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Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: Potential use in gas chromatography isotope ratio mass spectrometry

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The basis of a potential method for confirming intake of four natural androgens (testosterone, epitestosterone, dihydrotestosterone, and dehydroepiandrosterone is presented. The method relies on isolating from urine a steroid fraction containing androstenediol and androstanediol metabolites of these natural steroids and analyzing their ^{13}C content by gas chromatography, combustion, isotope ratio mass spectrometry. The steroids were recovered from urine by conjugate hydrolysis with a Helix pomatia *preparation (sulfatase and β-glucuronidase), Girard T reagent separation to obtain a nonketonic fraction, and Sephadex LH-20 chromatography for purification. Metabolites appropriate for all of the natural steroids could be separated (as diacetates) by gas chromatography on a DB-17 capillary column viz.: 5α (and β)-androstane-3α,17α-diol (epitestosterone as precursor); 5α (and β)-androstane-3α,17β-diol (testosterone as precursor); 5-androstene-3β,17β-diol (dehydroepiandrosterone precursor); and 5α-androstane-3α,17β- (and 17α-) diol (dihydrotestosterone precursor). Measurement of the ^{13}C content of the specific analytes after ingestion of the androgen precursors demonstrated a lowering of $\delta^{13}C‰$ value compared to normal values. Typically, in the male individual studied, $\delta^{13}C‰$ values for all components were −26 to −27 before drug administration and −29 to −30 at 6 h after, the latter values reflecting those obtaining for commercial synthetic steroid compared to in vivo synthesized steroid. While generally the metabolism of the steroids was as expected, this was not the case for 5α-dihydrotestosterone. A major metabolite was 5α-androstane-3α,17α-diol, which had presumably been formed by 17β/17α isomerization, a process previously known for unnatural anabolics but not for natural hormones. The isolation, purification, and isotope ratio mass spectrometry techniques described may form the basis of a general method for confirming natural steroid misuse by sports participants.* (*Steroids* **62**:665–673, 1997) © *1997 by Elsevier Science Inc.*

Keywords: gas chromatography combustion/isotope ratio mass spectrometry; doping; androgen; dihydrotestosterone; dehydroepiandrosterone; epitestosterone

Introduction

Recent communications by Southan et al.,[1] Becchi et al.,[2] Aguilera et al.,[3] Horning et al.,[4] and ourselves[5] addressed the use of isotope ratio mass spectrometry (IRMS) to detect testosterone (T) misuse in sports. Testosterone commercially synthesized from plant precursors has a lower ^{13}C content, reported as the $\delta^{13}C‰$ value,[2] compared to endogenously produced steroid. This is because synthetic testosterone tends to be made from a plant species (usually soy) with low ^{13}C content ($\delta^{13}C‰ < -30$) while our bodies (and their chemical products) originate from a variety of plants that are a mixture of relatively high ($\delta^{13}C‰$ −12 to −20) and low ($\delta^{13}C‰$ −23 to −35) values.[6] Typical values for human endogenous steroid products measured free or as acetate derivatives by different investigators[1–5] give $\delta^{13}C‰$

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values ranging from −24 to −28. The publications of groups who have worked in this field suggest that values of around −29 and below for urinary testosterone and its metabolites is conclusive evidence of exogenous testosterone administration.[1–5]

Testosterone is not the only natural androgen-like compound used by athletes. Others include epitestosterone (epiT), dehydroepiandrosterone (DHEA), and dihydrotestosterone (DHT). While epiT is not androgenic, it has been administered to mask the results of the commonly used T/epiT ratio. The greater than 6 value for the ratio adopted as proof of misuse[7] can be normalized by self-administering epiT as well as testosterone. DHT, the 5α-reductase-produced metabolite of testosterone, is itself a potent androgen which has in recent years been shown to be the subject of misuse.[8] DHEA oral administration is an interesting problem. While it has been banned by the International Olympic Committee (IOC), it is freely available at low cost in the United States marketed as a "nutritional supplement." The health and anti-aging properties of this steroid, although less than well proven, are being widely touted. DHEA itself is a weak androgen and only a minimal amount, probably ≤1, is converted to testosterone. While not even a C_{19} steroid, pregnenolone (in pill form) is also freely available in the United States and is being recommended for daily use by elements of the "health food industry." Pregnenolone has not been banned by the IOC but this may be anticipated if its ingestion is perceived to contribute to androgen synthesis.

Our previous study demonstrated the use of IRMS for confirming testosterone administration by measuring the ^{13}C content of its metabolites 5α- and 5β-androstane-3α,17β-diols. While these are not major steroid components, accounting for only about 4% of total testosterone metabolites,[9] they are structurally closely related to the precursor, particularly in contrast to the quantitatively important metabolites androsterone and etiocholanolone. Not only that but it is believed that in humans they are not formed to a significant extent by reduction of 17-oxosteroids.[9] The other natural steroids potentially misused also metabolize to androstane- or androstenediols (Figure 1), probably to a similar extent as does testosterone (3–5%), although diols are probably relatively more important in epitestosterone metabolism since little of this steroid is oxidized by 17α-hydroxysteroid dehydrogenase to 17-ketosteroids. Our previously described methodology[5] has been refined to encompass all of these metabolites, thus allowing comprehensive evaluation of natural hormone misuse through conducting a single gas chromatography (GC)/combustion/IRMS analysis. While this has been a pilot study, we believe that with further development and evaluation the methodology will be useful for routine screening use and may replace the T/EpiT ratio and other parameters currently used in doping control.

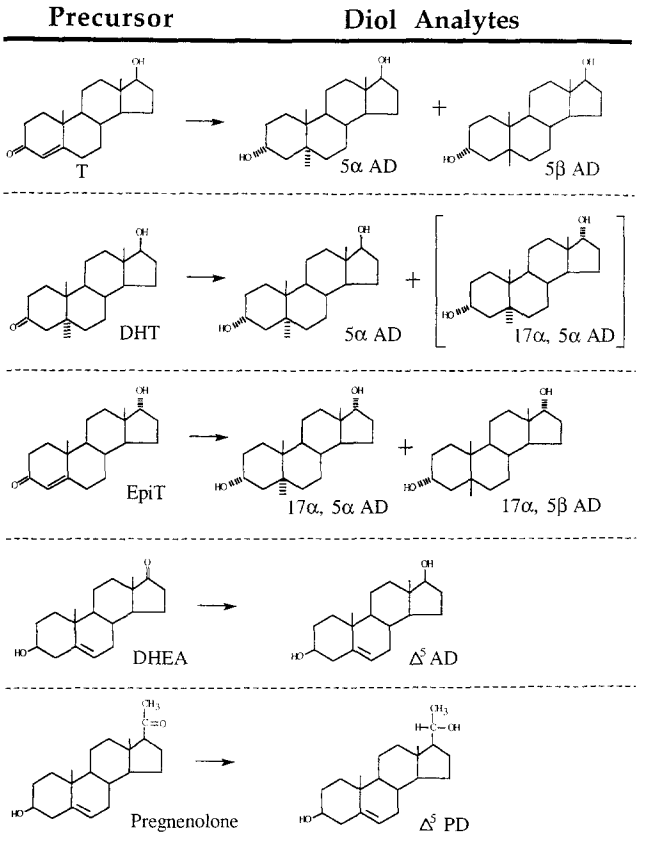

Figure 1 Diol metabolites of androgens and pregnenolone. The following abbreviations are used: 5αAD, 5α-androstane-3α,17β-diol; 5βAD, 5β-androstane-3α,17β-diol; 17α,5αAD, 5α-androstane-3α,17α-diol; 17α,5βAD, 5β-androstane-3α,17α-diol; Δ^5AD, 5-androstene-3β,17β-diol; and Δ^5PD, 5-pregnene-3β,20α-diol. 5α-Androstane-3α,17α-diol as a DHT metabolite is still controversial, so it has been bracketed. Not shown are the minor but significant testosterone, DHT, or epiT metabolites 5α-androstane-3β,17β (and 17α)-diols.

Experimental

Materials

DHT, epitestosterone, androstanediols, androstenediols, and other authentic steroids were obtained from Sigma Chemical Company (St. Louis, MO). DHEA and pregnenolone in "dietary supplement" tablet form were marketed by Natron (Chatsworth, CA) and Optimal Nutrients (Foster City, CA), respectively. The $\delta^{13}C‰$ values of acetates of these reference steroids were between −30 and −32. All solvents were of analytical grade.

Urine sample analysis

A representative urine sample was prepared for method evaluation. It was composed of an equal proportion of daily 24-h samples of urine from 15 male and 15 female individuals.

In addition, steroid metabolites excreted by a male adult after steroid ingestion were studied. This individual gave written informed consent. In separate experiments conducted 2 weeks apart, 50 mg of testosterone, DHT, epiT, DHEA, and pregnenolone were taken orally for 2 days. Urine was voided 3 and 6 h after the second administration, but only the second sample was collected and analyzed. In addition, collections were made 3 and 6 h after co-administration of 50 mg of testosterone and 5 mg of epitestosterone. The 6-h time period was chosen because Schänzer et al.,[10] found maximal excretion of androstanediols at that period after steroid ingestion.

Preparation of steroid extracts for GC/combustion/IRMS

The basic methodology was described previously although modifications have been made for simplicity.[5] The principal change has been deletion of a bismuthate oxidation step. In brief, 30 ml of urine were extracted by Sep-pak[30]11 and hydrolyzed by *Helix pomatia* enzymes. Whereas in the method reported previously,[5] Girard separation was undertaken on the hydrolysate directly, in this study the steroids were first extracted by Sep-pak cartridge. To the steroid extract was added 0.5 ml of acetic acid and 200 mg of Girard reagent T (Sigma Chemical Company, St. Louis, MO). The reaction was allowed to proceed for 30 min at 100°C whereupon the reaction tube was cooled and low polarity nonketonic steroids extracted with 2 × 3 ml of isooctane/methylene chloride (2:1). The extract was washed with 0.5 ml of 10% NaOH, and 0.5 ml of water and finally dried with a few milligrams of anhydrous sodium sulfate. Once dried, the steroid extract was purified on mini-Sephadex LH-20 columns (Pharmacia Biotech AB, Uppsala, Sweden) prepared in glass wool-plugged Pasteur pipettes (dry mass 0.5 g, bed size 60 × 5 mm inside diameter). The Sephadex LH-20 was stored in the solvent system (cyclohexane/ethanol, 4:1) to allow it to maintain a swollen state. The extract was applied in 0.2, 0.2, and 0.4 ml of the solvent system, allowing each application to absorb into the bed. Another 1 ml of solvent was added directly to the absorbent bed, and all of this initial eluant (1.8 ml) was discarded. The nonketonic androstanediol fraction was recovered by eluting the column with 1.4 ml of the same solvent. The steroid-containing fraction was dried and following addition of 1 μg of 5α-androstan-3β-ol internal standard was acetylated overnight with 100 μl of pyridine and 200 μl of acetic anhydride. The derivative was dried, dissolved in cyclohexane, and analyzed by GC/mass spectrometry (MS) and GC/combustion isotope mass spectrometry.

Gas chromatography/mass spectrometry

Authentication of the structures of the steroids present in the steroid fraction was achieved by GC/MS utilizing an HP 5971 MSD instrument (Hewlett-Packard, Palo Alto, California, USA) with a DB-17 column (30 m, 0.25 mm inside diameter, 0.25 μm film thickness; J&W Scientific, Folsom, California, USA).

For separation of the steroid acetates the following instrument conditions were utilized: injector 260°C, interface 280°C, splitless injection. Program: after injection at 50°C a 1-min hold, then 30°C/min to 270°C and 0.5°C/min to 282°C. Mass spectra were obtained by scanning a 50–650-amu range. Component structures were confirmed by comparing urinary and authentic steroid spectra. Molecular ions of the androstanediols and androstenediols were small but major ions were formed by —OAc removal (M-60 and M-60-60).

Gas chromatography/combustion/isotope ratio mass spectrometry

This was performed on a Micromass isochrom isotope ratio mass spectrometer (Micromass UK Ltd., Wythenshawe, Manchester, UK) as previously described.[5] Steroid separations were conducted on a DB-17 column identical with that used for GC/MS. Analysis of the steroid containing fraction took 20 min. ^{13}C isotope content (measured as $\delta^{13}C‰$) was determined for androstanediols and androstenediols and the endogenous reference compounds (ERCs), pregnanediol, and pregnanetriol. In our previous communication we used pregnanediol as the ERC, but pregnanetriol has an advantage because it is in a region of the chromatogram less subject to contamination and larger amounts (than pregnanediol) are excreted in adult males and in women during the greater part of their menstrual cycles.

Results and discussion

The analytes and their separation

Androstanediol and androstenediol metabolites have for some years been included in studies on the detection of endogenous steroids.[12–15] We have detected three androstenediols and six androstanediols in urine extracts (Table 1). The probable origins of these steroids are as follows: 5-androstene-3β,17β-diol is a metabolite of DHEA; 5-androstene-3β,17α-diol may be a side product of conversion of pregnenolone to 17-deoxysteroids[16] or be formed by 17α-hydroxysteroid dehydrogenase acting on DHEA; 5β-

Table 1 Retention indices and relative quantitative importance of urinary androstanediol and androstenediol components

Steroid	RI	Relative importance (% total)		
		HP	βGlucA	βGlucB
5α-Androstane-3β-ol (IS)	2866			
Androstenediol (unident.)	3132	10.0	9.7	NM[c]
5β-Androstane-3α,17α-diol	3192	7.3	11.1	1.0
5β-Androstane-3α,17β-diol	3217	32.0	33.1	52.0
5α-Androstane-3α,17α-diol	3230	4.7	3.1	10.0
5α-Androstane-3α,17β-diol	3241	14.0	16.8	15.7
5-Androstene-3β,17α-diol	3279	8.7	23.8	20.2
5α-Androstane-3β,17α-diol	3285	1.0	2.2	NM
5-Androstene-3β,17β-diol	3293	22.0	1.3	1.4
5α-Androstane-3β,17β-ol	3300	~1.0	~1.0	~1.0
Pregnanediol (ERC)	3406			
5-Pregnene-3β,20α-diol	3483			
Pregnanetriol (ERC)	3645			

Retention times and approximate proportions of androstanediols and androstenediols in urine extracts produced in this study by *H. pomatia* (HP) and β-glucuronidase hydrolysis (β-Gluc A). Results are also presented for Dehennin's data also obtained using β-glucuronidase hydrolysis (β-Gluc B). The values given assume a total of 100%. IS, internal standard; ERC, endogenous reference compounds.

androstane-3α,17β-diol and 5α-androstane-3α,17β-diol are testosterone metabolites and the latter is also a DHT metabolite; 5α-androstane-3β,17β-diol is a DHT and testosterone metabolite; epitestosterone gives rise to 5α (and 5β)-androstane-3α,17α-diols and 5α-androstane-3β,17α-diol.

Separation of all these steroids from urine as diacetate derivatives is shown in Figure 2, and the chromatogram can be considered average because the sample was prepared from equal proportions of 24-h urine collections from 15 male and 15 female adults. The chromatogram, the best that could be achieved within a practical time (30 min), shows that almost all steroids can be baseline separated. The earliest eluting androstenediol has not been identified although it was shown not to be 5-androstene-3α,17β-diol, 4-androstene-3β,17α (or β) -diol, or 4-androstene-3α,17α (or 17β) diols. 5-Pregnene-3β,20α-diol is a pregnenolone metabolite and the ERCs pregnanediol and pregnanetriol are metabolites of progesterone and 17-hydroxyprogesterone, respectively. Regarding the elution properties of the group, the following elution order applies: 3α-hydroxy-5β, 3α-hydroxy-5α, 3β-hydroxy-Δ^5, and 3β-hydroxy-5α. Superimposed on this order 17α-hydroxylated steroids elute before 17β.

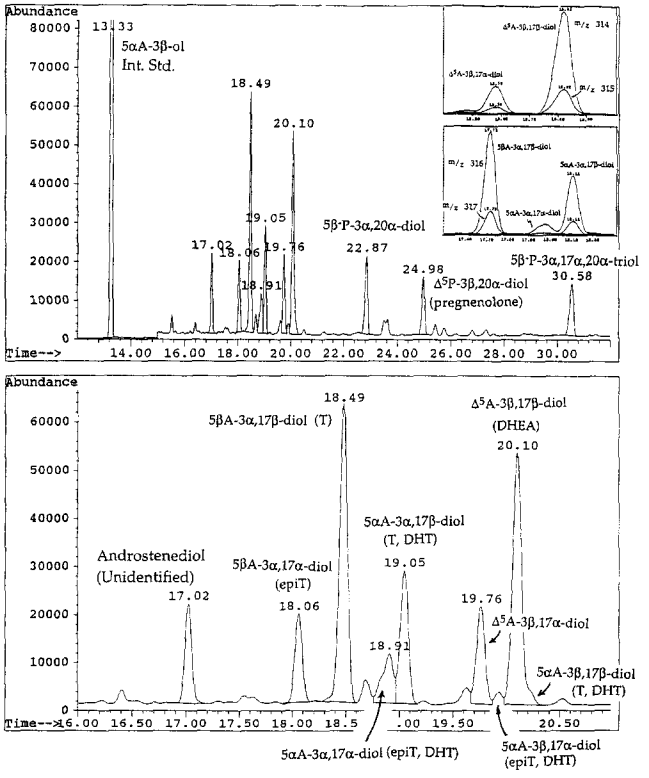

Figure 2 The contents of the diol fraction of normal urine. The upper panel shows the second half of the full steroid diacetate chromatogram including the internal standard and ERCs. The lower panel focuses on the androstanediols and androstenediols. The hormonal precursors of the individual metabolites are listed in parentheses. The inset shows selected ion monitoring results of M-60 and M-60 + 1 for the androstenediols and androstanediols, indicating that ^{12}C- and mono-^{13}C-labeled steroids exactly co-eluted.

Table 1 gives the retention indices of the androstanediols and androstenediols. The approximate amount of each metabolite (obtained after *H. pomatia* hydrolysis) relative to the total is also given and compared to values we obtained using *Escherichia coli* β-glucuronidase and those reported by Dehennin,[13] who also used the *E. coli* preparation. The obvious difference between β-glucuronidase and *H. pomatia* results is that 5-androstene-3β,17β-diol is a minor component of the β-glucuronidase profile, representing about 1/15 of the 17α-epimer. With *H. pomatia* hydrolysis, the 17β-epimer dominates reflective of its largely sulfate conjugation. The results and those of Dehennin[13] broadly agree; an exception is the low excretions of 5β-androstane-3α,17α-diol found in the latter study. The pregnenolone metabolite 5-pregnene-3β,20α-diol, an important component found after *H. pomatia* hydrolysis, was essentially absent from extracts hydrolyzed with β-glucuronidase. The comparative enzyme studies confirmed the advantage of using *H. pomatia* mixed β-glucuronidase and sulfatase for the current studies.

A desirable feature of the GC separation on the DB-17 column was that there was no apparent isotope fractionation of the intact steroids that would compromise IRMS measurement. The inset in Figure 2 shows *m/z* 316 and 317 peaks (M-60 and M-60 + 1) for androstanediols and *m/z* 314 and 315 for androstenediols, and it is quite evident that diol molecules with one ^{13}C label are not even partially resolved from completely ^{12}C-containing steroids.

Steroid excretion and δ% values after DHT administration

It is evident from the chromatogram illustrated in Figure 3 that two steroids, 5α-androstane-3α,17β-diol and 5α-androstane-3α,17α-diol, are dramatically increased in size relative to normal values (cf. Figure 2). Although increase in the former metabolite was expected, this was not the case for the 17α-diol, partly because its production from the 17β-diol seemed unlikely and partly because it has not previously been reported as 5αDHT metabolite.[8,10–15] Evi-

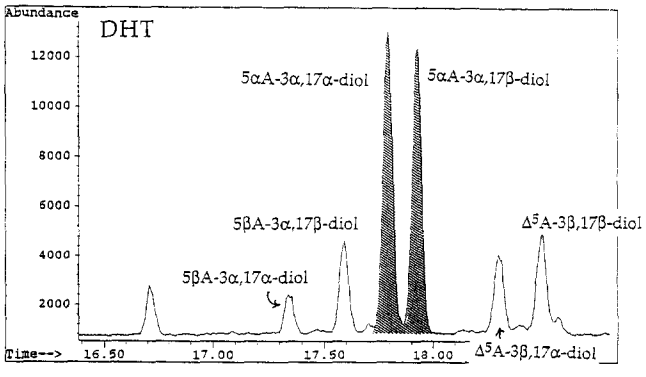

Figure 3 The steroid fraction following DHT administration. Only the central part of chromatogram is illustrated. The major metabolites are highlighted and were characterized as 17α- and 17β-androstanediol epimers. In contrast to undrugged state and to the situation following testosterone administration, the ratio of the amount of 5α-androstane-3α,17β-diol to 5β-androstane-3α,17β-diol is reversed.

dence that both compounds were metabolites of ingested 5αDHT comes from the fact that they both have lowered IRMS δ values (Table 2) whereas normal values (~ −26) were obtained for 5β-androstane-3α,17β-diol, 5-androstene-3β,17α-diol, and 5-androstene-3β,17β-diol. The finding that a 17α-androstanediol is a significant metabolite (at least in the individual studied) suggests that 17-hydroxyl isomerization is an important feature of 5αDHT metabolism. Quantitatively, and from the δ measurement (Table 2), it is unlikely that 5α-androstane-3α,17α-diol could have originated by serial activity of 17β-(oxidative) and 17α-hydroxysteroid (reductive) dehydrogenase on DHT. Previous studies on testosterone and nandrolone (19-nor-testosterone) have shown little if any reduction of 17-oxo-steroid metabolites following their production.[17–19] Although human 17β/17α isomerization has previously been reported for synthetic anabolics, including trenbolone, which has a conventional (not 17α-methylated) 17β-hydroxy D-ring,[20–22] this form of transformation has not been reported in natural hormone metabolism. Confirmation that isomerization must have occurred comes from an experiment (to be separately reported) where 7 mg of [16,16,17-$^2H_3$]DHT were administered and androstanediol metabolites were isolated. Both 5α-androstane-3α,17β-diol and 5α-androstane-3α,17α-diol metabolites had major components retaining three deuteriums. Although production of a 17α-hydroxylated product was significant, it must be borne in mind that it is still a quantitatively minor metabolite since the total amount of androstanediols represents only 10% of the urinary metabolites found after DHT ingestion.[10]

Epitestosterone administration

Although there are no IOC procedural guidelines governing epiT administration to athletes, it has been recommended that excretions >150 μg/liter should be further investigated. Such high excretions together with lowered δ value of metabolites may well provide definitive proof of misuse.

In our experiment, separate analysis of the total steroid fraction (before Girard separation) by GC/MS indicated that a large proportion of epitestosterone was excreted unchanged (with possible exception of conjugation) as reported in other studies.[14] This was expected because the relatively low activity of 17α-hydroxysteroid dehydrogenase in humans would not contribute to androstenedione and its metabolites androsterone and etiocholanolone greatly. Although 17-oxidation was not substantial, the excretion of androstanediol metabolites isolated after a 50-mg epiT administration was significant, and the chromatogram contained large peaks with retention times appropriate for 5β-androstane-3α,17α-diol, 5α-androstane-3α,17α-diol, and a lesser amount of 5α-androstane-3β,17α-diol (Figure 4). Isotope ratio measurement of the first two peaks showed they had highly negative δ values. The large amounts of 17α-androstanediols present in this experiment rendered the measurement of δ values for the other androstanediol and androstanediol compounds difficult and inaccurate, but they are of course likely to be normal. The amount of epiT administered was excessive considering its natural production rate and would not represent a typical amount taken by an athlete attempting to evade a positive T/epiT ratio. A secondary experiment was performed with a better model study, and chromatograms in Figure 4 (lower panel) show separation of steroids following a 50-mg testosterone administration and 6 h after a combination of 50 mg of testosterone and 5 mg of epitestosterone. When epitestosterone is taken in sports it is taken only to normalize the T/epiT ratio in cases where T is being administered. Thus the amount taken of epiT would be small compared to that of testosterone, particularly since epiT resists metabolism. An increase in the relative peak size of the 17α-androstanediols after T + epiT administration was evident but IRMS measurement was not conducted, so it is not known whether drug administration at this level could be confirmed by the isotope ratio technique.

DHEA administration

DHEA is rapidly metabolized by reduction to 5-androstene-3β,17β-diol or conversion to androsterone and etiocholanolone. A chromatogram of metabolites excreted after DHEA ingestion is shown in Figure 5 and illustrates a major increase in 5-androstene-3β,17β-diol peak size relative to the other constituents, and IRMS measurement (Table 2) showed lower δ values for this compound. Interestingly, the peak of 5-androstene-3β,17α-diol was not increased in size nor did it show reduced δ value, implying that this compound is not a metabolite of ingested DHEA. 5-Androstene-3β,17α-diol may not even be a major metabolite of in vivo produced DHEA since Weusten et al.[16] suggest that it is biosynthesized as a byproduct of the direct conversion of

Table 2 $\delta^{13}C‰$ values for androstenediol and androstanediol components following steroid ingestion

Steroid	Baseline	DHT	epiT	DHEA
5β-Androstane-3α,17α-diol	−25.90	NM	[−30.96]	−27.67
5β-Androstane-3α,17β-diol	−26.48	−26.51	NM	[−29.60]
5α-Androstane-3α,17α-diol	NM	[−30.53]	[−29.60]	NM
5α-Androstane-3α,17β-diol	−25.64	[−30.80]	NM	[−29.14]
5-Androstene-3β,17α-diol	−24.33	−27.19	NM	−26.39
5-Androstene-3β,17β-diol	−25.86	−27.88	NM	[−29.25]
Pregnanetriol (ERC)	−25.85	−26.50	−26.43	−26.06

IRMS $\delta^{13}C‰$ values obtained by measurement of diol fractions obtained 6 h after administration of 50 mg of synthetic androgens. The values "boxed" represent those showing significant reduction which indicates that the compounds were largely metabolites of exogenous synthetic steroids. NM, not measured.

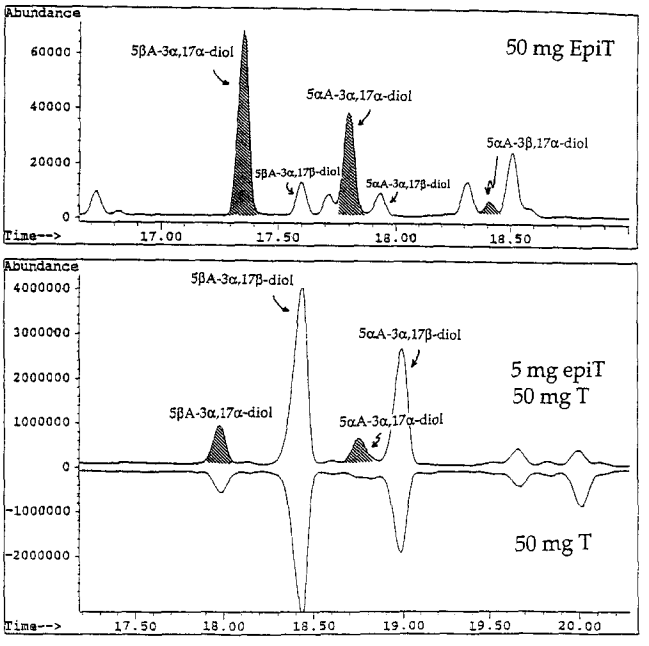

Figure 4 GC profiles following epiT administration. Only the central parts of the chromatograms are illustrated. The upper panel shows dominance of 17α-metabolites following a 50-mg epiT dose and these were shown to have significant reduction of $\delta^{13}C‰$ values. The experiment was repeated following 5 mg of epiT administration in conjunction with 50 mg of testosterone, providing a chromatogram (middle panel) more typical for actual use of epiT as a steroid taken to normalize the urinary T/epiT ratio. The lower ("mirror image") panel shows profile following testosterone administration alone.

pregnenolone to 5,16-androstanedien-3β-ol. Despite the prevailing notion that once oxidized, 17β-hydroxysteroids are not reduced to a significant extent,[17–19] 5α- (and 5β)-androstanediols did give lowered δ values.

Pregnenolone administration

Pregnenolone was primarily metabolized by 20α-hydroxysteroid dehydrogenase to 5-pregnene-3β,20α-diol. In contrast to the situation after DHEA administration, the peak in the chromatogram (Figure 5, bottom), representing 5-androstene-3β,17α-diol, when measured against its 17β-epimer, doubled in size. This would support the concept that the 17α-diol is a pregnenolone metabolite, but not one of 17α-hydroxypregnenolone or DHEA.[16] IRMS measurements were not made on the samples collected in the study.

Practicality of method and comparison to other studies

Optimum IRMS data require baseline separation of GC components so that (1) there is no secondary component that may alter the measured δ value and (2) integration can be accomplished over the whole peak to avoid a chromatographic isotope effect.[23] The requirements therefore differ greatly from GC/MS (in selected ion-monitoring mode) where 100% overlap of components is perfectly acceptable, so a major simplification of urinary steroid extracts is necessary before IRMS analysis to allow complete resolution of analytes. Another differing requirement pertains to derivatization. While in GC/MS trimethylsilyl ether (TMS) or methyloxime-TMS derivatives are those of choice because of their rich mass spectra, providing many ions suitable for selective ion monitoring, these derivatives are unsuitable for IRMS. It has been found that SiO_2 formed during combustion soon inactivates the oxidation catalyst. It would be preferable to avoid the use of derivatization altogether, but if more than one or two analytes are present in the GC chromatogram this is impossible because of increased peak width and tailing. While acetylation may appear to be undesirable because it could potentially contribute to isotope fractionation during derivative formation,[2,3] in practice this has not been shown to be significant in steroid analyses, so the acetates have become the derivative of choice.[2,3] Although any additional carbons are undesirable, acetylation only adds four to the diols whereas silylation would add six, a secondary reason for not using the latter reaction. Choice of GC chromatography column is also important. For GC/MS steroid analysis of TMS and MO-TMS derivatives, nonpolar OV-1 (methylsilicone) type columns (e.g., DB-1) are usually chosen because derivatized urinary steroids of increasing complexity are spaced almost equidistantly within a linearly programmed chromatogram. This is not the case for separation on polar or medium polarity columns where the polarity and stereochemistry of individual steroids plays a greater part in separation. It appears, however, that nonpolar columns give rise to greater isotope fraction-

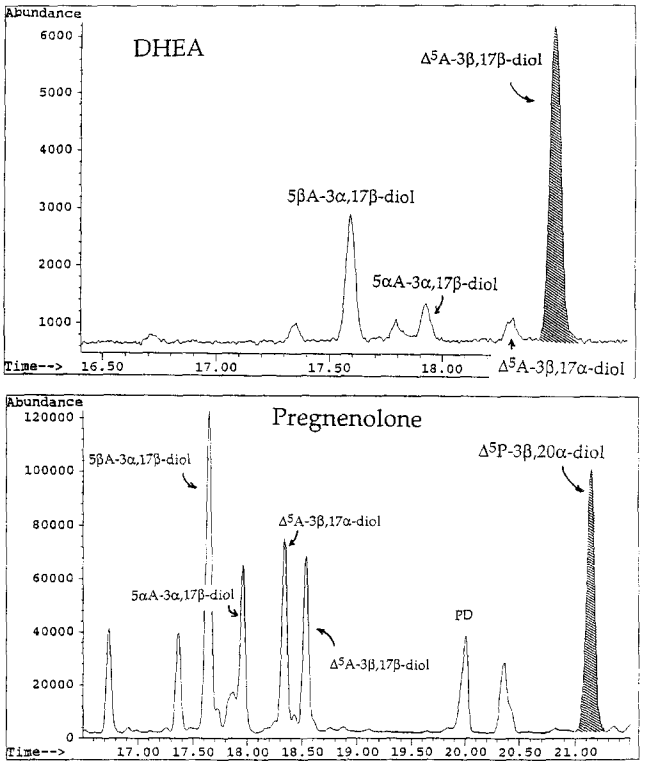

Figure 5 Diol profiles following the administration of the Δ^5 steroids DHEA and pregnenolone highlighting the appropriate analytes for IRMS measurement. Only central parts of chromatograms illustrated.

ation between isotopomers than do polar or medium polarity columns.[23] This is an undesirable feature for IRMS and can lead to compromised results, particularly if peaks are not baseline separated or completely integrated from beginning to end. In our studies, there was no discernible separation between ^{12}C and ^{13}C peaks containing androstanediol, indicating suitability of the column chosen. It must be borne in mind that the greatest degree of isotope fractionation occurs for CO_2 and $^{13}CO_2$ between combustion chamber and mass spectrometer collector.

There have been three major approaches to use of the IRMS technique in natural steroid doping confirmation. In their pioneering study, Becchi et al.[2] utilized β-glucuronidase hydrolysis of urinary steroids with low resolution silica cartridge fractionation and preparative high performance liquid chromatography (HPLC) resolution of testosterone, DHEA, and androstanediol fractions. Urinary cholesterol, separately extracted, was used as internal standard (ERC). Acetylation was carried out preceding GC/IRMS. In an adapted method,[3] they carried out acetylation prior to HPLC analysis to improve purity of the isolated components but this change had the disadvantage that DHEA could not be separately isolated, although 5-androstene-3β,17β-diol could be resolved. This latter compound provided the "baseline" value for δ since it is a precursor of testosterone, not a catabolic product. Use of this ERC would not be possible if the method was used on an individual who had been taking DHEA in addition to testosterone since the androstanediol is a major metabolite. However, the fact that 5-androstene-3β,17β-diol was separately isolated means that the method, essentially unchanged, could be utilized for proving DHEA administration through $\delta^{13}C‰$ measurement of the androstenediol. In their studies 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol diacetates co-eluted, preventing separate δ value measurement, but these epimers could certainly be separated by choosing a polar GC column. Horning et al.[4] have also described a technique for IRMS of urinary testosterone and metabolites. Their methodology also involved β-glucuronidase hydrolysis, but a novel immunoaffinity column (with selectivity towards 17β-hydroxy steroids) was employed, simplifying the steroid extract by primarily retaining testosterone and the androstanediols. Testosterone, 5α- and 5β-androstane-3α,17β-diol fractions were individually collected following HPLC of the immunoaffinity column fraction and these were subjected to GC/IRMS analysis underivatized. Because the 5α- and 5β-androstanediols were separately determined, this method can be used to distinguish testosterone from DHT misuse (S. Horning, personal communication, 1997). Presumably, their method could also be used to extract and analyze another 17β-steroid, the DHEA metabolite 5-androstene-3β,17β-diol. However, as is also the case in Aguilera's method,[3] this steroid could be obtained only from the glucuronide fraction unless a different hydrolytic enzyme preparation was used. Sensitivity of detection may therefore be a problem since the androstenediol is predominantly excreted as mono- and disulfate conjugates, not as a glucuronide (Table 1). The androstanediol products of epiT metabolism could not be isolated by this method unless a separate 17α-immunoaffinity column was developed and utilized.

Our approach to the use of IRMS has been somewhat different in that we have endeavored to produce a single high resolution GC chromatogram separating all analytes required to definitively establish whether any of four exogenous androgens was being administered. Prior to the GC analysis, we have sought to keep procedures low resolution to minimize the possibility of isotope fractionation, curtail instrument costs and facilitate batch processing. To minimize GC isotope fractionation, we utilize a medium polarity DB-17 column,[24] which fortuitously also allows the separation of 5α- and 5β-androstane-3α,17β-diols, unlike DB-1-like phases. In our methodology the low resolution fractionations include 1) removal of the urinary ketonic steroids which amount to about 75% of the total, 2) selection of an extraction solvent which only removes steroids equal to and below the polarity of pregnanetriol (a further 15% steroids and sterols removed), and 3) chromatography mini-LH-20 columns (which removed about another 2% of original urinary steroids and a major part of nonsteroidal impurities). The final extract of diols and pregnanetriol contains about 8% in mass of the original urinary steroids. We elected to use *H. pomatia* hydrolysis for deconjugation since this improves recovery of 3-monosulfates of 5-androstene-3β,17β (and 17α)-diol and 5-pregnene-3β,20α-diol. A criticism in the use of *H. pomatia* in analyses associated with doping control is that the enzyme preparation contains 3β-hydroxysteroid dehydrogenase which may result in the production of testosterone and androstenedione from DHEA and 5-androstene-3β,17β-diol, respectively.[24,25] However, minimal oxidation of Δ^5 steroids occurs if the hydrolysis is undertaken for a short period at high temperature (55°C), and in addition, any oxidative products formed would not remain in the analyte fraction since they would be discarded as Girard hydrazones.

Overall, the data presented by different investigators are remarkably similar in spite of the different methodologies utilized. Typically, in each study $\delta^{13}C‰$ values for testosterone or metabolites which would be considered negative for misuse run around −25 to −27 and those positive −28 to −31. It would seem that a consensus on discriminant values could be readily achieved and such values be adopted as regulatory.

Influence of peak contamination

Regarding contamination of chromatographic peaks, it was previously stated that IRMS requires homogenous peaks and complete baseline separation for the technique to be useful. While this is highly desirable, some degree of a secondary component in a peak is tolerable; the question is how much? In Figure 6 we provide models of contamination that suggest that up to 20% of an extraneous (not originating from administered drug) component may be permissible while still giving drug-positive test results. In the inset to Figure 6, we have assumed that a hypothetical δ −30 "exogenous" analyte is contaminated with a δ −25 endogenous component. This component can be of four principal types: (1) the identical analyte, but of endogenous origin; (2) a co-eluting different analyte; (3) the tail of a different component; or (4) chemical noise often associated with extended solvent front or column bleed. We believe our

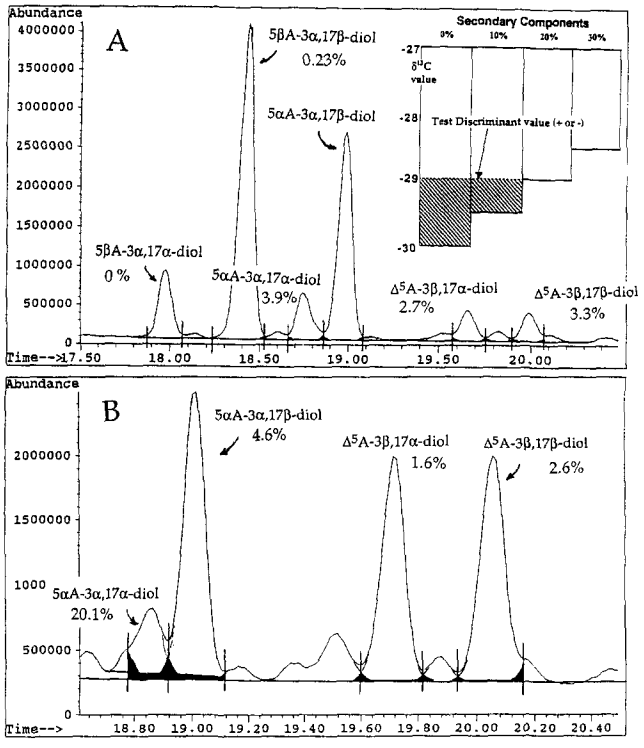

Figure 6 Influence of contamination of peak on major components. The inset illustrates how hypothetically contaminating a δ −30 with a δ −25 contaminant affects the overall δ value of a peak. At 20% contamination, the analyte value rises above a putative δ −29 value proposed for a positive drug test. Approximations are shown in chromatograms for negligible contamination (upper panel A, 50 mg T + 5 mg epiT administration) and more serious (lower panel B, no drug administration). The lower chromatogram had greater background remaining from "solvent front." The measured contaminant contributions are given as percentages on the chromatograms.

methodology excludes Item 2, and Item 1 must be accepted to some degree dependent on the amount of suppression of endogenous steroids by the administered hormone. Items 3 and 4 are real problems dependent on the quality of the samples and chromatographic separation. In Figure 6A we show an almost ideal chromatogram with the following strengths: it is from a drug-positive sample so ample steroid is present, reducing the relative amounts of chemical noise. Such a sample is also likely to have reduced amount of endogenous analyte because of hormone suppression. In this situation, contamination is limited to the minimal tails of adjacent peaks. In an example, we calculate peak contaminations from 0.23% for the major peak to 3.9% for 5α-androstane-3α,17α-diol, quite acceptable values within the context of the method.

Figure 6B illustrates a more compromised situation. It is from a control (no steroid drug) sample, but the lower quality chromatogram should not be considered typical for all control chromatograms. Quantitatively much less steroid is present in control samples so chemical noise and extraneous peaks contribute to a greater extent. The chromatographic baseline was sloping, indicating that nonspecific compound elution also contributed to the peak contents. In this case, we calculate extraneous material contributes between 1.6 and 20% for the different components. In reality, in this run only the result of the epitestosterone metabolite 5α-androstane-3α,17α-diol may be too compromised; the remaining components still show contaminations of $<10\%$. Use of this androstanediol analyte is also not essential since the other epiT metabolite 5β-androstane-3α,17α-diol is always baseline separated. This chromatogram illustrates that the major contaminant may well be nonspecific solvent front components ("chemical noise") rather than tails of adjacent peaks. In doping control practice it may well be possible to detect δ values compromised because of this form of contamination by incorporating an internal standard of known δ value which is eluted before the elution of the androstanediol analytes. The current ERCs elute late in the chromatogram when all solvent front components have eluted. We propose using 5α-androstane-3β-ol as the internal standard (see Figure 2).

Conclusion

In summary, we have in this paper produced evidence that our testosterone metabolite methodology[5] could be improved and expanded to include metabolites of three other natural C_{19} steroids potentially misused in sport. The metabolites can be separated in a single chromatogram and ^{13}C could be individually measured by IRMS. For testosterone, proof of administration would be a lowered δ value for 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol; for DHT, proof would be lowered δ for 5α-androstane-3α,17β-diol and 5α-androstane-3α,17α-diol but not 5β-androstane-3α,17β-diol; and for epitestosterone, lowered δ for 5α-androstane-3α,17α-diol and 5β-androstane-3α,17α-diol. In the latter case, lowered ^{13}C content may also be expected for the 17β-epimers as anyone misusing epiT is also likely to be self-administering testosterone. DHEA administration would be proved by decreased $\delta^{13}C‰$ value for 5-androstene-3β,17β-diol. Each steroid drug has at least one metabolite separated to baseline so mutual contaminations need not be a concern.

We propose that the comprehensive nature of the methodology and relative simplicity of workup procedure should allow it to be a candidate technique for doping control application. With development it could be used for screening, possibly in place of the classical T/epiT and other diagnostic parameters. Clearly, this study is preliminary and merely illustrative of potential methodologies: extensive controlled studies with variable oral and injectable doses of the natural steroids will be necessary to prove the efficacy of the technique in regulating drug abuse in sports.

Finally, we have shown that the pregnenolone metabolite 5-pregnene-3β,20α-diol can also be analyzed using this technique. While not yet proscribed by sports organizations, pregnenolone is freely available from United States health food stores and may be banned in the future. Forbidding its use would presumably depend on whether its administration increases androgen production. Although pregnenolone synthesized in vivo is the precursor of all gonadal and adrenal steroids, its ingestion is unlikely to significantly raise androgen titers. In our single experiment, we did note that of the C_{19} steroids examined, only 5-androstene-3β,17α-diol showed a significant increase in GC peak size,

a finding that would support evidence suggesting that the compound is a direct metabolite of pregnenolone (i.e., one formed not utilizing the accepted 17α-hydroxypregnenolone pathway). Clearly, detailed studies are necessary on the implications of regular pregnenolone administration to androgen production.

Note added at proof

Since acceptance of this manuscript, we have established that the unexpected DHT metabolite 5α-androstane-3α,17α-diol was essentially formed only after oral drug administration. Intramuscular (DHT heptanoate) and transdermal (Andractim® DHT cream) administration did not result in epimerization, 5α-androstane-3α,17β-diol being the only major nonketonic metabolite. The 17-epimerization occurring after oral anabolic steroid administration remains fascinating, and we are attempting to determine the site of this transformation.

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