding these results, it is very difficult for an antidoping laboratory to make conclusions regarding a positive case in relation to the nandrolone concentration found in urine, the “seriousness” of the case in relation to this concentration, the possible origin of the nandrolone found in urine in relation to the concentration found, and finally whether the sanction given to the athlete should be decided in relation to the nandrolone concentration found in urine. All of these questions need to be discussed by the sport federations together with the antidoping laboratories.

In addition, even when the two oral intakes of labeled nandrolone were very close in time (<24 h apart), no accumulation phenomenon was observed. This can be explained by low absorption in the digestive tract, no body storage, and a rapid elimination in urine. Some studies have shown that an intramuscular injection of nandrolone (e.g., Deca-Durabolin) could be a better way to increase absorption and consequently provide long-term anabolic effects [15].

Finally, the NE:NA ratio was not constant over the excretion study. In fact, in opposition to several published studies, the NE peak was sometimes higher than the NA peak in the chromatogram (Fig. 6). Of the 22 treated volunteers, only 5 had a “classic” NE:NA ratio, i.e., <1, during the excretion study. The other 17 volunteers had a ratio <1 during most of the elimination period for the nandrolone metabolites, but this ratio inverted near the end of the excretion study. When we analyzed the endogenous steroid profiles of the 22 treated volunteers, we observed no significant difference among individuals that could have explained these disparities concerning NE:NA ratios. In the same way, we observed no significant difference in the excretion patterns and kinetics among the volunteers. To our knowledge, this observation on NE:NA ratios has not been discussed and established in the literature.

We hope that this study will help clarify some incompletely explained problematic points or observations and will lead to better discussion between sport federations and antidoping laboratories when faced with a positive nandrolone case.

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