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Short communication

Urinary excretion of 19-norandrosterone of endogenous origin in man: quantitative analysis by gas chromatography–mass spectrometry

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Abstract

A GC–MS method, using deuterium-labelled 19-noretiocholanolone as internal standard and following an extensive LC purification prior to selected ion monitoring of the bis(trimethylsilyl) ethers at ion masses *m/z* 405, 419, 420 and 421, allowed the quantitation of subnanogram amounts of 19-norandrosterone present in 10-ml urine samples at *m/z* 405. Thirty healthy men, free of anabolic androgen supply, delivered 24-h urine collections in 4 timed fractions. Accuracy was proven by the equation, relating added (0.05–1 ng/ml) to measured analyte, which had a slope not significantly different from 1. Precision (RSD) was 4% at a concentration of 0.4 ng/ml, and 14% at 0.04 ng/ml. Analytical recovery was 82%. The limit of quantitation was 0.02 ng/ml. The excretion ranges were 0.03–0.25 μg/24 h or 0.01–0.32 ng/ml in nonfractionated 24-h urine.Taking into account inter-individual variability and log-normal distribution, a threshold of 19-norandrosterone endogenous concentration of 2 ng/ml, calculated as the geometric mean plus 4 SD, was established. This value corresponds to the decision limit advised by sport authorities for declaring positive (anabolic) doping with nandrolone.

Keywords: 19-Norandrosterone; Steroid

1. Introduction

The principal urinary metabolites of 19-nortestosterone (nandrolone, 17β-hydroxy-4-estren-3-one) in the human species are glucuronides of 19-norandrosterone (NA, 3α-hydroxy-5α-estran-17-one) and 19-noretiocholanolone (NE, 3α-hydroxy-5β-estran-17-one) [1–3]. Low-dose, oral administration of 19-norandrostenedione (4-estrene-3,17-dione) has recently been shown to produce similar urinary metabolites (Dehennin and Bonnaire, unpublished data).

Official doping analysis of illicit nandrolone administration is based on the detection of both metabolites above a provisional decision limit of 2 ng/ml. This leads sometimes to contest by athletes declared positive for nandrolone, and who denied having misused this drug. According to Björkhem and Ek [4], who developed an isotope dilution–mass spectrometric method for NA quantitation after i.m. nandrolone ester administration, some untreated subjects displayed NA levels below 5 ng/ml. Debruyckere et al. [5], however, reported three male volunteers with basal urinary NA concentrations between 9 and 37 ng/ml and they excluded that such

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levels might have been generated by the ingestion of nandrolone residues present in first-quality beef, which is the kind of meat consumed by sportsmen [6].

It has repeatedly been proven that in vitro enzymatic conversion of aromatisable androgen (testosterone and androstenedione) to estrogen (respectively estradiol-17β and estrone) by various aromatase preparations (mainly from human placenta) is accompanied by nonaromatising 19-demethylation with production of small amounts of 19-norsteroids (product ratio of *19-nortestosterone/estradiol* <10%). The human in vivo production of 19-nortestosterone and 19-norandrostenedione by aromatase-rich tissues has been reported in the ovarian follicle [7] and during pregnancy by the placenta [8]. It thus can be anticipated that other tissues, containing less aromatase (for instance, adipose tissue, skin, testis, adrenal, liver and muscle), may contribute to the production of trace amounts of 19-norsteroids, which should then be reflected in the urine by excretion of NA and NE at low concentrations. In any case, the average 19-norsteroid urinary excretion in men should be well below the corresponding total estrogen excretion.

Recently, controversy has arisen among some sports administrators concerning the presence or the absence of nandrolone metabolites in the urine of untreated male athletes, submitted to current screening procedures for anabolic steroids. We, therefore, felt the need to perform a specific study on the detection and quantitation of NA and NE in a small reference population of healthy men.

2. Experimental

2.1. Urine collection

Thirty healthy male volunteers, ages 18–60 (mean 30, SD 10), sedentary or with moderate but regular sports activity and free of any previous androgen supply, delivered 24-h urine collections in four timed fractions, corresponding to the time intervals 8–10, 10–12, 12–18 and 18–8 h. Fraction volumes were measured and an aliquot was stored at 4°C until analysis within 48 h after collection.

2.2. Materials

3α-Hydroxy-5α-estran-17-one was purchased from Research Plus (Bayonne, NJ, USA), 3α-hydroxy-5β-estran-17-one was kindly donated by Schering (Berlin, Germany), 3α-hydroxy-[3β,4β,5β-$^{2}H_{3}$]estran-17-one (NE-d_{3}) was obtained from Radian International (Austin, TX, USA). DEAE-Sephadex A25 in the chloride form and Sephadex LH-20 were produced by Pharmacia (Uppsala, Sweden). Solid-phase extraction cartridges loaded with 500 mg Bond-Elut C18-HF (octadecyl silica) were obtained from Varian Associates (Harbor City, CA, USA).

β-Glucuronidase (EC 3.2.1.31) from *Escherichia coli* (200 I.U./ml) was supplied by Boehringer Mannheim (Mannheim, Germany). Trimethylsilyliodosilane (TMSI) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) came from Fluka (Buchs, Switzerland). Organic solvents and other usual chemicals were obtained from common chemical suppliers; they were of analytical grade and used without further treatment.

2.3. Gas chromatography–mass spectrometry

GC–MS analyses were carried out using a Hewlett-Packard 6890 gas chromatograph (HP Analytical Division, Waldbronn, Germany), equipped with a HP 7673 autosampler and coupled to a HP 5973 mass selective detector (MSD). Instrument control and data processing were performed with a HP Vectra XA Computer and ChemStation software. GC separation was achieved on a HP-5 Trace (5% phenylmethyl-polysiloxane) fused silica column (25 m× 0.20 mm I.D., 0.33 μm film thickness) operated with a helium inlet pressure of 108 kPa and temperature programming: 130°C for 0.5 min, ramped at 15°C/min to 230°C, ramped at 4°C/min to 285°C, ramped at 30°C/min to 310°C and held for 5 min. Injections of 2 μl-samples were made at 275°C in the splitless mode (0.75 min) into a split–splitless injection port with an inner silanised glass liner containing silanised glass wool, and the transfer line was heated at 280°C. The ion source was operated in the electron impact mode with 70 eV electron energy and the electron multiplier was set to 400 V above the automatic tuning voltage.

2.4. Sample preparation

2.4.1. Solid-phase extraction

Ten-milliliter aliquots of urine were spiked with NE-d_3 (10 ng dissolved in 50 μl ethanol) and divided in two equal parts. For practical reasons of instrumental set-up, and also in order to improve extraction yield, solid-phase extraction was performed with two cartridges of 500 mg Bond-Elut C18 mounted on a Benchmate Workstation (Zymark, Hopkinton, MA, USA). Conditioning, loading and elution were done as follows: 4 ml of methanol, 4 ml of water, sample aspiration, rinsing with 5 ml water and elution with 6 ml methanol. The two eluates were combined and methanol was evaporated at 60°C with a TurboVap VL Evaporator (Zymark).

2.4.2. Enzyme hydrolysis of glucuroconjugates

Extraction residues were dissolved in 1.5 ml phosphate buffer (0.1 *M*, pH 6.5) and incubated with 12 I.U. of β-glucuronidase during 60 min at 55°C. The deconjugated and free steroids were then extracted with 3 ml of a mixture of *n*-hexane-diethyl ether (8:2, v/v). The organic phase was dried with anhydrous sodium sulfate and the solvent was evaporated under a nitrogen stream at 60°C.

2.4.3. Purification by ion-exchange and partition chromatography

The free base form of DEAE-Sephadex was prepared according to Fotsis and Adlercreutz [9] and this support could be used for 2 weeks when stored in methanol at −20°C. Disposable minicolumns (10×5 mm I.D.) were made in Pasteur pipettes and the residues of enzymatic hydrolysis, dissolved in 0.5 ml methanol were deposited on top. The first 0.5 ml together with another 2.5 ml methanol for elution of neutral steroids were collected in a single tube and methanol was evaporated under nitrogen.

Monohydroxy-ketones were isolated on columns (200×5 mm I.D.) packed with Sephadex LH-20, swollen in a mixture of dichloromethane–methanol (95:5, v/v). After deposition on top of the column of the residue from the previous chromatography, dissolved in 0.25 ml of dichloromethane–methanol mixture, the first 4 ml of eluent were discarded. Androgens bearing one hydroxy- and one keto-group, with or without a double bound, were recovered in the next 1.5 ml. Thereafter, columns were rinsed with 6 ml eluent ; they could be reused several times when stored at 4°C and covered with eluent.

2.4.4. Derivatisation and selected ion monitoring

The 3,17-bis(trimethylsilyl) ethers were made by dissolving final dry residues, which had been transferred into appropriate injection vials, in 30 μl derivatisation reagent (MSTFA containing 0.2% TMSI, v/v) and by subsequent heating at 70°C for 30 min. Fragment ions m/z 405 and 408 were used for quantitative analysis; ions m/z 420 (M^+), 421 and 419 were monitored as an aid for detection of possible interferences.

2.5. Quantitative analysis

No certified reference standards of NA and NE were commercially available. The analytical grade of NA, specified by the supplier (Research Plus), was considered sufficient. Our NE standard belonged to the batch preparation kindly distributed by Schering to many laboratories in the world. Stock solutions were made by weighing 2 mg of NA and NE with 0.01 mg precision and by dissolving each in 5 ml ethanol. The internal standard NE-d_3 was only available as a solution (100 μg/ml in 1,2-dimethoxyethane, Radian Code RE 911) and no commercial source for deuterium-labelled NA could be found. Therefore, NE-d_3 was used as an isotopic analogue for quantitation of NE by isotope dilution, and as a heterologous internal standard for NA estimation. Stock solutions were stored at 4°C and working dilutions at 1 and 10 μg/ml were used for preparation of calibration mixtures, for standard additions or for spiking.

Two different procedures were found suitable for quantitation. The first one used a standard curve relating concentration ratios (analyte/internal standard=NA/NE-d_3 or NE/NE-d_3) to corresponding peak-area ratios (ion 405/ion 408). Concentration ratios in the 0.01–0.50 range afforded typically for NA a standard curve equation of $y=0.75x-0.03$, where y was the peak-area ratio and x the concentration ratio. A similar equation for NE was $y=0.70x-0.04$. Intra- and inter-assay RSDs were in the 5–6% range.

In the second procedure, response factors of the

mass selective detector for a standard mixture containing equal amounts of NA, NE and NE-d_3 were measured. Corrections for isotope contributions were: 3% contribution of NE (m/z 405) to NE-d_3 (m/z 408) and similar reverse contribution. However, NE-d_3 contained approx. 55% NE-d_2, therefore typical response factors were well below 1 (0.50 for NE-d_3/NA and 0.55 for NE-d_3/NE). Variabilities of replicate analyses were similar to those of the first procedure. Further calculation of quantitative data was done according to equations outlined previously [10]. No statistically significant differences could be observed between results from the two procedures.

Concentrations are expressed in nanograms of free steroid/ml. Excretions (μg/24 h) in nonfractionated 24-h urine were calculated by taking into account the urine volumes collected during the four time intervals and the corresponding concentrations. The average 24-h urinary concentrations (ng/ml) were obtained by dividing 24-h excretion levels (ng/24 h) by corresponding diureses (ml/24 h). Excretion rates (ng/h) were calculated by dividing the amounts excreted during each time interval by the corresponding duration in hours.

Methodological blanks, where urine was replaced by phosphate buffer, displayed undetectable NA and NE.

2.6. Accuracy, precision,recovery and limit of quantitation

Accuracy was evaluated by standard additions of NA and NE (range 0.25–5.0 ng) made to 5-ml urine samples from a pool, containing approx. 0.04 ng/ml of endogenous NA and NE. The equation relating added (x) to found (y) NA (in ng/ml) was $y=0.96(\pm0.08)x+0.036(\pm0.006)$, $r>0.99$, $n=3$. The slope was not significantly different from 1. A similar equation for NE was $y=0.93(\pm0.11)x+0.044(\pm0.011)$. Intercepts corresponded to endogenous concentrations.

Precision was evaluated by replicate analyses of timed urine collections made after an oral administration of 10 μg nandrolone or 10 μg 19-norandrostenedione to male volunteers. This allowed the constitution of pools containing different concentration levels of NA and NE glucuroconjugates. Inter-assay variabilities (RSD, $n=4$) were: (1) at the 0.4 ng/ml level, for NA 3.9%, for NE 5.1%; (2) at the 0.1 ng/ml level, for NA 5.6%, for NE 7.2%; and (3) at the 0.04 ng/ml level, for NA 14%. In most urine samples, NE could not be quantified at the 0.04 ng/ml level and below.

The average recovery of NA was 82±2% ($n=3$). Therefore, NA was determined in a urine pool (approx. 2 ng/ml) with internal standard addition, either at the beginning of the analytical procedure, or just before derivatisation. Recovery corresponded to the percentage ratio of both results.

The limits of quantitation (defined as peak heights corresponding to 10 times the baseline noise) were established, with 10-ml sample sizes, in the ranges 0.01–0.02 ng/ml for NA and 0.04–0.05 ng/ml for NE.

2.7. Statistical analysis

Analytical data from the reference population were log transformed and tested for normality of distribution. Comparison of geometric means was done by t-test at the $P<0.05$ significance level. Replicate analytical results are arithmetic means±SD.

3. Results and discussion

Accurate quantitative trace analysis of endogenous steroids requires, either highly specific sample treatment coupled with capillary GC and low resolution mass spectrometry, or less extensive sample treatment compensated by higher resolution of mass spectrometry, and perhaps a combination of both procedures in some special cases. Based on the outstanding gas chromatographic and mass spectrometric characteristics of the trimethylsilyl-enol trimethylsilyl ethers of NA and NE, which were fully described by Massé et al. [11], the latter derivatives were retained for quantitative analysis.

Typical selected ion chromatograms obtained for NA and NE in urine (Fig. 1) illustrate the performance of the analytical method. This was achieved by thoroughly optimized sample clean-up with chromatographic techniques using specific gels with very low irreversible adsorption, which were found essential for the quantitative analysis of the present trace amounts of NA and NE. Fast anion-

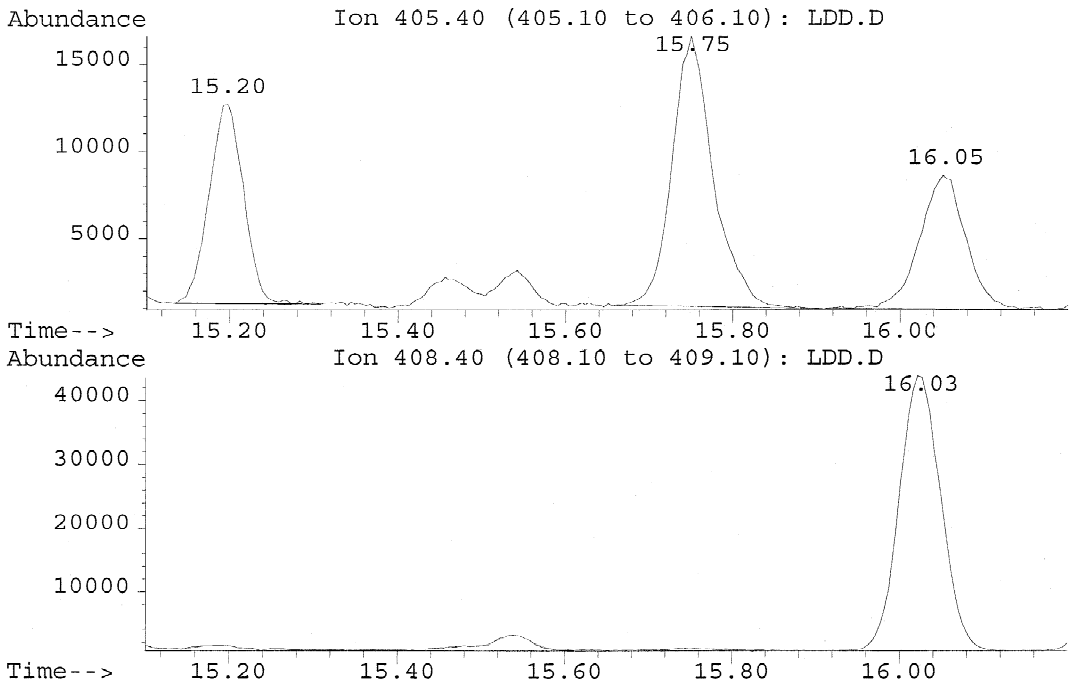

Fig. 1. Selected ion chromatograms at m/z 405 and 408 of a 10-ml urinary extract containing 0.11 ng/ml of 19-norandrosterone (retention time 15.20 min), 0.09 ng/ml of 19-noretiocholanolone (retention time 16.05 min) and 1 ng/ml of deuterium-labelled 19-noretiocholanolone as internal standard (retention time 16.03 min).

exchange chromatography on DEAE-Sephadex, bearing the diethylamino-group in the free base form, was particularly efficient for the isolation of a neutral steroid fraction with the entire, specific removal of urinary chromogens and other weakly acidic compounds (including vitamin E metabolites and estrogens, which could eventually be recovered in an ultimate chromatographic fraction). Further group-fractionation by LC on Sephadex LH-20 was required in order to isolate monohydroxy-ketones. High-resolution GC with adequate temperature programming and selected ion monitoring at m/z 405 and 408 were the final requirements before quantitation. Four fractionated urine collections from four subjects could be handled in 2 working days by one individual.

The most prominent quantitative data obtained in this small reference population are summarised in Table 1. Concentrations were not corrected for variable diureses (range 370–2490 ml) in order to

Table 1
Urinary excretion of 19-norandrosterone in men: circadian variation of excretion rates and concentrations (geometric mean, GM, $n=30$) with corresponding concentration thresholds established at a confidence interval of 4 SD (GM+4 SD), and highest individual concentrations (maximum)

Time interval (h)	Rate (ng/h)	Concentration (ng/ml)	Threshold (GM+4 SD) (ng/ml)	Maximum (ng/ml)
8–10	5.0[a]	0.084[a]	2.6	0.54
10–12	4.5	0.074	2.2	0.63
12–18	3.9	0.080	1.4	0.31
18–8	2.9[a]	0.072[a]	1.8	0.25

[a] $P<0.05$.

report data comparable with those generated by doping analysis of spot urine collections. As expected, the highest concentrations were measured in the lowest excretion volumes. The statistical distribution of excretion data was positively skewed, but logarithmic transformation allowed normalisation. NE concentrations were not measurable at most of the endogenous levels.

Fractionated 24-h urine collections were analysed in order to detect possible nycthemeral variations, which may be considered as a support for the endogenous origin of an analyte. Decrease of NA output could only be evidenced at night, when the average concentrations (ng/ml) and excretion rates (ng/h) were significantly lower than during the first time interval of the day. In that respect, excretion rates may be considered as a better indicator since they are less dependent on urine flow. Ranges of individual episodic variation of NA excretion, expressed as the ratio of *maximum/minimum* concentration or rate, were respectively 1.1–6.5 (mean 2.5, SD 1.3) or 1.1–9.8 (mean 3.4, SD 2.6). These variations should be related to up and down regulation of both, the 19-norsteroid production and the renal clearance of their conjugated metabolites.

Excretion rates of NA, measured in nonfractionated 24-h urine, were in the range 0.03–0.25 μg/24 h, corresponding to a concentration range 0.01–0.32 ng/ml (Fig. 2). This is lower than the total excretion rate (range 3–10 μg/24 h) of the principal estrogens (estrone and estradiol-17β) in men [9], by one or two orders of magnitude. Moreover, it can be extrapolated from previous data on the 19-norsteroid/estrogen ratio found at equilibrium in the follicular microenvironment [7], and from the average urinary excretion of estrone and estradiol in men (approx. 5 μg/24 h), that the global concentration of all urinary metabolites of endogenous nandrolone and 19-norandrostenedione (mainly glucuronides of NA and NE, but also minute amounts of corresponding sulfates, and estranediol isomers), should be around 0.4 ng/ml for normal diureses (approx. 1.2 l). The present urinary NA levels are corroborated by this extrapolated data.

A ‘decision limit of about 1–2 ng/ml for nandrolone metabolites’ has been advised in 1996 to laboratories, accredited by the International Olympic Committee, for declaring positive anabolic doping. This limit is supported by the range of threshold data (1.4–2.6 ng/ml; Table 1 and Fig. 2), which include a

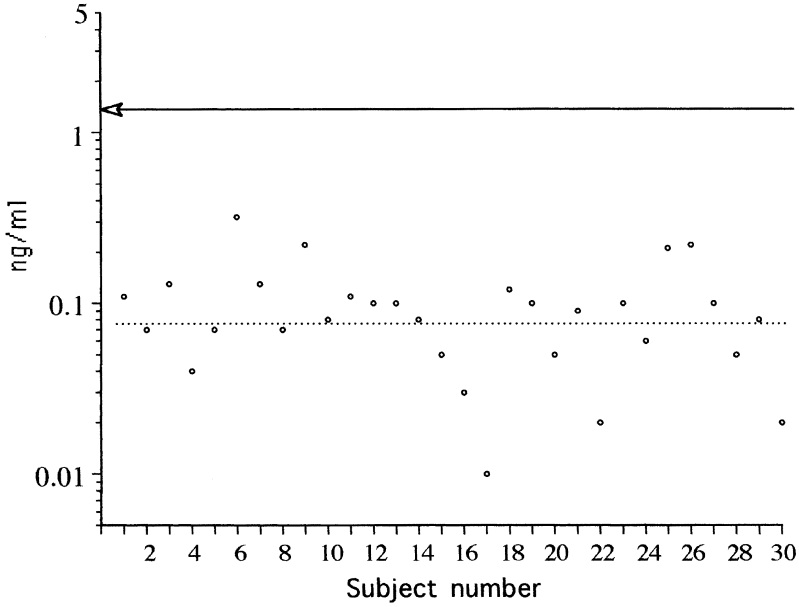

Figure 2. Concentrations of 19-norandrosterone in nonfractionated 24-h urine samples of 30 male subjects: range 0.01–0.32 ng/ml; geometric mean 0.078 ng/ml (dotted line); geometric mean+4 SD at 1.4 ng/ml (arrow).

confidence interval of 4 SD with an approx. 1/10 000 risk of false positive. However, such a statistical agreement should be considered with caution owing to the low number of subjects in the present reference population.

Concerning the possible ingestion of nandrolone-contaminated meat, it should be mentioned that, according to the statistics issued by Official Reference Laboratories of the European Union, nandrolone residue levels in meat of treated animals do not exceed 100 ng/kg. Thus it can be inferred that the amount of contaminated meat which would have to be consumed in order to attain the levels reported here, would be in the kg-range.

In conclusion, this study has proven that men, untreated with anabolic steroids, excrete daily in the urine NA of endogenous origin in the subnanomolar range. This finding constitutes only an initial step towards some insight into very low 19-norsteroid production, and ensuing metabolism, in man. It should be confirmed in a larger cohort of normal male subjects, including athletes at training and after competition. The effects of possible, but yet unknown, physiological stimuli of 19-norsteroid biosynthesis in vivo will have to be investigated in order to find out if the decision limit for positive nandrolone doping can be exceeded in a non surreptitious way.

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