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Gas chromatography–combustion–isotope ratio mass spectrometry analysis of 19-norsteroids: application to the detection of a nandrolone metabolite in urine

Jean-Charles Mathurin*, Valérie Herrou, Emmanuel Bourgogne, Laurent Pascaud, Jacques de Ceaurriz

Laboratoire National de Dépistage du Dopage, CREPS, 143 avenue Roger Salengro, 92290 Chatenay Malabry, France

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Abstract

Determination of whether the major metabolite of nandrolone in urine, 19-norandrosterone (19-NA), is exogenous or endogenous in origin is one of the most exciting challenges for antidoping laboratories. Gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) can be used to differentiate these two origins by carbon isotopic ratio analysis. A complete method for purification of 19-NA in urine has been established. Acetylated ketosteroids, and in particular 19-NA, are isolated from the urine matrix before analysis after hydrolysis and purification of urine by reversed-phase and normal solid-phase extraction. The limit of detection for 19-NA was about 60 ng with recoveries of 54–60%. Evidence of exogenous administration of 19-NA may be established from isotope ratio determination from the $^{13}C/^{12}C$ ratios of several synthetic 19-norsteroids compared to those obtained for endogenous steroids. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: 19-Norsteroids; Nandrolone

1. Introduction

The International Olympic Committee (IOC) first prohibited anabolic androgenic steroids for the Montreal Olympic games in 1976. Nandrolone or 19-nortestosterone (19-NT) belongs to this class of compounds and is one of the most widely used anabolic steroids. Administration of Nandrolone (or other 19-nonsteroid precursors such as norandrostendione or norandrostendiol) can be detected in urine from the presence of their two major glucuronic metabolites, 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) [1,2] (Fig. 1). Other metabolites have been described but are presented in urine in smaller amounts [3]. By the IOC anti-doping code, an adverse report will be issued and may lead to sanction if a concentration of at least one metabolite in excess of 2 ng/ml or 5 ng/ml of urine is found in a male or female competitor, respectively. This threshold has been established in males in order to exclude low endogenous production of 19-NA which is well known in stallions [4] and boars [5,6]. Different authors have recently reported such natural production in human beings [7–11]. No evidence of

*Corresponding author. Tel.: +33-146-60-2869; fax: +33-146-60-3017.

E-mail address: jc.mathurin@wanadoo.fr (J.-C. Mathurin).

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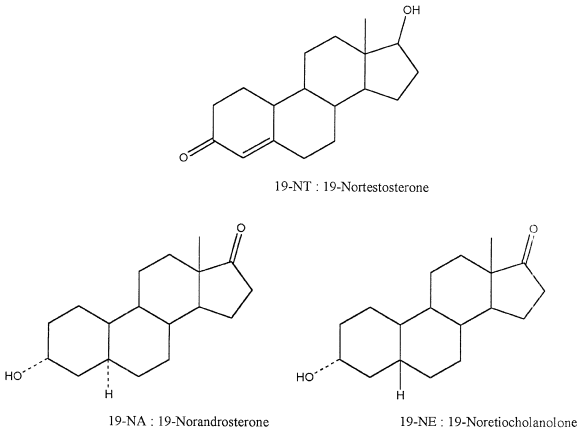

Fig. 1. Structures of 19-nortestosterone (19-NT) and its two mains metabolites: 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE).

endogenous production of 19-NA producing a level of >2 ng/ml was found in males in these previous studies. The problem is more complex for females as nandrolone has been found in human ovarian follicular fluid [12] and in the placenta during pregnancy [13]. Mareck-Engelke et al. [14] reported that the concentration of 19-NA may reach 20 ng/ml in urine during pregnancy. These cases are, however, unusual in the context of anti-doping testing and are easily detected by a test for HCG. It has also been reported that some certain norsteroid (e.g. norethisterone) contained in contraceptive pills can be metabolised to 19-NA and 19-NE. In these cases, however, other relevant metabolites (tetrahydro-metabolites of norethisterone) are also present in urine.

Various sources of contamination have also been put forward in order to explain the presence of 19-NA in the urine of sports players who have claimed to be innocent. Food contaminated by endogenous production of 19-NA [15] or by exogenous administration of nandrolone used as a growth promoter animals [16] has been investigated, as have permitted nutritional supplements which contain 'unexpected' traces of anabolic steroid. In this context, differentiation between 19-NA in urine of endogenous and exogenous origin represents an important challenge for anti-doping laboratories. Since the introduction of gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) into the field of anti-doping testing, a similar problem which arises with testosterone abuse is in the process of being resolved. It has been shown that exogenous administration of testosterone can be differentiated from naturally high production by measuring the $^{13}C/^{12}C$ ratio [17–23]. Discrimination is made from the lower level of the ^{13}C stable isotope in the source material used in the chemical synthesis of testosterone compared to endogenous testosterone production. Such differentiation between synthetic and endogenous origins has also been reported for other hormones such as DHEA [24] and corticosteroids [25,26]. This report presents the first results of $^{13}C/^{12}C$ ratio measurement in 19-norsteroids. We describe a purification procedure for 19-NA in urine for use in GC–C–IRMS analysis and discuss the reliability of carbon isotope results obtained by GC–C–IRMS.

2. Experimental

2.1. Chemicals and reagents

All the solvents and reagents were analytical grade purity (SDS, Villeurbanne, France; Carlo Erba, Val de Reuil, France; Merck, Darmstadt, Germany; Sigma, Saint Quentin, Fallavier, France).

Escherichia coli β-glucuronidase was supplied by Roche Molecular Diagnostic.

The synthetic reference codes were: 19-norandrosterone or 5α-estran-3α-ol-17-one (Promochem, Molsheim, France, ref. E-910), 19-noretiocholanolone or 5β-estran-3β-ol-17-one (Promochem, ref. 908), nandrolone or 19-nortestosterone (Sigma), 5α-tetrahydronorethisterone (Steraloids, Wilton, NH, USA, ref. E202), 5β-tetrahydronorethisterone (Steraloids, ref. E268), testosterone acetate (Sigma).

5α-Androstan-3βol (Sigma) was used as internal standard (I.S.). 5α-Androstan-3βol acetate was prepared using the derivatisation procedure described below.

Solid-phase extractions were carried out on solid-phase extraction (SPE) columns filled with reversed-phase octadecyl (Bond-Elut® C_{18}, 200 mg/3 ml and 500 mg/3 ml, Varian, Les Ulis, France) or silica-gel phase (column 500 mg/3 ml, Alltech, Templemars, France).

Immunoaffinity chromatography gels for 19-nor-

testosterone were obtained from CER (Laboratoire d'Hormonologie, Marloie, Belgium) and the chromatography column (Econo column, 14 cm length×1.5 cm I.D.) from Bio-Rad (Glattbrugg, Switzerland).

2.2. Extraction and purification of 19-NA

2.2.1. Immunoaffinity chromatography

Immunoaffinity chromatography (IAC) was evaluated to isolate 19-NA and 19-NE from urine as described by Robinson et al. [27]. The gel was loaded in a column containing 10 ml of PBS (9 g/l NaCl, 0,75 g/l KH_2PO_4, 79 g/l $Na_2HPO_4 \cdot 2H_2O$, 1 g/l NaN_3). We used synthetic solutions of 19-NA (100 ng), 19-NE (100 ng) and 19-NT (50 ng) which were dried, dissolved in 150 μl of methanol and diluted with 1.5 ml of PBS. Extraction involved the following steps: washing the column with 2×5 ml of PBS buffer, applying the sample to the top of the gel, washing the gel with 3×3 ml of water, washing the gel with 3 ml of MeOH–H_2O (10:90), eluting the bound fraction with 3 ml of MeOH–H_2O (80:20), rinsing the column with 1 ml of the elution solution and regenerating the column with 2×5 ml of PBS buffer. The elute phase was dried, acetylated and injected into the GC–MS system.

2.2.2. Solid-phase extraction and acetylation

Urines were extracted by SPE on a C_{18} column (200 mg) and the conjugated steroids were washed with water (3 ml) and eluted in methanol (4 ml). The dried methanolic residue was reconstituted in 1.5 ml of phosphate buffer (pH 6.5) and 30 μl of β-glucuronidase were added. Incubation was carried out at 55°C for 1 h. The extract was incubated at 55°C for 1 h and then centrifuged at 4000 cycles/min for 10 min. Deconjugated 19-NA was extracted by SPE on a C_{18} column (200 mg) by washing with 2 ml of MeOH–H_2O (10:90), and eluting with *tert*.butylmethylether (TBME) (4 ml). The dried ether extract obtained above was redissolved into 2 ml of a mixture of toluene–ethyl acetate (90:10) and then applied to a silica-gel SPE column (500 mg). The 19-NA fraction was eluted with 7 ml of toluene–ethyl acetate (90:10). Acetylation was performed by incubating at 80°C for 1 h with 100 μl of pyridine and 100 μl of acetic anhydride. The dried acetylation residue was redissolved in 1 ml of acetonitrile and 1 ml of water. The extract was purified by SPE on a C_{18} 500 mg column. The fraction containing 19-NA was obtained by elution with 6 ml of CH_3CN–H_2O (75:25) after washing with 8 ml of CH_3CN–H_2O (50:50). The I.S. (20 μl of a solution containing 20 ng/ml of 5α-androstan-3β-ol acetate) was then added. The extract was then evaporated and dissolved in 50 μl of acetonitrile, then injected into the GC–MS system. For the GC–C–IRMS injection the extract was dissolved in 30 μl of acetonitrile.

2.3. GC–EI-MS analysis of acetate derivatives

All of the extraction yields and optimisation of purification were performed by GC–EI-MS. The GC–EI-MS analyses were performed on a Hewlett-Packard (HP) 5970 mass-selective detector linked to an HP 5890 gas chromatograph, equipped with an DB-17 (J&W Scientific) fused-silica capillary column (30 m×0.25 mm I.D.; 0.25 μm film thickness). The injections were made in the splitless mode (1 μl). The carrier gas was helium. The injection temperature was 280°C and the oven temperature started at 70°C (1 min), increased at 30°C/min to 265°C, then at 3°C/min to 300°C and stayed at the final temperature for 0.5 min. Semiquantification of 19-NA was carried out in SIM mode (*m*/*z* 258 for NA and *m*/*z* 248 for the I.S.).

2.4. GC–C–IRMS analysis of acetate derivatives

An HP 6890 series GC instrument (Hewlett-Packard, Palo Alto, CA, USA) was equipped with a DB-17 capillary column (30 m×0.25 mm I.D., 0.25 μm film thickness) and 2 μl of the final extract were injected in the splitless mode using a 6890 autosampler. The injection temperature was 280°C and the oven temperature started at 70°C (initial hold 1 min) and was increased at 30°C/min to 271°C, at 0.6°C/min to 280°C, and then at 5°C/min to 300°C (final hold 5 min). The carrier gas was helium at a flow-rate of 1 ml/min. The outlet of the GC column was connected to a microglass column packed with oxide pellets placed in a combustion furnace maintained at 850°C. The combustion gases were passed through a cryotrap to remove water and the abundance ratios between the dried $^{13}CO_2$ and $^{12}CO_2$ were analysed on an Isoprime™ isotope ratio mass

spectrometer (Micromass, Manchester, UK). An electron impact ion source was used with an ionisation current of 400 μA. The δ^{13}C‰ value of carbon atoms is defined as parts per thousand deviation of isotopic compositions from that of PeeDee Belemnite (PDB) carbonate, and is calculated from the following equation

$$\text{Carbon Isotopic Ratio (CIR)} = {}^{13}CO_2/{}^{12}CO_2$$

$$\delta^{13}C‰_{\text{sample}} = [(CIR_{\text{sample}} - CIR_{PDB})/CIR_{PDB}] \times 1.000$$

Negative shifts of the δ^{13}C‰ value due to the formation of an acetate were corrected as follows

$$D_{OH} = D_{OAC} + 2m(D_{OAC} - D_{AC})/n$$

where D_{OH} is the δ^{13}C‰ for the underivatised compound, D_{OAC} is the δ^{13}C‰ for the acetylated compound, D_{AC} is the δ^{13}C‰ for the acetylating reagent, n is the number of carbon atoms in a molecule and m is the number of hydroxyl groups to be acetylated.

All subsequent δ^{13}C‰ values have been corrected for this negative shift if necessary (the D_{AC} value of our acetylating reagent was calculated to be −50.1).

3. Results and discussion

3.1. IAC extraction

In order to test the selectivity of the IAC, different fractions were collected during the different steps of the chromatography (loading, washing and elution). Large amount of 19-NA and 19-NE were found in the first two fractions (loading and washing) with the final yield in the eluted fraction was only 17% for 19-NA and 3% of the 19-NE. These results have been confirmed using two other gels obtained from the same supplier. These yields are insufficient for our purposes and do not corroborate a previous experiment performed by Robinson et al. [26] on same IAC gels. From our experience, however, it appears that cross reactions with 19-NA and 19-NE are too low to enable selective purification of these norsteroids using specific antibodies directed against 19-NT.

3.2. SPE extraction

Free steroids can easily be extracted using liquid–liquid extractions with a polar or an apolar solvent. In these cases, however, considerable interferences are also extracted from the urine matrix and upsets the GC–C–IRMS analysis. We therefore applied a normal-phase SPE onto silica gel [28] after pre-purifying the hydrolysed urine by reversed-phase (C_{18}) SPE. This procedure allowed us, amongst other things, to eliminate a large proportion of etiocholanolone, an abundant hormone in urine which can interfere in isotopic analysis. As recommended by several authors, the compounds are acetylated before injection in order to protect the alcohol groups and to enhance chromatographic performance. At this step, we observed a residual interference close to the 19-NA chromatogram peak (Fig. 2). From its mass spectrum (not shown here) this interference probably corresponds to an isomer of androstendiol. When this hormone is present in urine in large quantities, it is not possible to obtain full baseline separation from 19-NA by our GC–C–IRMS method. Aguilera et al. [23] have described a reversed-phase SPE procedure on acetylated mono-keto (e.g. 5α-androstane-3a-ol-17-one, 5β-androstane-3a-ol-17-one . . .) and diol steroids (e.g. 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol . . .), in order to separate these two classes of compounds. Firstly, the most polar interferences and derivatisation residues are removed by rinsing the column with a 8 ml CH_3CN–H_2O (50:50) washes. Then acetylated mono-keto steroids are then eluted with 6 ml of CH_3CN–H_2O (75:25). Under these conditions, residual diacetylated steroids (diols) are retained on the C_{18} phase and discarded. The chromatogram of the final extract containing mainly acetylated mono-keto steroids is shown in Fig. 3. This final clean-up procedure produces an improved signal-to-noise ratio. The entire procedure yields have been calculated at three concentrations of 19-NA from 5 ml of urine extracted in three replicates and are shown in Table 1.

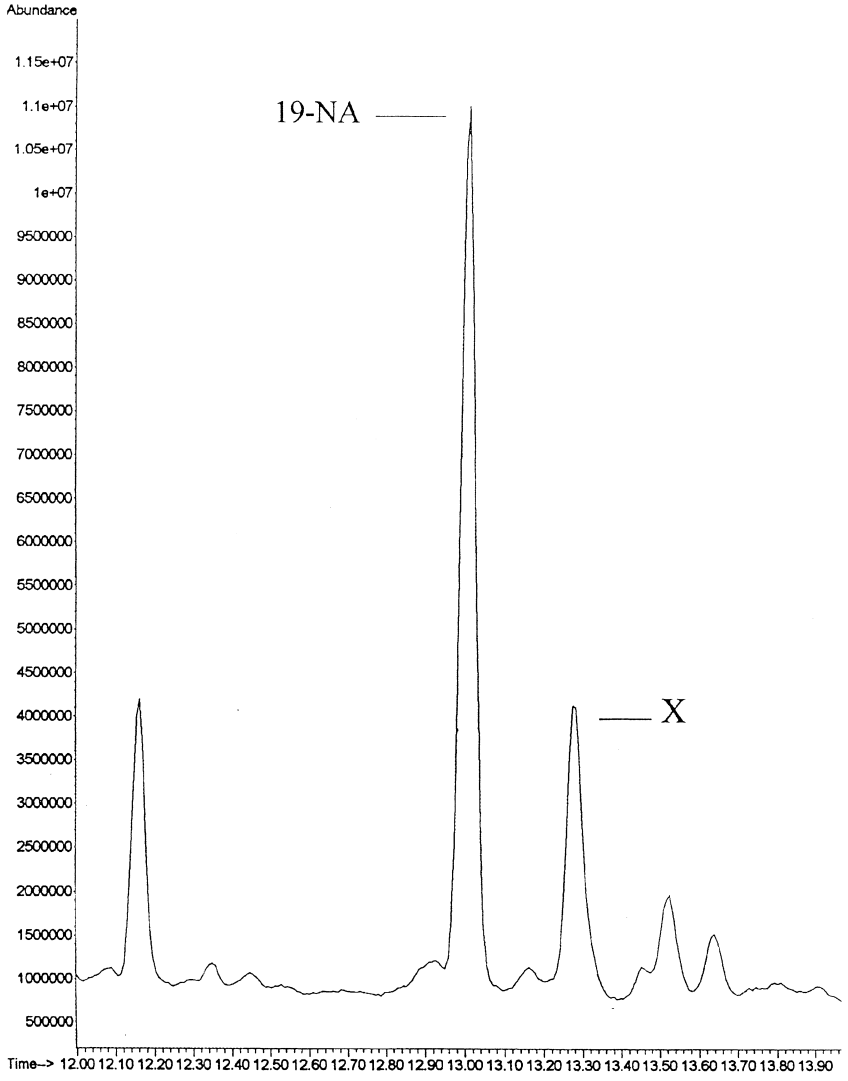

Fig. 2. GC–MS measurement in full scan mode of an urine spiked with 19-NA after purification without the last inverse phase SPE (see Section 2.2). The interference (X), while not formally identified, would probably correspond to one isomer of androstendiol.

3.3. Measurements of $\delta^{13}C$‰ values of commercially available synthetic norsteroids

Commercially available norsteroid reference chemicals or active ingredients of pharmaceutical preparations were analysed by GC–C–IRMS without chemical transformation, except for Keratyl® where a methanolysis [29] procedure was carried out in order to inject free nandrolone (Table 2). The identity of the norsteroids was checked by GC–EI-MS prior to injection into the GC–C–IRMS system and each norsteroid reference was diluted in order to inject approximately 500 ng of norsteroids onto the column. All $\delta^{13}C$‰ values are given with an error of ± 0.4‰ (approximately 2σ). The values obtained agreed closely with those found for synthetic steroids [24] or corticosteroids [25,26], indicated that the starting material for synthesis was of C3-plant origin. We found that the pharmaceutical preparation had a lower ^{13}C abundance (between -28.7 and -30.5)

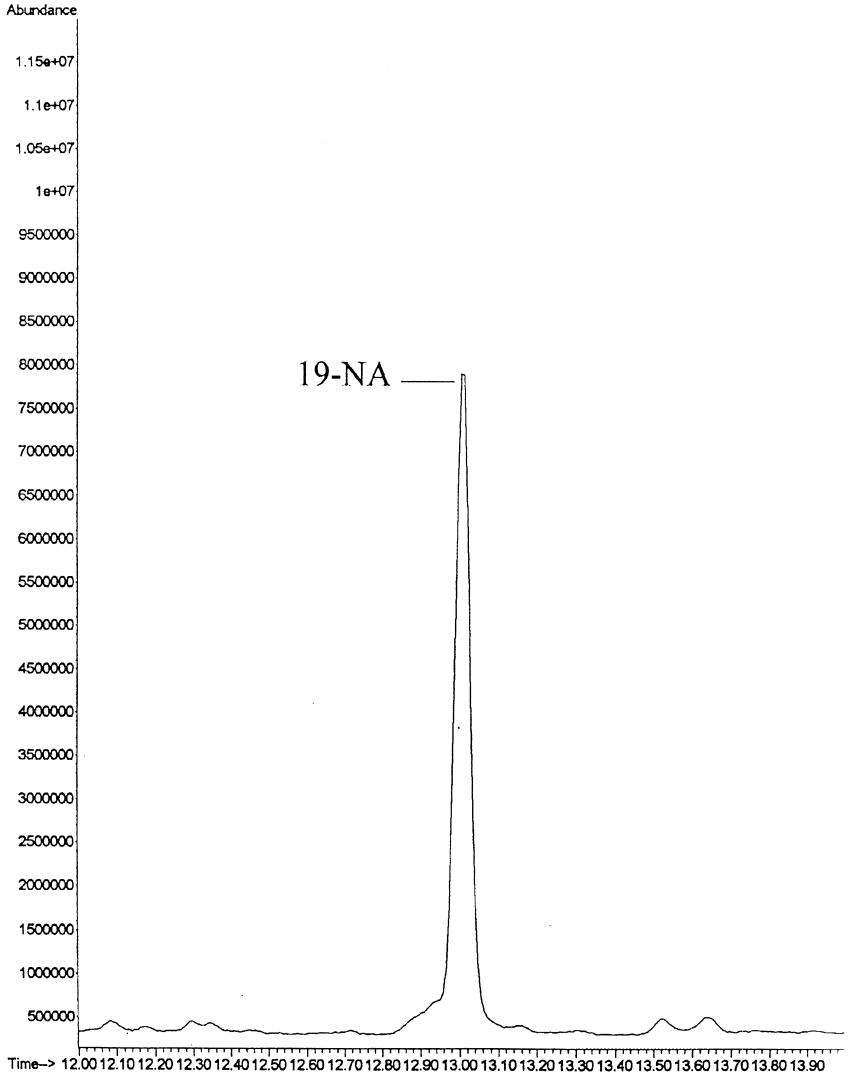

Fig. 3. GC–MS measurement in full scan mode of an urine spiked with 19-NA after purification with the three solid-phase extractions as described in Section 2.2.

Table 1
Extraction yield of 19-NA in urine (5 ml extracted)

Concentration (ng/ml)	Extraction yield (%)	C.V. (%)
10	54	13
50	57	9
100	60	8

than the chemical standards (between −27.5 and −28.5), expected for 5β-tetrahydronorethisterone (−31.3). The endogenous value for natural steroids such as pregnanediol, testosterone and its metabolites (androsterone, etiocholanolone, androstandiols) [22,24] and corticosteroids [26,30] were found between −15 and −26. As endogenous 19-NA is suspected to be produced by a 19-demethylation of an endogenous precursor, its δ^{13}C‰ values should be within the same range of values. The low values

Table 2
δ^{13}C‰ values for synthetic 19-norsteroids

Generic name	Product name	Manufacturer	δ Value
5α-Estran-3α-ol-17-one	19-Norandrosterone (19-NA)	Promochem	−27.5
5β-Estran-3α-ol-17-one	19-Noretiocholanolone (19-NE)	Promochem	−27.7
Estr-4-en-17-β-ol-3-one	19-Nortestosterone (19-NT) (Nandrolone)	Sigma	−28.0
5α-Tetrahydronorethisterone	5α-Tetrahydronorethisterone	Steraloïds	−28.5
5β-Tetrahydronorethisterone	5β-Tetrahydronorethisterone	Steraloïds	−31.3
Norethisterone acetate	Miniphase®	Schering	−30.3
Norethisterone acetate	Milligynon®	Schering	−30.5
Norethisterone acetate	Kliogest®	Specia, RPR	−30.4
Norgestrienone	Ogyline®	Roussel Diamant	−28.7
Norethisterone	Triella®	Janssen-Cilag	−29.2
Nandrolone sulfate sodic	Keratyl 1%®	Chauvin	−29.0
19-Nor-5-androsten-3,17-dione	Norandrostendione	Internet supplier	−29.7

of δ^{13}C‰ for 19-norsteroids in pharmaceutical preparations should facilitate differentiation between endogenous and exogenous sources.

3.4. Estimation of the linearity and limit of detection of 19-NA by GC–C–IRMS

One of the drawbacks of GC–C–IRMS is that it is difficult for the user to define and control an acceptable accuracy and reproducibility for the determination of δ^{13}C‰ values. Differentiation between endogenous and exogenous sources is established from a low variation in δ^{13}C‰ values. Any variation in the tuning of the instrument or in measurements can have considerable influence on how the final results are interpreted. The first step is therefore to control the ability of the instrument to provide accurate and reproducible δ^{13}C‰ values for a compound. In our case, the accuracy and reproducibility of the instrument is controlled by injected the VG mix (a mixture of four alcane standards: decane, undecane, dodecane and methyl decanoate) obtained from the manufacturer (Micromass). Theoretical δ^{13}C‰ values are compared to those measured on the instrument and results are accepted if the standard deviation of five injections and if the bias meet the manufacturer's specification (bias <0.3‰ and $\sigma<0.3$ ‰). An internal laboratory testosterone acetate standard is injected daily at the start of each sequence to check for any drift in the δ^{13}C‰ measurement and sensitivity of the instrument. The typical standard deviation of between-day measurements for these compounds is 0.16‰ for a δ^{13}C‰ of -30.19 and for an ion current (I^{44}) of approximately $5\cdot10^{-9}$ A (representing the intensity of the ion beam at m/z 44).

Another source of error in δ^{13}C‰ measurement is that isotope ratio mass spectrometers are limited by their poor linearity in the accurately determining the ^{13}C:^{12}C ratio. In order to test against the manufacturer's specifications, the VG mix was injected to obtain an I^{44} ion current of between $1\cdot10^{-9}$ and $1\cdot10^{-8}$ A. Below this level, accuracy and the precision are greatly affected. This non-linear effect has recently been studied by Hall et al. [31] and appears to be compound dependent. For these reasons we decided to investigate the limit of concentration of acetylated 19-NA at which precision is preserved in order to provide reliable measurement of δ^{13}C‰.

A 3-μl volume of acetylated 19-NA diluted in acetonitrile was injected in splitless mode at four concentration levels in three replicates: 10, 20, 50, 200 ng/μl in 19-NA. The δ^{13}C‰ values are shown against the ion current in Fig. 4. The theoretical isotopic value of -27.55 ($\sigma=0.17$ ‰) is given by the average of the δ^{13}C‰ values for the three standards at 200 ng/μl. At a concentration of 20 ng/μl this value is shifted to -28.02 with a standard deviation of 0.37‰. The I^{44} ion current at this concentration is about 1 nA. This corresponds to the lower limit of linearity of the instrument. Beneath this limit, accuracy (-28.93) and repeatability ($\sigma=$ 0.96‰) of measurement are greatly affected. We

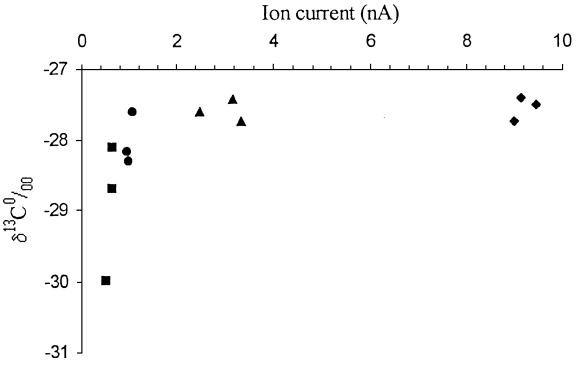

Fig. 4. Ion current (I^{44}) versus δ^{13}C‰ values measured at four concentrations of 19-NA (■: 10 ng/μl, ●: 20 ng/μl, ▲: 50 ng/μl, ♦: 200 ng/μl).

therefore consider that the lower acceptable concentration limit for determining the δ^{13}C‰ value for 19-NA is 60 ng when injected in splitless mode, equivalent to 30 ng/ml of urine (and a 20-ml aliquot of urine). In view of the purification yield described above (~50%), 60 ng of 19-NA are required per millilitre of urine (using a 20-ml aliquot of urine) in order to determine the δ^{13}C‰ values with an accuracy of ±0.8‰. This level of accuracy will avoid any misinterpretation, provided the precautions described above are observed. From our results obtained from pharmaceutics preparations of 19-norsteroids, none of these may be confused with an endogenous origin. A difference of at least 3‰ would be observed (see above). Interpretation of the result may be improved by comparing the δ^{13}C‰ value for 19-NA to the value for an endogenous reference compound (ERC) in the same subject [23]. This would correct for inter-individual variation and the $\delta^{13}C‰_{19\text{-NA}}/\delta^{13}C‰_{ERC}$ would be used as the positive identification threshold after the determining the range of natural variation of the ratio $\delta^{13}C‰_{19\text{-NA}}/\delta^{13}C‰_{ERC}$. With our method, androsterone, a major testosterone metabolite is present in the purified fraction together with 19-NA following the final SPE and it could be used as the ERC.

4. Conclusion

The method described here offers considerable potential to resolve cases of suspected nandrolone doping. At this time however, analytical limitations restrict its field of application to 'high' concentration of 19-NA in urine (over 60 ng/ml). Recent cases in antidoping testing have shown that even at these concentrations (more than 50 ng/ml) athletes and lawyers use the hypothesis of high endogenous levels of production to refute doping accusations. IRMS methodology can be of considerable assistance to antidoping laboratories in these cases.

Further development is required to improve the method, and in particular to optimise sample preparation in order to reduce losses of 19-NA. Although IAC columns with specific antibodies directed against 19-NA are not currently available, the development of this type of column is one of the most promising solutions to overcome the unavoidable losses which occur in multi-SPE purification procedures. As the relatively low sensitivity of instrumentation is one of the main limits of the technique, manufacturers will also need to become involved in improving instrument technology. By increasing instrument sensitivity by a factor of 10 and improving the extraction of 19-NA from urine to a recovery of around 90%, IRMS analysis would be able to unambiguously differentiate between endogenous and exogenous urinary 19-NA even at low levels.

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