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JOURNAL OF CHROMATOGRAPHY B
Journal of Chromatography B, 810 (2004) 157–164
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Urinary analysis of 16(5α)-androsten-3α-ol by gas chromatography/combustion/isotope ratio mass spectrometry: implications in anti-doping analysis

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Received 11 June 2004; accepted 27 July 2004
Available online 26 August 2004

Abstract

We present a method for the analysis of urinary 16(5α)-androsten-3α-ol together with 5β-pregnane-3α,20α-diol and four testosterone metabolites: androsterone (Andro), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αA), 5β-androstane-3α,17β-diol (5βA) by means of gas chromatography/combustion/isotopic ratio mass spectrometry (GC/C/IRMS). The within-assay and between-assay precision S.D.s of the investigated steroids were lower than 0.3 and 0.6‰, respectively. A comparative study on a population composed of 20 subjects has shown that the differences of the intra-individual δ^{13}C-values for 16(5α)-androsten-3α-ol and 5β-pregnane-3α,20α-diol are less than 0.9‰. Thereafter, the method has been applied in the frame of an excretion study following oral ingestion of 50 mg DHEA initially and oral ingestion of 50 mg pregnenolone 48 h later. Our findings show that administration of DHEA does not affect the isotopic ratio values of 16(5α)-androsten-3α-ol and 5β-pregnane-3α,20α-diol, whereas the isotopic ratio values of 5β-pregnane-3α,20α-diol vary by more 5‰ upon ingestion of pregnenolone. We have observed δ^{13}C-value changes lower than 1‰ for 16(5α)-androsten-3α-ol, though pregnenolone is a precursor of the 16-ene steroids. In contrast to 5β-pregnane-3α,20α-diol, the 16-ene steroid may be used as an endogenous reference compound when pregnenolone is administered.
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Keywords: Androstenol; Pregnenolone; Isotope ratio mass spectrometry (IRMS); Doping control

1. Introduction

16(5α)-Androsten-3α-ol (Andro) is excreted in human urine with mean values of 1 mg/24 h and 0.3 mg/24 h by men and women, respectively. The amounts excreted by prepubertal children and by elderly men and post-menopausal women are considerably lower than these mean values [1]. The precise biosynthesis of androstenol has been subject for debate. Recent studies revealed that androstadienol (5,16-androstadien-3β-ol) is synthesized from pregnenolone in a single step (Fig. 1) by a 16-ene-synthase enzyme system in human testicular homogenates [2]. Very recently, it has been demonstrated that P450c17 possesses a 16-ene-synthase activity capable to transform pregnenolone into androstadienol without the formation of a precursor [3]. Further transformation of androstadienol into androstenol involves the classic enzymes of the steroidogenic pathway [4]. Although it is known that androstenol has a pheromonal activity in pigs, the role of this steroid in humans is still ill-defined [1]. It has been suggested recently that menstrual synchrony is related to the ability to perceive the odour emitted by androstenol [5]. A study reports also the ability of androstenol to bind and deactivate the nuclear receptor CAR-β [6].

Isotope ratio mass spectrometry (IRMS) allows measurements of slight differences in the carbon isotope ratio (^{13}C/^{12}C) of the exogenous and endogenous steroids. Synthetic steroids are generally produced from precursors derived from plants with low ^{13}C content, whereas the ^{13}C and ^{12}C content in the natural endogenous form depends on the

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doi:10.1016/j.jchromb.2004.07.029

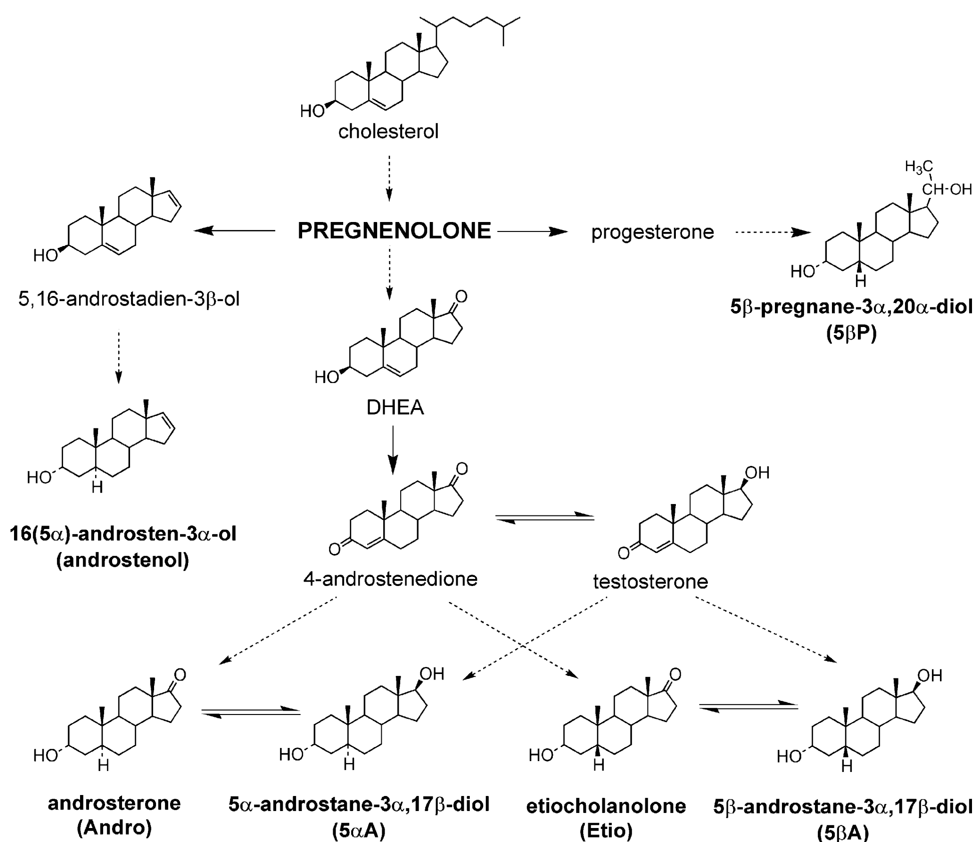

Fig. 1. Simplified metabolic pathways of pregnenolone.

isotopic carbon composition of the food diet and is influenced by additional effects of human biological processing [7]. Such differentiation between synthetic and endogenous origins has been reported for misuse in sport of steroids such as testosterone [7–9], DHEA [10], epitestosterone [11], DHT [12,13], nandrolone [14] and corticosteroids [15]. For IRMS measurements, where the inter-individual variability and instrument calibration may significantly influence the results, it is recommended to relate the difference of the steroids isotopic value to that of an endogenous reference compound [16]. For that purpose, cholesterol and 5β-pregnane-3α,20α-diol (5βP) are usually used as an endogenous reference compound in IRMS measurements because the isotopic values should not be affected by the administration of exogenous steroids such as testosterone [7,8], DHEA [10], DHT [12] and androstenedione [17]. However, the origin of urinary cholesterol is not well understood [7] and it has been also demonstrated that the use of 5β-pregnane-3α,20α-diol as endogenous reference compound is not longer applicable when pregnenolone is ingested [18].

In this paper, we present a method for the analysis of urinary androstenol together with 5β-pregnane-3α,20α-diol (5βP) and four testosterone metabolites: androsterone, etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αA), 5β-androstane-3α,17β-diol (5βA) by means of gas chromatography/combustion/isotopic ratio mass spectrometry (GC/C/IRMS). We show that androstenol may be used as an endogenous reference compound and to what extent the administration of pregnenolone will affect the isotopic ratio values of both androstenol and 5β-pregnane-3α,20α-diol. Indeed, pregnenolone is a precursor of 5β-pregnane-3α,20α-diol and androstenol (Fig. 1) and is a freely available compound in the United State, on internet and may be found in nutritional supplements with other pro-hormones. It is supposed that pregnenolone has supporting benefits for memory, cognitive function, joint regeneration, skin conditions and firmness of skin.

2. Experimental

2.1. Chemicals

All solvents and reagents of analytical grade purity were purchased from Fluka (Buchs, Switzerland). 5α-Androstan-3β-ol acetate and 5β-pregnane-3α,20α-diol were obtained from Steraloids Inc. (Newport, RI, USA). 5α-Androstane-3α,17β-diol, 5β-androstane-3α,17β-diol and 16(5α)-androsten-3α-ol were supplied by Sigma (St. Louis, MO, USA). Etiocholanolone and androsterone were purchased from NARL (Pymble, Australia). Bakerbond spe™ 500 mg or 1000 mg octadecyl C_{18} disposable extraction columns were obtained from JT Baker (Phillipsburg, NJ, USA). β-Glucuronidase from *Escherichia coli* in a 50% glyc-

erol solution (pH 6.5, 140 U/ml at 37 °C) was supplied by Roche Diagnostics GmbH (Manheim, Germany). The reference carbon dioxide gas was purchased from Carbagaz (Domdidier, Switzerland). The mixture of three alkanes, C_{15} (*n*-pentadecane), C_{20} (*n*-eicosane) and C_{25} (*n*-pentacosane) was supplied by Chiron AS (Trondheim, Norway).

2.2. Dosage and collection of urine samples

Two 25 mg DHEA capsules (Schiff®, Salt Lake City, UT, USA) were self-administered orally initially and one 50 mg pregnenolone capsule (Metabolic Response Modifier™, San Clemente, CA, USA) was self-administered orally 48 h later by a Caucasian healthy male volunteer. Baseline urine samples were obtained before initial administration, and subsequent spot urine samples were collected over a period of 155 h after the first capsule administration. Reference urine samples were collected from 20 Caucasian male and female subjects living in Switzerland. The positive quality control urine (QC-pos) was obtained from an excretion study after ingestion of a 40 mg testosterone undecanoate capsule (Panteston®, Organon SA, France) whereas the negative quality control urine (QC-neg) was obtained from a healthy subject. Each urine was aliquoted into 20 ml cryogenic tubes and stored without additives at −20 °C until analysis.

2.3. Sample preparation

The urine sample (10 ml) was centrifugated in glass tubes at 2500 rpm for 5 min and was applied onto a C_{18} column (500 mg). The conjugated steroids were eluted with methanol (8 ml) and the eluate evaporated to dryness. To hydrolyze the conjugated steroids, the residue was dissolved in 1 ml of 0.2 M phosphate buffer (pH 7.0) and 50 μl of β-glucuronidase was added as supplied. After incubation at 50 °C during 1 h (or at 37 °C overnight) in a thermostated water bath, the deconjugated steroids were extracted on C_{18} column (500 mg) by washing with 6 ml acetonitrile:H_2O (20:80 (v/v)) and subsequently by 6 ml acetonitrile:H_2O (35:65 (v/v)) and finally eluted with 12 ml acetonitrile. The eluate was evaporated to dryness under a nitrogen stream (Turbo Vap LV evaporator, Zymark, Hopkinton, MA, USA) and then the residue was dried over phosphorus pentoxide for 20 min. Acetylation of the extract was carried out in 50 μl of pyridine and 50 μl of acetic anhydride at 60 °C for 1 h incubation. The reaction medium was evaporated to dryness and subsequently the residue was redissolved in 3 ml of acetonitrile:H_2O (50:50 (v/v)). The solution was applied onto a C_{18} column (1000 mg). The fraction F1 containing androsterone and etiocholanolone was obtained by elution with 15 ml acetonitrile:H_2O (75:25 (v/v)) after washing with 12 ml of acetonitrile:H_2O (50:50 (v/v)). Finally, the fraction F2 with the androstandiols, the pregnanediol and the androstenol was obtained by eluting with 15 ml of acetonitrile. The derivatized steroids of fractions F1 and F2 were spiked with 80 and 10 μl of an internal standard (IS, 5α-androstan-3β-ol acetate, 0.20 mg/ml in methanol), respectively. Then, both fractions F1 and F2 were evaporated to dryness and finally dissolved in 500 and 40 μl of cyclohexane, respectively.

2.4. Determination of androstenol and 5βP concentrations

Urinary concentration of androstenol and 5βP were quantified in the reference urine samples collected from 20 Caucasian male and female subjects. Quantitative analyses were performed on a Hewlett-Packard 5890 Serie II Plus chromatograph (HP Analytical Division, Waldbronn, Germany) equipped with a HP 7673 auto-sampler and coupled with a HP 5971 mass selective detector (MSD). GC separation was achieved on a ZB-5 column (15 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA) operated with a helium inlet pressure of 103 kPa. The oven temperature was increased from 80 (1 min) to 270 °C (6.0 min) at 15 °C/min, then to 300 °C at 15 °C/min. Injections of 1 μl-samples from fraction F2 were made at 280 °C in the splitless mode. The analyses were performed in single ion monitoring mode (SIM) with ions $m/z = 316$ for androstenol acetate, $m/z = 344$ for 5βP acetate and $m/z = 204, 258$ for 5α-androstan-3β-ol acetate (IS). Regression curves with 30, 60, 120, 300, 600 ng/μl androstenol ($R^2 = 0.994$) and 5β-P ($R^2 = 0.993$) solutions were used for the quantification of both steroids concentrations. EI mass spectra were recorded by continuous scanning in the mass range 50–450 at an ionization potential of 70 eV.

2.5. GC/C/IRMS analysis

The carbon isotope measurements were performed on a Delta^{Plus} IRMS system (Thermo Finnigan MAT, Bremen, Germany) coupled to an Agilent 6890A gas chromatograph (HP Analytical Division, Waldbronn, Germany) via a Finnigan GC Combustion III interface (Thermo Finnigan MAT, Bremen, Germany) and a CTC Analytics CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland). The mass spectrometer consisted of an electron impact source held at 3 kV acceleration voltage for CO_2 gas. Chromatographic separations were achieved on a HP cross-linked 50% phenylmethylsiloxane fused silica capillary column (30 m × 0.25 mm i.d., 0.15 μm film thickness) from J&W Scientific. The injector temperature was set to 280 °C. The combustion and reduction oven temperatures were set to 940 and 600 °C, respectively. Reference carbon dioxide gas pulses (20 s durations) were introduced at four different times during the course of the chromatographic separation. For the calibration of the reference gas with the alkane mixture of known $\delta^{13}C$-values, the oven temperature was increased from 80 (1 min) to 270 °C (7.0 min) at 15 °C/min. For the analysis of the androsterone and etiocholanolone acetates (fraction F1), the oven temperature was increased from 80 (1 min) to 270 °C (8.3 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. For the analysis

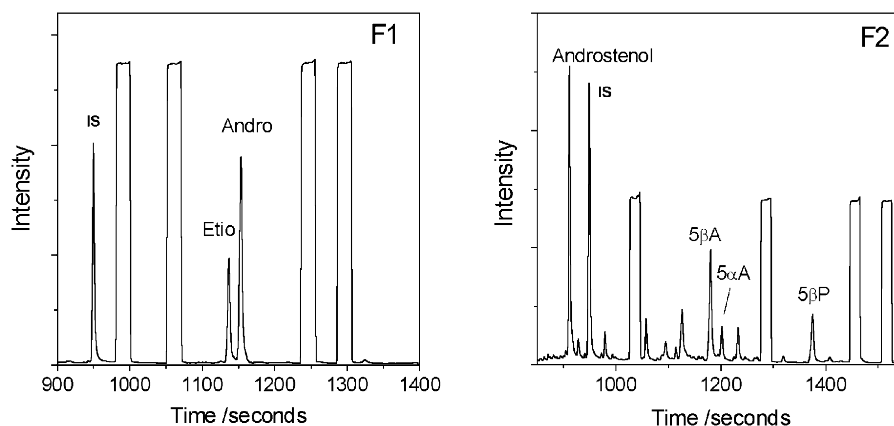

Fig. 2. Typical GC-C-IRMS *m/z*44 mass chromatograms of fractions Fl (androsterone and etiocholanolone acetates) and F2 (5αA, 5β5A, 5β5P and androstenol acetates) spiked with an internal standard (IS, 5α-androstan-3β-ol acetate). The square-topped peaks represent pulses of CO_2 reference gas.

of the fraction F2 containing the androstandiols, pregnanediol and androstenol acetates, the oven temperature was increased from 80 (1 min) to 270 °C (11.5 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. The volume of injection was 1–2 μl, depending on the concentrations of the compounds of interest. The extracts were injected once in splitless mode.

The symbol δ is the standard notation for expressing carbon isotope ratios. It is defined as parts per thousand deviation of isotopic compositions from that of Pee Dee Belemnite (PDB), and is calculated according to:

$$\delta^{13}\mathrm{C}/\ \text{‰} = \frac{(^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{sample}} - (^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{standard}}}{(^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{standard}}} \times 1000$$

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

$$D_{\mathrm{OH}} = D_{\mathrm{OAC}} + 2m\frac{D_{\mathrm{OAC}} - D_{\mathrm{AC}}}{n}$$

where D_{OH} is δ^{13}C-value for the underivatized steroids, D_{OAC} the δ^{13}C-value for the acetylated steroids, D_{AC} the δ^{13}C-value for the acetylating reagent, n the number of carbon atom in a molecule and m the number of hydroxyl groups to be acetylated.

All subsequent δ^{13}C-values have been corrected for this negative shift. The D_{AC} value of our acetylating reagent was determined to be –48.8‰ according to a published procedure [19].

3. Results and discussion

3.1. Extraction method, isotope measurements, quality control and precision

The reference carbon dioxide gas which is pulsed several times during the course of the chromatographic separation is calibrated by a mixture of three alkanes, C_{15} (*n*-pentadecane), C_{20} (*n*-eicosane) and C_{25} (*n*-pentacosane) from Chiron AS with δ^{13}C-values of −30.22, −33.06 and −28.21‰, respectively.

The extraction method allows the separation of androsterone and etiocholanolone acetates from the 5α-androstane-3α,17β-diol (5αA), 5β-androstane-3α,17β-diol, 5β-pregnane-3α,20α-diol and androstenol acetates on the C_{18} column by eluting with different ratio mixture of acetonitrile/H_2O [18]. Before the GC/C/IRMS analysis, an internal standard (5α-androstan-3β-ol acetate) is spiked in both fractions. Fig. 2 shows an example of GC/C/IRMS chromatograms of *m/z* 44 for fractions F1 and F2. The chromatograms show symmetrical peaks for the compounds of interest, no tailing, and no evidence of co-eluting compounds. The peaks of GC/C/IRMS chromatograms were attributed to the assigned compounds after comparison of their retention time and mass spectra by GC/MS with those of authentic samples of the compounds of interest.

Three urines (6 ml) of a 3-year-old boy spiked with a 10 μl solution of the steroids of interest (1 mg/ml in methanol) were used to determine the recovery yields and the possible isotopic discrimination of the steroids during the separation and analysis procedure. We have obtained recovery yields of 95, 90, 92, 88, 80 and 58% for Andro, Etio, 5αA, 5βA, 5βP and androstenol, respectively. The isotopic ratio value of the spiked androstenol in urine ($\delta^{13}\mathrm{C}_{\mathrm{androstenol}} = -29.92$‰) was very similar to the standard value ($\delta^{13}\mathrm{C}_{\mathrm{androstenol}} = -30.40$‰). For the other steroids, we have obtained $\Delta\delta^{13}$C-values lower than 0.5‰ between the δ^{13}C-values of the spiked substances in the urine and the standards. Although the recovery yields of the steroids of interest are not quantitative, our findings tend to show that there is no significant isotopic discrimination during the separation and analysis procedure.

Each batch includes positive (QC-Pos) and negative (QC-Neg) quality controls and the measurements are performed in the linear range of the IRMS. The IRMS response of the steroids of interest was found linear for injected quantities

Table 1
Within-assay precision for etiocholanolone, androsterone, 5βA, 5αA, 5βP and androstenol (n = 5)

	QC-Neg						QC-Pos					
	Etio	Andro	5βA	5αA	5βP	Androstenol	Etio	Andro	5βA	5αA	5βP	Androstenol
Mean	−24.12	−23.98	−24.37	−24.17	−23.57	−24.13	−27.57	−27.54	−28.20	−28.14	−23.61	−23.98
S.D.	0.19	0.20	0.20	0.22	0.29	0.11	0.16	0.17	0.19	0.25	0.29	0.24
CV (%)	0.8	0.9	0.8	0.9	1.2	0.4	0.6	0.6	0.7	0.9	1.2	1.0
Minimum (‰)	−24.39	−24.42	−24.47	−24.47	−23.93	−24.31	−27.83	−27.91	−28.46	−28.63	−24.02	−24.33
Maximum (‰)	−23.82	−23.82	−23.93	−23.86	−23.08	−23.95	−27.35	−27.42	−27.83	−27.78	−23.12	−23.76

Table 2
Between-assay precision for etiocholanolone, androsterone, 5βA, 5αA, 5βP and androstenol (n = 16)

	QC-Neg						QC-Pos					
	Etio	Andro	5βA	5αA	5βP	Androstenol	Etio	Andro	5βA	5αA	5βP	Androstenol
Mean	−24.23	−24.08	−24.37	−24.39	−23.75	−24.18	−27.67	−27.93	−27.89	−28.46	−23.69	−24.34
S.D.	0.16	0.17	0.27	0.34	0.32	0.34	0.25	0.35	0.31	0.59	0.26	0.28
CV (%)	0.7	0.7	1.1	1.4	1.4	1.4	0.9	1.2	1.1	2.1	1.1	1.2
Minimum (‰)	−24.59	−24.35	−24.83	−25.00	−24.27	−24.70	−28.13	−28.66	−28.52	−29.30	−24.30	−25.07
Maximum (‰)	−23.84	−23.75	−23.80	−23.81	−23.02	−23.73	−27.46	−27.43	−27.12	−27.13	−23.17	−23.74

ranging from 20 to 200 ng. The purpose of the quality controls is to verify the reproducibility of the extraction procedure and GC/C/IRMS measurements. The within-assay precision was determined by extracting five aliquots of QC-Pos and QC-Neg and injecting each once within the same day. For the determination of the between-assay precision, 16 aliquots of QC-Pos and QC-Neg were extracted by four different technicians over a period of 12 months, and injected once each. The within-assay precision and the between-assay precision for all steroids of interest in QC-Pos and QC-Neg are listed in Tables 1 and 2, respectively. These results indicate that the S.D.s for each compound measured with QC-neg and QC-pos are slightly higher in the case of the between-assay precision (range: 0.16–0.59‰) compared to the within-assay precision (range: 0.16–0.29‰). Although this method allows for the δ^{13}C-value determination of six steroids, the S.D.s and CVs are comparable to existing IRMS methods used for two or three steroids [20,21]. For QC-Pos in the within-assay precision, it is worth noting that the isotopic mean values of androstenol and 5βP are comparable and differ by at least 3.5‰ with the values of the T metabolites (Table 1). These findings tend to indicate that the isotopic values of androstenol are likely not affected by testosterone ingestion.

3.2. *Concentration and δ^{13}C-values of androstenol and 5βP in a reference population*

To investigate the potential use of androstenol as an endogenous reference compound, the concentration of both androstenol and 5βP were determined and compared in fraction F2, without correction for the recovery yields. The limits of detection (LOD) and quantification (LOQ) of the method used for quantification of androstenol and 5βP in fraction F2 were of 0.3 and 1.2 ng/μl, respectively. Fig. 3 shows the concentration of androstenol and 5βP extracted from the urines of a healthy population composed of 10 females and 10 males of Caucasian origin living in Switzerland. The concentration ratio androstenol/5βP vary from 1.3 to 9.7 with a mean of 4.5 (S.D. = 2.8, CV = 61%) for the male population with an age range of 22–34 years, whereas the ratio values range from to 0.1 to 1.4 with a mean of 0.6 (S.D. = 0.4, CV = 59%) for the female population with an age range of 21–35 years. Although more data are needed to have a more descriptive statistic, these findings significantly show that the concentration of androstenol is higher than 5βP in the male population. Except for one subject, the ratio is lower than 1.0 in the female population. For two subjects, the ratio androstenol/5βP is of 0.1, with a much higher concentration of 5βP compared to the other subjects. Indeed, it is known that a strong increase of the 5βP-excretion can be observed in the second part of the female menstrual cycle due to the progesterone production of the corpus luteum with consecutive metabolism of progesterone to 5βP [22].

As mentioned previously, 5β-pregnane-3α,20α-diol is used as an endogenous reference compound in steroid IRMS measurements. To validate the use of androstenol as an endogenous reference, the δ^{13}C-values of androstenol and 5βP were therefore compared for the urine of the reference population. The δ^{13}C-values of androstenol and 5δP for the male and female populations are depicted in Fig. 4. The differences of the intra-individual δ^{13}C-values for androstenol and 5δP are less than 0.9‰ for both populations. In the male population, the mean δ^{13}C-values are of −23.29 and −23.18‰ for androstenol and 5βP, respectively, whereas −23.69 and −23.71‰ are obtained for the female population. These findings show that the mean δ^{13}C-values are similar for both population and are comparable to isotopic values of endogenous substances of healthy subjects living in Europe [15].

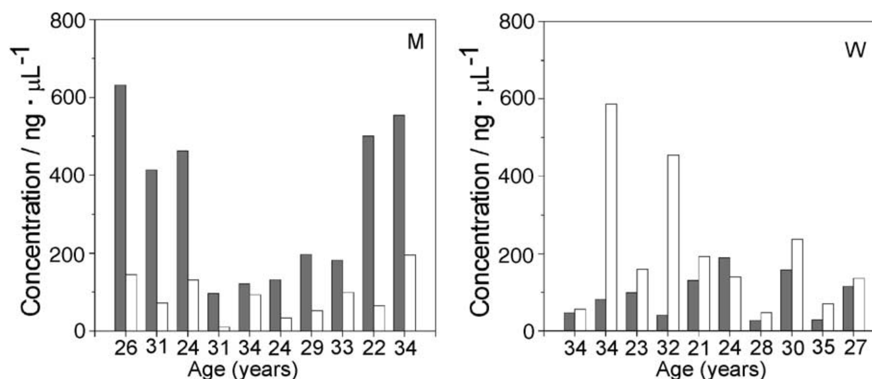

Fig. 3. Concentration in fraction F2 of androstenol (dark column) and 5βP (white column) extracted from the urines of a healthy population composed of 10 females (W) and 10 males (M) of Caucasian origin. The dark and white columns depict the androstenol and 5βP concentrations, respectively.

The S.D.s for androstenol and 5βP are comparable in the male population ($S.D._{androstenol}$ = 0.45‰, $S.D._{5βP}$ = 0.46‰) and slightly lower than the S.D.s of both steroids in the female population ($S.D._{androstenol}$ = 0.83‰, $S.D._{5βP}$ = 0.79‰).

Since the 5βP δ^{13}C-values are not affected by testosterone or DHEA administration [7,10], this compound is frequently used as an endogenous reference. The comparison of the concentration and isotopic values between androstenol and 5βP in a control group has shown no significant isotopic discrimination for androstenol and sufficient amounts for IRMS analysis. Therefore, these findings together with evidences of androstenol biosynthesis demonstrate the potential use of this compound as an endogenous reference for detecting doping with testosterone or DHEA.

3.3. δ^{13}*C-values following oral DHEA and pregnenolone administration*

The method was applied in the frame of an excretion study following oral ingestion of 50 mg DHEA initially and oral ingestion of 50 mg pregnenolone 48 h later by a Caucasian healthy male volunteer. The time course of isotopic ratio values in urine for the steroids of interest is shown in Fig. 5. Baseline δ^{13}C-values were obtained from two urines collected 26 and 2 h before DHEA administration.

As reported in a previous study [10], the δ^{13}C-value of 5βP was not affected by administration of DHEA. In the seven urines measured during the DHEA excretion study (t = 0–48 h), we determined a mean δ^{13}C-value of −23.71‰ with S.D. = 0.23‰, range: −23.38 to −24.05‰ for 5βP. The S.D. value is similar to what obtained for the between-assay of QC-neg and QC-pos and shows that the δ^{13}C-values for 5βP remain constant during the time course of the DHEA excretion. During this time course, the δ^{13}C-values of androstenol range from −23.54 to −24.10‰ with a mean δ^{13}C-value = −23.89‰ and a S.D. = 0.20‰. These findings show similar results for androstenol and 5βP, and therefore no significant variation of the isotopic values of both steroids upon DHEA ingestion. However, significant changes of the isotopic values of the T metabolites were observed after ingestion of DHEA. Fig. 5 illustrates these changes with comparable minimum δ^{13}C-value of −29.98, −30.41, −29.73 and −30.19‰ for androsterone, etiocholanolone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol, respectively. Thus, a difference higher than 6‰ is detected for the T metabolites with respect to the mean δ^{13}C-value of androstenol and 5βP after ingestion of DHEA. For the spot urine at t = 36 h, it may be still observed a difference of 0.4, 1.6, 3.8, 4.4‰ for androsterone, etiocholanolone, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, respectively, com-

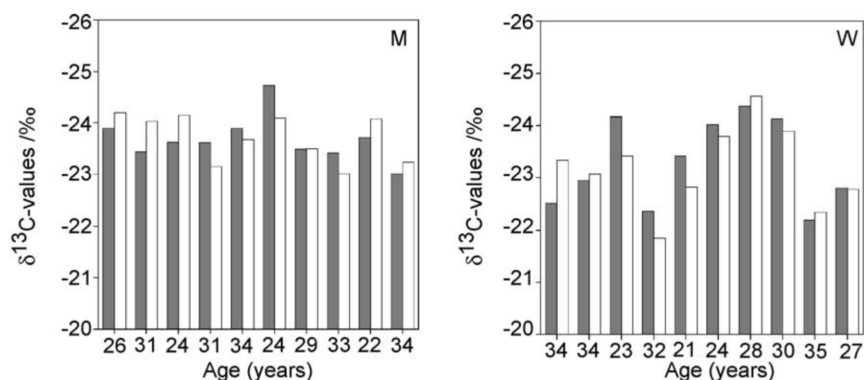

Fig. 4. The δ^{13}C values of androstenol (dark column) and 5βP (white column) extracted from the urines of a healthy population composed of 10 females (W) and 10 males (M) of Caucasian origin. The dark and white columns depict the androstenol and 5βP δ^{13}C-values, respectively.

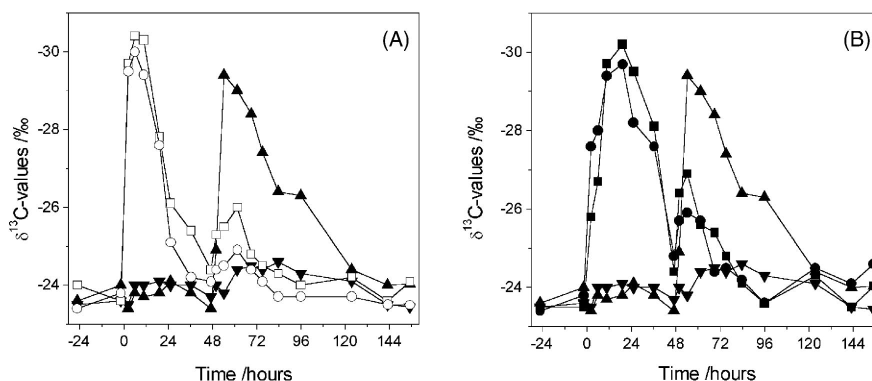

Fig. 5. Excretion study of DHEA (50 mg) and pregnenolone (50 mg), ingested initially (t = 0) and 48 h later (t = 48 h), respectively. Panel A represent the time course of the urinary δ^{13}C-values of andro (□), etio (○) together with 5βP (▲) and androstenol (▼), whereas panel B shows the time course of the urinary δ^{13}C-values of 5αA (■) and 5βA (●) together with 5βP (▲) and androstenol (▼).

pared to the δ^{13}C-value of the endogenous reference 5βP.

After ingestion of 50 mg of pregnenolone, 10 spot urines were collected over a period of 105 h. The δ^{13}C-values of 5βP were significantly affected with a difference of 5.69‰ compared to the mean δ^{13}C-value of 5βP of the urines collected during 48 h after DHEA ingestion. Similar results with δ^{13}C-values variations of 5βP higher than 4‰ were obtained on four subjects upon oral administration of 50 mg of pregnenolone [18]. As depicted in Fig. 5, the δ^{13}C-values returned to the baseline 75 h after pregnenolone ingestion. Such a difference in the carbon isotope ratio value reveals the synthetic origin of the steroid and it tends to demonstrate that 5βP is not longer applicable as endogenous reference compound. As represented in Fig. 1, pregnenolone may be also transformed to androstenol and to testosterone through the pathways leading to the formation of 16-ene steroids and sex steroids, respectively. The results show that the difference in the isotopic values of androstenol were lower than 1‰ compared to the mean value obtained after DHEA ingestion (from t = 0 to 48 h), while for androsterone, etiocholanolone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol it may be observed minimum δ^{13}C-values of −24.95, −26.00, −25.87 and −26.86‰, respectively. Thus, the isotopic values of the T metabolites have changes lower than 3‰ after oral administration of 50 mg pregnenolone.

After pregnenolone ingestion, the largest variation of the δ^{13}C-values was obtained with 5βP. Although pregnenolone is a precursor for all investigated steroids, similar variation of the δ^{13}C-values for all of them were not observed. In a previous study, it has been reported no influence of the isotopic values of androsterone, etiocholanolone and 5β-androstane-3α,17β-diol upon administration of pregnenolone [18]. Hence, these findings tend to demonstrate a major conversion of pregnenolone to 5βP compared to the T metabolites (sex steroids pathway) or androstenol (16-ene steroids pathway). Although other excretion studies on different subjects with multiple ingestions at variable dose schedules would be needed to test this effect, our results show that androstenol may serve as endogenous reference compound after ingestion of pregnenolone.

In conclusion, we have presented an IRMS method for the determination of the δ^{13}C-values of four T metabolites together with 5βP and androstenol. This method gives consistent and reproducible δ^{13}C-values for the analyzed steroids. Our findings show that 5βP and androstenol may be used as endogenous reference compounds when DHEA or testosterone has been administered. However, administration of pregnenolone will significantly affect the isotopic values of 5βP, with much smaller variations of the androstenol δ^{13}C-values. Therefore, it may be anticipated that the differences between the δ^{13}C-values of androstenol and 5βP could be an indication of pregnenolone administration.

Acknowledgements

This study was supported by a grant from the Swiss Olympic Association. We thank A. Maître for fruitful discussions and S. Cruchon for technical assistance.

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