ELSEVIER

Journal of Chromatography B, 780 (2002) 145–153

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Evaluation of human hepatocyte incubation as a new tool for metabolism study of androstenedione and norandrostenedione in a doping control perspective

Jean-François Lévesque*, Mireille Gaudreault, Robert Houle, Nathalie Chauret

Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe-Claire-Dorval, Québec, H9R 4P8, Canada

Received 7 May 2002; received in revised form 27 June 2002; accepted 1 July 2002

Abstract

Human hepatocyte incubations were used to study the metabolism of precursors of testosterone and nortestosterone and to evaluate qualitatively the correlation between in vitro and published in vivo urinary metabolic profiles. Both phase I and phase II biotransformations were observed in vitro: oxidoreduction at C-3 and C-17, reduction at C-4,5, hydroxylation at C-6β and C-16, glucuronidation and sulfation. All major metabolites detected in urine following oral administration of androstenedione and norandrostenedione were present in human hepatocyte incubations. The good correlation between in vitro and in vivo metabolic profiles indicates that hepatocyte incubations can be a useful tool to identify and characterize metabolites that could be potential urinary markers for detection of steroid abuse by athletes.

Keywords: Hepatocyte; Androstenedione; Norandrostenedione; Steroids

1. Introduction

The Medical Commission of the International Olympic Committee (IOC) prohibits the use by athletes of over 100 doping agents classified in five categories including the anabolic agents [1]. From synthetic anabolic steroids (e.g. stanozolol) to natural hormones (e.g. testosterone), athletes use different types of Anabolic–Androgenic Steroids (AAS), mainly injectables, to increase their muscular mass and to enhance the intensity and duration of training sessions [2].

The misuse of anabolic steroids is detected by evaluating the presence of banned substances or their metabolites in urine samples. Metabolism studies of anabolic steroids are usually performed in vivo in humans. After oral or intra-muscular administration of the doping agent, urine samples are collected over a certain period of time. Standard extraction and derivatization methods are followed by GC–MS analysis for detection and quantification [3–5]. Good correlation between in vivo studies and so-called urinary positive steroid profiles is observed. However, urine samples contain many interfering substances complicating the detection and identification of new urinary markers. Moreover, metabolites of

*Corresponding author. Tel.: +1-514-428-3726; fax: +1-514-428-4900.

E-mail address: jeanfrancois_levesque@merck.com (J.-F. Lévesque).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00444-0

interest are often present at low concentrations in the urine, leading to detection problems and characterization difficulties.

For the past 20 years, pharmaceutical and academic research groups have been using in vitro systems to study the metabolism of drugs and potential drug candidates to ultimately predict their in vivo profile in humans [6–8]. In vitro incubations are usually performed in sub-cellular systems such as microsomes under oxidative, reductive or phase II conjugation conditions or in whole cells such as hepatocytes. These in vitro systems provide the opportunity to form higher concentrations of metabolites, detect intermediates, isolate metabolites for further characterization, evaluate kinetics, study tissue specificity and maximize safety by reducing the need for in vivo studies. Veterinary agencies use in vitro bovine hepatocyte incubations to study metabolism of steroids, considered as growth promoter agents in animal production [9–11]. To our knowledge, limited work using such in vitro tools has been done over the years in the human doping control field. Rendic et al. studied the implication of different cytochrome P450s in the oxidative metabolism of steroids using human recombinant enzymes [12]. In vitro incubations could be useful tools for the identification and characterization of new metabolites as potential urinary markers for the detection of steroid abuse.

Androstenedione and norandrostenedione (Fig. 1) were selected to evaluate the potential of in vitro incubations to study the metabolism of anabolic androgenic steroids in humans. These steroids are precursors of testosterone and nortestosterone respectively and are prohibited by the medical commission of the IOC [1]. Over the past 5 years, scientific studies have been done to determine the in vivo metabolism profiles of these steroids [13–16]. Following oral administration, similar biotransformations were observed for both compounds: reduction of double bond at C-4,5, reduction of ketones at C-3 and C-17, hydroxylation and conjugation of phase I metabolites with glucuronic acid or sulfate groups. Knowing that these steroids are likely to be administered per os as dietary supplements, their absorption by the digestive track system should lead to first pass metabolism by the liver [17]. Therefore, hepatocytes isolated from human liver represent a good alternative to in vivo studies to look at the metabolism profile of such compounds. In order to confirm the usefulness of human hepatocyte incubations, we first studied the in vitro metabolic profiles of androstenedione and norandrostenedione and evaluated qualitatively the correlation with published in vivo urinary metabolic profiles. Then, the major biotransformations observed in vitro were compared to known in vivo metabolic pathways.

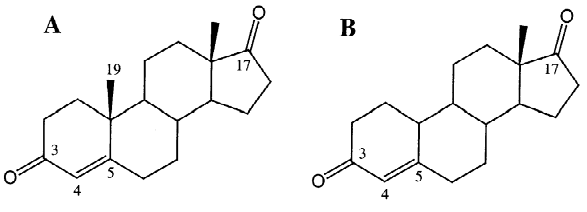

Fig. 1. Structures of androstenedione (A) and 19-norandrostenedione (B).

2. Experimental

2.1. Standards and chemicals

All the following steroid standards were purchased from Steraloids (Newport, RI, USA): androst-4-ene-3,17-dione, testosterone, androst-4-ene-17α-ol-3-one (epitestosterone), 5α-androstan-3α-ol-17-one (androsterone), 5β-androstan-3α-ol-17-one (etiocholanolone), 5α-androstane-3β-ol-17-one (epiandrosterone), 5α-androstan-3α,17β-diol, 5β-androstane-3α,17β-diol, 5α-androstan-3β,17β-diol, androst-4-ene-6β-ol-3,17-dione, androst-4-ene-6α-ol-3,17-dione, estr-4-ene-3,17-dione (norandrostenedione), estr-4-ene-17β-ol-3-one (nortestosterone), estr-4-ene-17α-ol-3-one (norepitestosterone), estrone, 5α-estran-3,17-dione, 5α-estran-3α-ol-17-one (norandrosterone), 5β-estran-3α-ol-17-one (noretiocholanolone), 5α-estran-3β-ol-17-one (norepiandrosterone) and estr-4-ene-6β-ol-3,17-dione.

Sodium acetate, anhydrous dibasic sodium phosphate and sodium carbonate were obtained from American Chemicals (Montreal, Canada) and monobasic sodium phosphate from Anachemia (Montreal, Canada). Acetonitrile, methanol, diethyl ether, hexane and dichloromethane were purchased from Merck KGaA (HPLC grade; Darmstadt, Germany).

Anhydrous THF 99.9%, sulfuric acid, ethanethiol 97%, triethylamine and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA, derivatization grade) came from Aldrich Chemicals (Milwaukee, WI, USA). Trimethylsilyliodide was obtained from Fluka Chemica (Steinheim, Germany).

2.2. Hepatocyte isolation and incubation conditions

For human hepatocytes, fresh human liver tissues were obtained from local hospitals from consenting donors as previously described [18]. Hepatocyte isolation was conducted by a two-step collagenase perfusion (90 units/ml) of the liver sample as described by Li et al. [19] and Silva et al. [18]. Viability of freshly isolated hepatocytes was assessed by trypan blue uptake (0.2%) [20]. Hepatocyte preparations with cell viability <80% were rejected. In a 48-well plate, 1×10^6 hepatocytes in 0.5 ml of Kreibs–Henseleit buffer were pre-incubated for 20 min at 37 °C under a 95:5-atmosphere of oxygen and carbon dioxide. Two sets of human hepatocyte incubations were performed using cells from two different donors. After pre-incubation, androstenedione, norandrostenedione, nortestosterone and norandrost-4-ene-3β,17β-diol were added in separate wells (final concentration of 100 μ*M*) and the plate was incubated for 0.5–4 h under the above mentioned conditions. Samples were quenched with 0.5 ml of acetonitrile and centrifuged for 10 min at 14 000 rpm. Control incubations with quenched hepatocytes were also run to evaluate the presence of undesired metabolites at $t = 0$.

2.3. Metabolite isolation

A three-step isolation procedure, as reported by Ayotte et al. [3], was used in order to isolate subsequently free, glucuroconjugated and sulfoconjugated steroids from the same incubation mixtures. Since direct analysis of steroid conjugates by GC–MS is not feasible [21], selective hydrolysis of the glucuronide and the sulfate groups were performed prior to derivatization and analysis.

2.3.1. Free fraction

To a 0.75-ml in vitro aliquot, 5 ml of acetate buffer (pH 5.2) and 5 μ*M* of internal standard (17α-methyl-5α-androstane-3β,17β-diol) were added and the sample was applied to a pre-washed (10 ml methanol followed by 5 ml water) Oasis HLB 6 ml extraction cartridge from Waters (Milford, MA, USA). The cartridge was washed with 5 ml water and 3 ml hexane and elution was carried out with 7 ml of methanol. Sample was evaporated to dryness under nitrogen at 45 °C, and then solubilized in 1 ml of phosphate buffer (pH 6.9) and 0.1 ml sodium acetate 2*M*. A double extraction of non-conjugated compounds was performed using 5 ml diethyl ether. The organic layer was evaporated to dryness under nitrogen, and the residue was dissolved in methanol and transferred to conical vials. After evaporation, the sample was derivatized as described later.

2.3.2. Glucuronide fraction

After the extraction of non-conjugated steroids, the aqueous phase was acidified at pH 5. Then, 5 ml of acetate buffer (pH 5.2) and the internal standard (17α-methyl-5α-androstane-3β,17β-diol) were added. As described earlier, the sample was again applied on an extraction cartridge, followed by washing and elution steps. After evaporation of methanol, the residue was dissolved in 1 ml of phosphate buffer (pH 6.9) and 2000 units of β-Glucuronidase from E.Coli type IX-A (Sigma, St Louis, MO, USA) were added. The selective hydrolysis of glucuroconjugated steroids was completed after 60 min at 50 °C. After addition of 0.1 ml of 2 *M* sodium carbonate, a double extraction was performed with 5 ml of diethyl ether. The organic layer was evaporated to dryness under nitrogen and the residue was dissolved in methanol and transferred to new conical vials. After evaporation, each sample was derivatized as described later.

2.3.3. Sulfate fraction

The procedure used for the isolation of sulfoconjugated steroids was similar to the one described earlier for glucuroconjugated steroids, the main difference being the hydrolysis conditions. Instead of using an enzymatic hydrolysis, a solvolysis was performed to hydrolyze the sulfate group. After evaporation of methanol, 1 ml of THF and 2 μl of 4 *M* sulfuric acid were added, and the sample was

heated at 50 °C for 60 min [22]. Then, 1 ml of phosphate buffer (pH 6.9) and 0.1 ml of 2 *M* sodium carbonate were added and a double extraction was carried out with 5 ml diethyl ether. The organic layer was evaporated and the residue derivatized.

2.4. Trimethylsilyl derivatization

The ketone and hydroxy groups of all steroids were derivatized as TMS-enol and TMS-ether respectively using the following protocol. Conical vials were capped under nitrogen and 50 μl of a mixture of MSTFA/TMSI (3.0 ml MSTFA, 60 μl ethanethiol and 60 μl of TMSI 0.1 *M*) were added. Vials were heated for 30 min at 70 °C and then analysed by GC–MS. Some samples were also derivatized as TMS-ether by adding sequentially 50 μl of pyridine, 40 μl of TMCS and 10 μl of pure MSTFA in a conical vial capped under nitrogen. After 60 min at 70 °C, samples were evaporated and residues were solubilized in 50 μl of hexane.

2.5. GC–MS analysis

Analysis of in vitro samples were performed using an Agilent 5973 Mass Selective Detector coupled to an Agilent 6890 gas chromatograph equipped with a model 7683 autosampler (Mississauga, Canada). Instrumentation control and data handling were performed with a Hewlett-Packard Kayak XM600 computer using Agilent Enhanced MS ChemStation software (G-1701CA version C.00.00). One microliter of derivatization mixture was injected in pulsed splitless mode into a split/splitless liner packed with silanized glass wool. Separation of steroids was achieved using a HP-1MS (15 m long, 0.25 mm internal diameter and 0.25 μm film thickness) from Agilent (Mississauga, Canada). The following oven temperature program was used: 150 °C (held 0.5 min) to 200 °C (20 °C/min), to 220 °C (2 °C/min) to 320 °C (30 °C/min) and the final temperature was held for 5 min. Helium (5.0 grade) was used as carrier gas (BOC Gases; Montreal, Canada) and all acquisitions were performed in scan mode (from *m/z* 50 to 600).

3. Results

3.1. In vitro metabolism of androstenedione

Metabolic profiles of androstenedione after 4 h incubations in human hepatocytes from the first donor are presented in Fig. 2. Characteristic $[M\text{-}15]^+$ and molecular ions of reduced metabolites of androstenedione ($C_{19}O_2$) were selected to build chromatograms ($[M]^{+\cdot}$ at *m/z* 430, 432, 434, 436, and $[M\text{-}15]^+$ at *m/z* 415, 417, 419 and 421). Androsterone, epiandrosterone, testosterone and androstenedione were present in free fraction whereas androsterone, etiocholanolone, epitestosterone, androstenedione and testosterone were observed as glucuroconjugates. In the sulfate fraction, androsterone, epiandrosterone, 5α-androstane-3β,17β-diol, androstenedione and testosterone were present. Epiandrosterone was the most abundant peak in sulfate fraction, whereas androsterone and testosterone were the major metab-

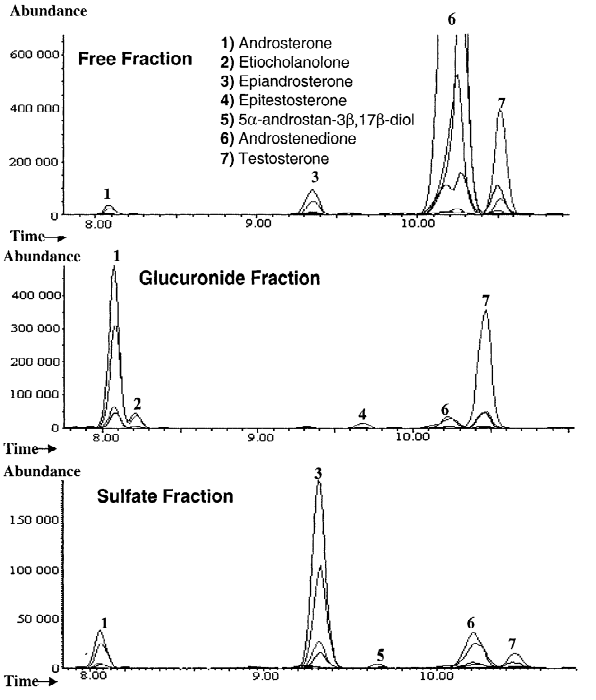

Fig. 2. Metabolic profiles of androstenedione after 4 h incubations in human hepatocytes from donor #1: Free, glucuronide and sulfate fractions. Extracted ions: *m/z* 430, 432, 434, 436, 415, 417, 419 and 421.

olites in glucuronide and free fractions respectively. A second set of human hepatocyte incubations was also performed using cells from a second donor. All metabolites observed in the first experiment were also present in the second set of incubations in their respective fractions (results not shown). In addition, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol were both observed as glucuroconjugates and sulfoconjugates.

Different incubation times ($t = 0.5$, 1 and 4 h) were also tested in order to evaluate qualitatively the in vitro rate of formation of the different metabolites. In all fractions, profiles at $t = 0.5$ and 1 h were similar to those discussed earlier ($t = 4$ h) but the extent of metabolism was lower (results not shown).

In vitro formation of hydroxylated metabolites ($C_{19}O_3$) of androstenedione was also observed in all fractions. Part of the numerous $C_{19}O_3$ metabolites present were identified based on authentic standards, retention times and mass spectrum previously published in literature [13,15]. Formation of 6β-hydroxyandrostenedione, 6β-hydroxyandrosterone, 6β-hydroxyetiocholanolone, 6β-hydroxyepiandrosterone and 6β-hydroxytestosterone was observed in in vitro incubations for both hepatocyte batches. All five hydroxylated metabolites were mainly observed in the free form after 4 h incubations. Only 6β-hydroxyepiandrosterone was present in sulfate fraction whereas 6β-hydroxyandrostenedione, 6β-hydroxyandrosterone and 6β-hydroxyetiocholanolone were also glucuroconjugated. Concentrations of 6β-hydroxyandrostanes increased in all fractions with incubation time ($t = 0.5$, 1 and 4 h) whereas levels of 6β-hydroxyandrostenedione and 6β-hydroxytestosterone decreased. The mass spectrum of TMS-enol TMS-ether 6β-hydroxyandrostenedione, 6β-hydroxyandrosterone, 6β-hydroxyetiocholanolone and 6β-hydroxyepiandrosterone are shown in Figs. 3 and 4.

3.2. In vitro metabolism of norandrostenedione

Metabolic profiles of norandrostenedione after 4 h incubations in human hepatocytes from the first donor are presented in Fig. 5. Characteristic $[M\text{-}15]^+$ and molecular ions of reduced metabolites of 19-norandrostenedione ($C_{18}O_2$) were selected to build

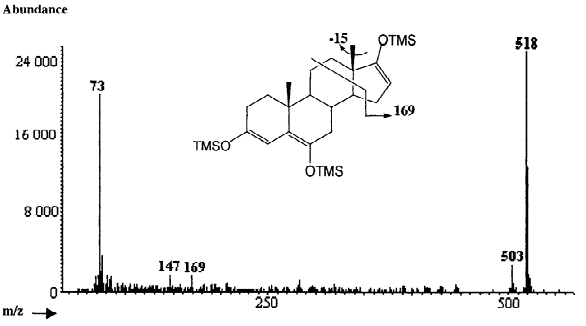

Fig. 3. Mass spectrum of 6β-hydroxyandrostenedione TMS-enol TMS-ether.

chromatograms ($[M]^{+\cdot}$ at m/z 416, 418, 420, 422, and $[M\text{-}15]^+$ at m/z 401, 403, 405 and 407). Noretiocholanolone, norepitestosterone, norandrosterone, norepiandrosterone, norandrostenedione and nortestosterone were all present in the sulfate frac-

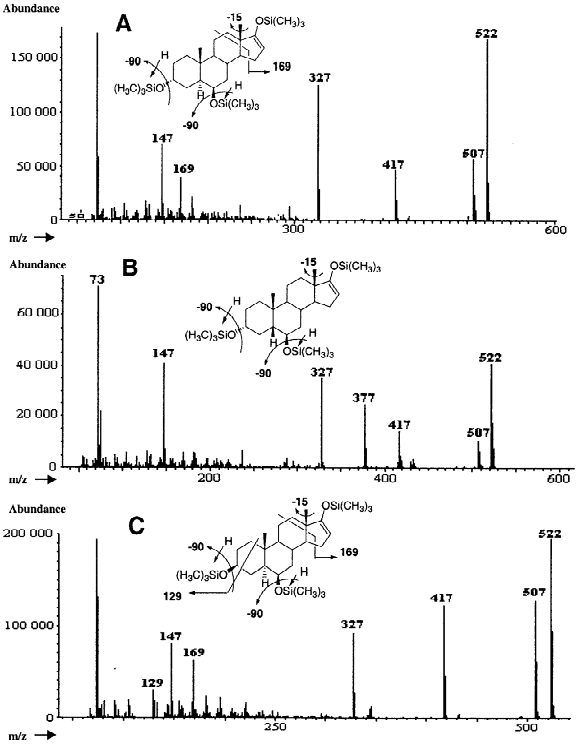

Fig. 4. Mass spectrum of the TMS-ether TMS-enol derivatives of 6β-hydroxyandrosterone (A), 6β-hydroxyetiocholanolone (B) and 6β-hydroxyepiandrosterone (C).

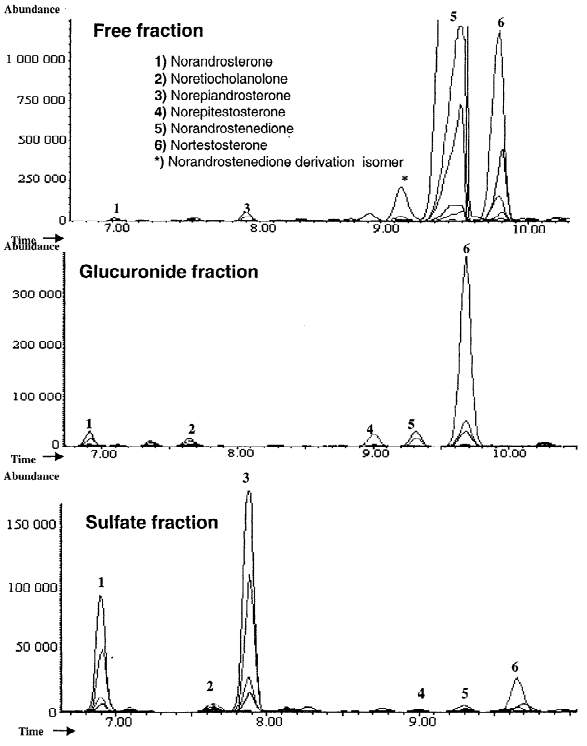

Fig. 5. Metabolic profiles of norandrostenedione after 4 h incubations in human hepatocytes from donor #1: Free, glucuronide and sulfate fractions. Extracted ions: *m*/*z* 416, 418, 420, 422, 401, 403, 405 and 407.

tion whereas only the later four were observed in their unconjugated form. Glucuroconjugates of norandrosterone, noretiocholanolone, norepitestosterone, nortestosterone and norandrostenedione were also detected. It was unclear if presence of norandrostenedione in sulphate and glucuronide fractions was due to conjugation via enol formation or to residual levels from the free fraction. All metabolites observed in the first experiment were also present in the second set of incubations in their respective fractions (results not shown). In addition, estrone was observed in the free and glucuronide fractions and 5α-estrane-3,17-dione in the unconjugated form.

By varying incubation time from 0.5 to 4 h, we observed that nortestosterone was predominant at early time point while the abundance of norandrosterone, noretiocholanolone and norepiandrosterone increases with time. In order to compare their metabolic profiles, norandrostenediol and nortestosterone were also incubated in human hepatocytes for 2 h. Profiles of both compounds were almost identical to the ones presented earlier for norandrostenedione (results not shown).

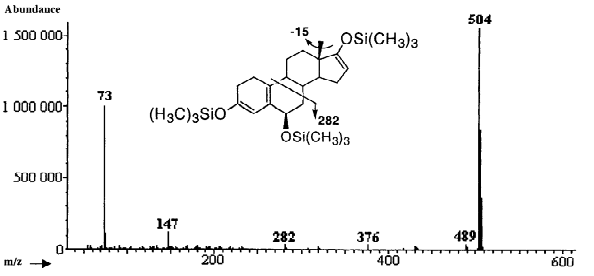

Fig. 6. Mass spectra of 6β-hydroxynorandrostenedione TMS-enol TMS-ether.

Over ten mono-hydroxylated metabolites of norandrostenedione ($C_{18}O_3$) were also detected in all fractions for both hepatocyte batches. The main $C_{18}O_3$ metabolite of norandrostenedione was observed in free, glucuronide and sulfate fractions and had a mass spectra (Fig. 6) and a retention time identical to 6β-hydroxynorandrostenedione bis-TMS-enol TMS-ether. In order to prove the configuration of the hydroxy group in C-6, the unknown in the incubation and the authentic standard of 6β-hydroxynorandrostenedione were derivatized as TMS-ether and co-injected on GC–MS. The two peaks perfectly co-eluted, which confirms that the unknown was 6β-hydroxynorandrostenedione. A peak with an intense loss of 15, an ion at *m*/*z* 147 and a molecular ion at *m*/*z* 508 (double reduction and single hydroxylation of norandrostenedione) was present in all fractions. Based on characteristic $[M-15]^+$ and *m*/*z* 147 ions and a mass spectra identical to the one reported in literature [16], this unknown might be 16α-hydroxynorandrosterone. Further characterization is required to confirm its structure.

4. Discussion

4.1. In vitro vs. in vivo metabolism of androstenedione

In vivo, the oral administration of androstenedione was reported to cause important increases of the

basal concentrations of androsterone, etiocholanolone and testosterone in the glucuronide fraction [13,14]. Abnormally high urinary concentrations of androgens and a testosterone glucuronide over epitestosterone glucuronide ratio (TG/EG) above the cut-off of 6 are the probes commonly used by doping control laboratories to detect the misuse of androstenedione [1,13,14]. In both human hepatocyte incubation experiments, the major glucuronides formed were also androsterone, etiocholanolone, and testosterone. Epiandrosterone was present but only in the free and the sulfate fractions. Since no data on sulfoconjugated steroids are reported in literature, it was impossible to correlate with in vivo data. Moreover, the 6β-hydroxyandrostanes identified in vivo [13,15] (6β-hydroxyandrosterone, 6β-hydroxyetiocholanolone and 6β-hydroxyepiandrosterone) were present in vitro either in the free, glucuronide or the sulfate fractions. The only major difference with in vivo studies was the absence of 6α-hydroxyandrostenedione in human hepatocyte incubations and the presence of its isomer, 6β-hydroxyandrostenedione. Considering structure similarity and metabolic profile variations with time, this hydroxylated metabolite is most likely to be the precursor of 6β-hydroxyandrosterone, 6β-hydroxyetiocholanolone and 6β-hydroxyepiandrosterone. Incubations of 6β-hydroxyandrostenedione standard in human hepatocytes led to formation of all three 6β-hydroxyandrostanes and confirmed the existence of this metabolic pathway. Although its presence was suspected, the 6β-hydroxyandrostenedione intermediate had never been reported in vivo. The proposed metabolism pathways of androstenedione in human hepatocytes are presented in Fig. 7.

Poor fragmentation of 6β-hydroxyandrostenedione, as observed in its mass spectra (Fig. 3), might be due to presence of three OTMS functions linked to sp_2 carbons. Major fragments observed are $[M-15]^+$ ion, m/z 169 ion and m/z 147 ion formed via bis-OTMS rearrangement [23] and the characteristic fragment at m/z 319. In the case of 6β-hydroxyandrostanes, their mass spectra all contain an initial loss of radical methyl followed by two successive losses of TMSOH groups. The main difference between these compounds is the presence of a characteristic ion at m/z 377 only for 6β-hydroxyetiocholanolone bis-TMS-ether TMS-enol. Similar

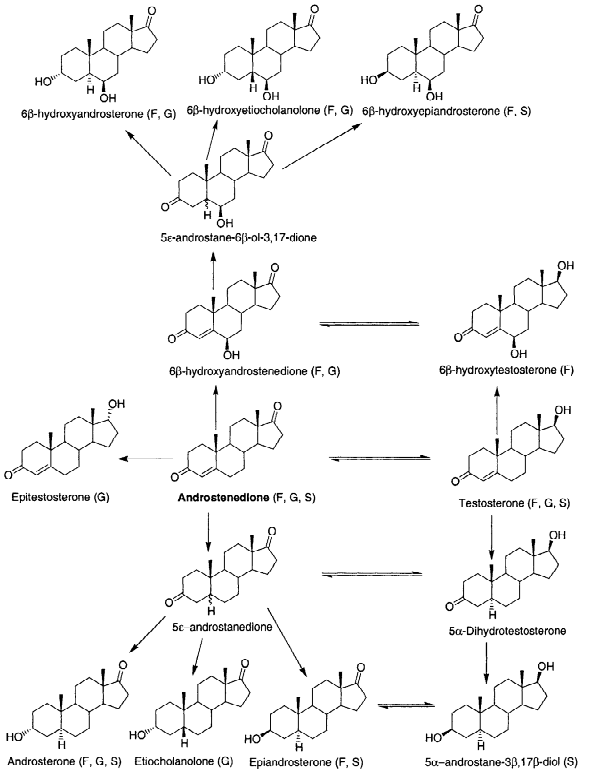

Fig. 7. Proposed in vitro metabolism pathways of androstenedione in human hepatocytes. Fractions where metabolites were observed are indicated in parentheses. (F = unconjugated; G = Glucuronide; S = Sulfate).

observations were seen with 5β-cholestane-3α,6β-diol bis-TMS-ether by Harvey and Vouros [24]. Our suggested mechanism leading to the formation of the ion at m/z 377 is presented in Fig. 8. Based on the 3-dimensional configuration of our proposed mechanism, only a 5β-androstane having OTMS ether groups in C-6β and C-3α positions could generate such a fragment. A similar mechanism was proposed in literature by Grupe and Spiteller for underivatized steroids [25].

4.2. In vitro vs. in vivo metabolism of norandrostenedione

Norandrostenedione abuse by athletes is monitored by detection of norandrosterone and noretiocholanolone in urine. After oral administration, the following metabolites were detected in vivo whether

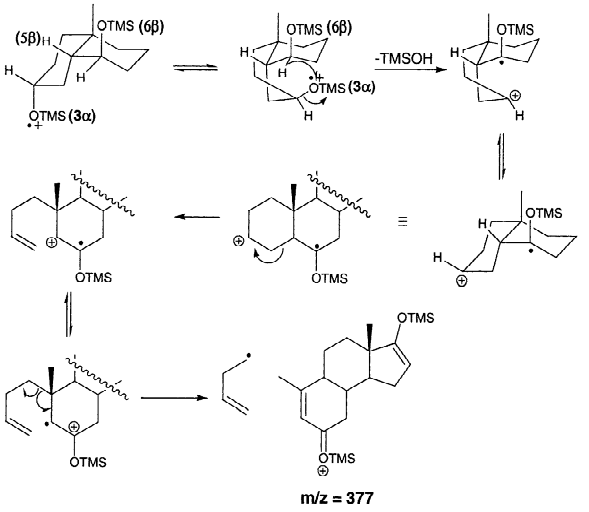

Fig. 8. Proposed mechanism leading to the formation of the characteristic ion at m/z 377 for the bis-TMS-ether TMS-enol derivative of 6β-hydroxyetiocholanolone.

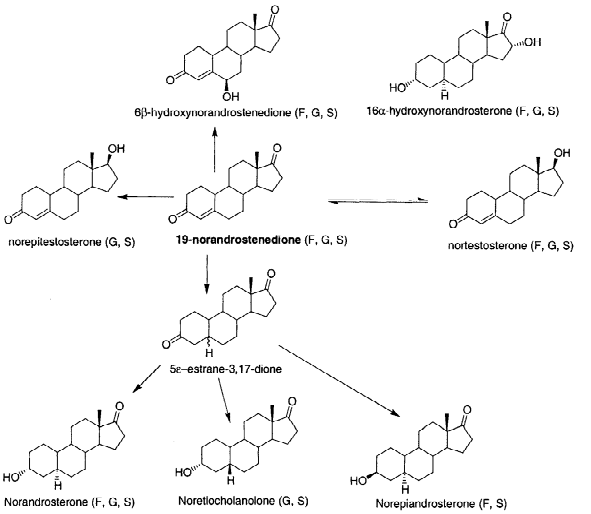

Fig. 9. Proposed in vitro metabolism pathways of norandrostenedione in human hepatocytes. Fractions where metabolites were observed are indicated in parentheses. (F = unconjugated; G = Glucuronide; S = Sulfate).

in the free, glucuronide or the sulfate fractions: norandrosterone, noretiocholanolone, norepiandrosterone, norepitestosterone, norandrostenedione, nortestosterone, estrone, 5ε-estran-3,17-dione and estr-4-ene-3ε-ol-17-one [14,16]. In vitro, all the above mentioned metabolites were present in both human hepatocyte incubation experiments except the later three steroids. Estrone and 5ε-estran-3,17-diones were observed only in the second set of incubations. The 17ε-ol-estrenes were mainly unconjugated and/or glucuroconjugated whereas the 3α-ol-estranes were present in all fractions. As observed previously for 3β-ol-androstane, 3β-ol-estrane was observed in the free and sulfate fractions. Hydroxylated metabolites ($C_{18}O_3$) identified in urine [16] were also detected in vitro. The 6β-hydroxynorandrostenedione and 16α-hydroxynorandrosterone (proposed structure) were detected in all fractions. The proposed metabolism pathways of norandrostenedione in human hepatocytes are presented in Fig. 9.

4.3. In vitro metabolism of AAS

Metabolic profiles of androstenedione, norandrostenedione, norandrostenediol and nortestosterone all showed both phase I and phase II metabolites. Major sites of oxidation and/or reduction were located at C-3, C-4,5 and C-17 on the steroids. Reductions of 4-ene and 3-keto functions were observed for all substrates and only three of the four possible reduced isomers were formed (5α-androstane-3α-ol, 5α-androstane-3β-ol and 5β-androstane-3α-ol or 5α-estrane-3α-ol, 5α-estrane-3β-ol and 5β-estrane-3α-ol). No 5β-androstane-3β-ol or 5β-estrane-3β-ol were detected. Mono-hydroxylated metabolites such as 6β-hydroxyandrostenedione were also metabolized by these reductive pathways. Reduction of the ketone groups at C-17 (androstenedione and norandrostenedione) and oxidation of the hydroxy functions at C-17 (nortestosterone and norandrost-4-ene-3β,17β-diol) were the most favored phase I biotransformations observed in vitro. Hydroxylation of AAS at C-6β and C-16 was also detected. Phase II conjugates of parent compounds and their phase I metabolites were formed in vitro. Steroids with hydroxy groups at C-17β or C-17α were glucuroconjugated in hepatocytes whereas C-3β-ol-steroids were mainly in the free and sulfate forms. The 3α-ol-androstanes were mainly glucuroconjugated or unconjugated whereas sulfoconjugation of 3α-ol-estranes seemed to be more favored. These

observations regarding major phase I and phase II biotransformations of AAS are similar to those reported in vivo in human urine by Schanzer [26].

In hepatocyte incubations, all major intermediates formed can easily be detected whereas in vivo, conjugated end products are the main metabolites excreted in urine. Intermediates such as unconjugated phase I metabolites (e.g. 6β-hydroxyandrostenedione) can be detected using in vitro systems. In vitro, free steroid fraction can be considered as a transitory state leading eventually to formation of end products and to phase II conjugation. Parent compounds and steroids with 17β-ol and 3β-ol functions were the main compounds present in the free fraction. High concentrations of unconjugated material could be related to high rate of phase I metabolism and to poor phase II conjugation.

4.4. Conclusions

Human hepatocyte incubations of androstenedione, norandrostenedione, nortestosterone and norandrostenediol have led to both phase I and phase II metabolism. Similar biotransformations to those reported in vivo in humans were observed in vitro for both donors: oxidoreduction at C-3 and C-17, reduction at C-4,5 and hydroxylation at C-6β and C-16. Steroids with hydroxy groups at C-17β or C-17α were conjugated as glucuronide whereas 3β-ol steroids were mainly unconjugated or in the sulfate form. Steroids with C-3α-ol were observed in all fractions. All major metabolites detected in urine following oral administration of androstenedione and norandrostenedione were observed in human hepatocyte incubations. The good correlation between in vitro and in vivo metabolic profiles confirms that hepatocyte incubations could be a useful tool to identify and characterize metabolites that could be potential urinary markers for detection of steroid abuse by athletes.

References

[1] Comité International Olympique, IOC Medical Code and Explanatory Documents, 2000.
[2] A. Noret, Le Dopage, Editions Vigot, Paris, 1981.
[3] C. Ayotte, D. Goudreault, A. Charlebois, J. Chromatogr. B 687 (1996) 3.
[4] K.J. De Cock, F.T. Delbeke, P. Van Eenoo, N. Desmet, K. Roels, P. De Backer, J. Pharm. Biomed. Anal. 25 (2001) 843.
[5] W. Schanzer, S. Horning, G. Opfermann, M. Donike, J. Steroid Biochem. Mol. Biol. 57 (1996) 363.
[6] S.A. Wrighton, M. Vandenbranden, J.C. Stevens, L.A. Shipley, B.J. Ring, A.E. Rettie, J.R. Cashman, Drug Metab. Rev. 25 (1993) 453.
[7] A.D. Rodrigues, Biochem. Pharmacol. 48 (1994) 2147.
[8] S. Ekins, J. Maenpaa, S.A. Wrighton, in: T.F. Woolf (Ed.), Handbook of Drug Metabolism, Marcel Dekker, New York, 1999, p. 363.
[9] A.S. Clouet, B. Le Bizec, F. Boerlen, F. Monteau, F. André, Analyst 123 (1998) 2489.
[10] N.G. Coldham, G. Biancotto, C. Montesissa, L.C. Howells, M.J. Sauer, Analyst 123 (1998) 2589.
[11] A.S. Clouet-Dumas, B. Le Bizec, M.A. Le Pape, D. Maume, F. Monteau, F. André, J. Steroid Biochem. Mol. Biol. 74 (2000) 57.
[12] S. Rendic, E. Nolteernsting, W. Schanzer, J. Chromatogr. B 735 (1999) 73.
[13] J.-F. Lévesque, C. Ayotte in: W. Schanzer, H. Geyer, A. Gotzmann, U. Marek-Engelke (Eds.), Proceedings of the 17th Cologne Workshop on Dope Analysis, Cologne, March 1999, 2000, p. 169.
[14] V.P. Uralets, P.A. Gillette, J. Anal. Toxicol. 23 (1999) 357.
[15] D. Goudreault, P. Bhérer, J.-F. Lévesque, D. Poirier, C. Ayotte in: W. Schanzer, H. Geyer, A. Gotzmann, U. Marek-Engelke (Eds.), Proceedings of the 19th Cologne Workshop on Dope Analysis, Cologne, March 2001, 2002 (in press).
[16] W. Schanzer, A. Breidbach, H. Geyer, C.V. Kuk, E. Nolteernsting, M. Thevis in: W. Schanzer, H. Geyer, A. Gotzmann, U. Marek-Engelke (Eds.), Proceedings of the 18th Cologne Workshop on Dope Analysis, Cologne, February 2000, 2001, p. 155.
[17] G. Gordon Gibson, Introduction To Drug Metabolism, Nelson Thornes, Cheltenham, UK, 2001.
[18] J.M. Silva, S.H. Day, D.A. Nicoll-Griffith, Chem. Biol. Interact. 121 (1999) 49.
[19] A.P. Li, M.A. Roque, D.J. Beck, D.L. Kaminski, J. Tissue Cult. Methods 14 (1992) 139.
[20] M.N. Berry, A.M. Edwards, C.J. Barrit, Isolated Hepatocytes Preparation, Properties and Applications, Elsevier Science, Amsterdam, Netherlands, 1991, p. 46.
[21] W.J.J. Leunissen, Quantitative aspects of the determination of steroid profiles from urine by capillary gas chromatography, Ph.D. Thesis, Eindhoven, Netherlands, 1979.
[22] J.-F. Lévesque, L'importance clinique de l'excrétion urinaire de stéroïdes sulfoconjugués lors de contrôle antidopage, Master Thesis, Pointe-Claire, Canada, 1998.
[23] S. Sloan, D.J. Harvey, P. Vouros, Org. Mass Spectrom. 5 (1971) 789.
[24] D.J. Harvey, P. Vouros, Biomed. Mass Spectrom. 6 (1979) 135.
[25] A. Grupe, G. Spiteller, Org. Mass Spectrom. 13 (1978) 448.
[26] W. Schanzer, Clin. Chem. 42 (1996) 1001.