Clinical Chemistry 48:4
629–636 (2002)

Drug Monitoring and Toxicology

Detection of Epitestosterone Doping by Isotope Ratio Mass Spectrometry

Rodrigo Aguilera,[1] Caroline K. Hatton,[1] and Don H. Catlin[1,2*]

Background: **Epitestosterone is prohibited by sport authorities because its administration will lower the urinary testosterone/epitestosterone ratio, a marker of testosterone administration. A definitive method for detecting epitestosterone administration is needed.**

Methods: **We developed a gas chromatography–combustion–isotope ratio mass spectrometry method for measuring the $\delta^{13}C$ values for urinary epitestosterone. Sample preparation included deconjugation with β-glucuronidase, solid-phase extraction, and semipreparative HPLC. Epitestosterone concentrations were determined by gas chromatography–mass spectrometry for urines obtained from a control group of 456 healthy males. Epitestosterone $\delta^{13}C$ values were determined for 43 control urines with epitestosterone concentrations ≥40 μg/L (139 nmol/L) and 10 athletes' urines with epitestosterone concentrations ≥180 μg/L (624 nmol/L), respectively.**

Results: **The log epitestosterone concentration distribution was gaussian [mean, 3.30; SD, 0.706; geometric mean, 27.0 μg/L (93.6 nmol/L)]. The $\delta^{13}C$ values for four synthetic epitestosterones were low (less than or equal to −30.3‰) and differed significantly ($P < 0.0001$). The SDs of between-assay precision studies were low (≤0.73‰). The mean $\delta^{13}C$ values for urine samples obtained from 43 healthy males was −23.8‰ (SD, 0.93‰). Nine of 10 athletes' urine samples with epitestosterone concentrations >180 μg/L (624 nmol/L) had $\delta^{13}C$ values within ± 3 SD of the control group. The $\delta^{13}C$ value of epitestosterone in one sample was −32.6‰ (*z*-score, 9.4), suggesting that epitestosterone was administered. In addition, the likelihood of simultaneous testosterone administration was supported by low $\delta^{13}C$ values for androsterone and etiocholanolone.**

Conclusions: **Determining $\delta^{13}C$ values for urinary epitestosterone is useful for detecting cases of epitestosterone administration because the mean $\delta^{13}C$ values for a control group is high (−23.8‰) compared with the $\delta^{13}C$ values for synthetic epitestosterones.**

Epitestosterone (E), the 17α epimer of testosterone, is a naturally occurring steroid found in urine in concentrations similar to those of testosterone. Although epitestosterone was first isolated from human urine in 1964 *(1)*, a physiologic role for epitestosterone has not been established. In humans it is produced in the testes *(2)* and probably by the ovaries *(3)* and adrenals *(4)*, but it has minimal or no androgenic activity *(5)*. Epitestosterone is not available as a pharmaceutical agent, but it can be purchased in bulk from chemical companies.

Epitestosterone is of great interest in the field of doping control because it is the denominator in the testosterone/epitestosterone (T/E)[3] ratio, an indirect marker of testosterone (T) administration. When testosterone is administered, the excretion rate of urinary testosterone increases, the excretion rate of epitestosterone declines *(6)*, and the T/E ratio increases. An increased T/E ratio is also an indirect marker of androstenedione and dehydroepiandrosterone administration *(7, 8)*. If the T/E ratio exceeds 6, doping control laboratories report the case to the sport authorities, who conduct an investigation of the cause of the increased T/E ratio *(9)*.

One technique for circumventing the T/E test is to self-administer epitestosterone *(10)*. Athletes who administer testosterone and therefore have an increased T/E ratio have been reported to administer epitestosterone to rapidly lower the T/E ratio. To thwart this activity, the International Olympic Committee (IOC) classifies epitestosterone as a urine-manipulating agent, and laboratories

[1] UCLA Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology, and [2] Department of Medicine, University of California, Los Angeles, Los Angeles, CA 90025-6106.

*Address correspondence to this author at: UCLA Olympic Analytical Laboratory, 2122 Granville Ave., Los Angeles, CA 90025. Fax 310-206-9077; e-mail dcatlin@ucla.edu.

Received October 18, 2001; accepted December 26, 2001.

[3] Nonstandard abbreviations: T/E, testosterone/epitestosterone; IOC, International Olympic Committee; GC/MS, gas chromatography–mass spectrometry; QC, quality control; and GC/C/IRMS, gas chromatography–combustion–isotope ratio mass spectrometry.

are required to report cases if the urine epitestosterone concentration exceeds 200 μg/L (693 nmol/L). However, unless the urine epitestosterone is exceedingly increased [>1000 μg/L (3467 nmol/L)], it has been difficult to prove in an administrative hearing that epitestosterone has been administered. In part this is attributable to the wide range in concentrations of urinary steroids *(11)*.

It has now been convincingly established that the deviation, in parts per thousand, in ^{13}C content between an unknown and an international standard ($\delta^{13}C$), calculated as {[($^{13}C/^{12}C$) sample − ($^{13}C/^{12}C$) standard]/($^{13}C/^{12}C$) standard} × 1000, where $^{13}C/^{12}C$ refers to the isotope ratio in the sample or an international standard, for pharmaceutical testosterone is low compared with endogenous testosterone and that administration of pharmaceutical testosterone lowers the $\delta^{13}C$ value of urinary metabolites of testosterone *(12–15)*. We therefore investigated the carbon isotope ratio of chemical epitestosterones and determined the $\delta^{13}C$ value of urinary epitestosterone in urine samples obtained from healthy controls and athletes. In addition, we describe the distribution of urine epitestosterone values in a large population of healthy males.

Materials and Methods

CHEMICALS

HPLC-grade methanol was purchased from Fisher Chemicals, acetonitrile from Pierce Chemical Company, and cyclohexane from Fluka. Bakerbond C_{18}, 500-mg solid-phase extraction columns were obtained from JT Baker and used on a 24-port vacuum manifold from Burdick and Jackson. β-Glucuronidase from *Escherichia coli* was supplied by Boehringer Mannheim. Chemical-grade epitestosterone was obtained from four of the six different US vendors: Sigma (lot 100H4021), Research Plus, Inc. (lot 09891), Steraloids, Inc (lot L951), and Alltech, Inc. (lot 163).

INDIVIDUALS PROVIDING URINE FOR EPITESTOSTERONE CONCENTRATIONS

Urines were collected from 456 male, first-year UCLA medical students between the ages of 20 and 38 from six consecutive classes (years 1996–2001), who were enrolled in a laboratory course in biological chemistry. The experiment was conducted under the oversight of the Human Subjects Protection Committee. The students had the option to give a medical and drug history and to declare their ethnicity. No student declared taking steroids or reported any chronic disease. The ethnicity choices were Caucasian, Asian, black, Hispanic, and other. The students collected 24-h urine samples beginning with the first voided morning urine. All urines were stored at 4 °C during collection and at −4 °C until analysis. The pH was measured to the nearest half-unit with a dipstick, the specific gravity to the nearest thousandth with a refractometer, and the creatinine by HPLC *(16)*. Two of the 24-h volumes were <500 mL, probably because one or more urinations were missed. However, the pH, specific gravity, and analytical data gave no reason to question the authenticity of the urines; thus, none were excluded. None of the urines in the study was found to contain exogenous androgens.

STUDENTS AND ATHLETES PROVIDING URINE FOR EPITESTOSTERONE $\delta^{13}C$ VALUES

The controls were a subset of the group described above and included 43 medical students between the ages of 21 and 31 from the 1999–2001 school years. The urines were selected because they contained ≥40 μg/L (≥139 nmol/L) epitestosterone. In addition, 10 urine samples from the athlete testing program that our laboratory conducts for various sport agencies were analyzed. These samples were selected because their epitestosterone concentrations were at least 180 μg/L (624 nmol/L) and they were available at the time of the study.

SAMPLE PREPARATION AND GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS FOR DETERMINATION OF URINE STEROID CONCENTRATIONS

The concentrations of testosterone, epitestosterone, and other endogenous steroids were estimated from a urine steroid screen *(13)*. Sample preparation included centrifugation if solids were present, the addition of [16,16,17-$^2H_3$]testosterone to 2.5 mL of urine to give a concentration of 40 μg/L (139 nmol/L), solid-phase extraction (Bond-Elut 57-μm C_{18} cartridge; 3 mL/500 mg; Varian Associates), enzymatic hydrolysis with 50 μL of β-glucuronidase from *E. coli* (Roche Diagnostics; lot 1585665; >200 kU/L at 37 °C), and extraction with diethyl ether. After evaporation of the solvent, the trimethylsilyl ethers and enol ethers of the compounds of interest were prepared by reconstituting in 50 μL of MSTFA–NH_4I–dithioerythritol (1000 μL:1.8 mg:4.5 mg) and incubating at 60 °C for 20 min. The gas chromatographic–mass spectrometric analysis was conducted with Hewlett-Packard gas chromatography–mass spectrometry (GC/MS) systems in the selected-ion monitoring mode as described previously *(13, 17)*. This analysis quantifies free steroids and glucuronides together. The T/E ratio was determined from the peak height ratio of testosterone/epitestosterone (*m/z* 432). The screen includes positive and negative quality-control (QC) samples whose estimated T/E ratios must fall within tolerance ranges and are monitored on QC charts. The concentration of epitestosterone was calculated from the peak height of *m/z* 435 of [16,16,17-$^2H_3$]testosterone ($T_{ph}435$) and the peak height of *m/z* 432 of epitestosterone ($E_{ph}432$) by the following formula: epitestosterone concentration = ($E_{ph}432$) (40 μg/L)/($T_{ph}435$). The overall recovery of epitestosterone in this method exceeds 90% *(18)*. Two QC urines containing testosterone at 10 and 100 μg/L, analyzed 150 times over 2 weeks, gave CVs of 10% and 13%, respectively. Steroid sulfates were not targeted.

sample preparation for epitestosterone gas chromatography–combustion–isotope ratio mass spectrometry (gc/c/irms) analysis

To 5 mL of urine we added 1.0 mL of 0.2 mol/L phosphate buffer (pH 7.0) and 100 μL of β-glucuronidase from *E. coli*. Following incubation for 1 h at 60 °C, the hydrolysate was poured into a solid-phase extraction column (Bakerbond C_{18} Octadecyl; 500 mg; 6 mL; Mallinckrodt Baker) conditioned with 6 mL of methanol followed by 6 mL of water, washed with 6 mL of water, and eluted with 6 mL of acetonitrile. The eluate containing the free steroids was dried under a nitrogen stream (Turbo Vap LV evaporator; Zymark), redissolved in 200 μL of acetonitrile, and injected on a semipreparative C_{18} column (Sphereclone ODS2; 250 × 10 mm; 5-μm bead size; Phenomenex). The acetonitrile–water (46:54 by volume) mobile phase was delivered by a HP 1090 liquid chromatograph at a flow rate of 3.8 mL/min. The eluate was monitored at 240 nm. The epitestosterone fraction was collected at the retention time observed for a calibrator (± 1 min; typically 19.5–21.5 min), evaporated to dryness, reconstituted in 30 μL of cyclohexane, and transferred to an autosampler vial. Nineteen of the samples (35%) were analyzed by GC/MS to check the identity and purity of the epitestosterone.

gc/c/irms analysis

The GC/C/IRMS analysis was performed on a Finnigan Delta Plus isotope ratio mass spectrometer connected to a HP Model 6890 gas chromatograph via a Finnigan Combustion III interface. The GC column, inlet, and oven temperatures were the same as for the above GC/MS system. The IRMS conditions have been described *(13)*. In addition, we measured the $\delta^{13}C$ values for acetylated androsterone and etiocholanolone as described previously *(13)*.

qc and precision

QC and precision studies were performed on two urine samples, designated negative QC (Neg-QC) and positive QC (Pos-QC). The epitestosterone concentration (free plus glucuronide) in the Neg-QC was 130 μg/L (451 nmol/L). The Pos-QC was prepared by extracting the steroids on a XAD-2 column from a 2-L pool of female urine and adding epitestosterone (Sigma; lot 18F-4057; $\delta^{13}C = -34.0‰$). The QC urines were prepared and stored in 5-mL aliquots at −20 °C until analysis. The within-assay precision of the method was determined by preparing three aliquots each of Pos-QC and Neg-QC and injecting each once. The between-assay precision of the method was determined by extracting one aliquot of Pos-QC and Neg-QC per day for 4 days and injecting each once.

statistics

The normality of the distributions was assessed with the Anderson–Darling (A^2) test, and the distributions were compared using the general linear model with the Duncan multiple range test *(19)*. The $\delta^{13}C$ values for the chemical-grade epitestosterones were compared using the general linear model. The log-power transformation *(20)* was applied to the concentrations of urine epitestosterone per milligram of creatinine.

Results

control group urine epitestosterone concentrations

The percentile distributions of epitestosterone concentrations are shown in Table 1. Of the 456 members of the control group, 36.4% were Caucasian, 21.7% were Asian, 19.3% were Hispanic, and 7.5% were black. Of the remaining 15% (n = 69), 46 gave their ethnicity as "other", and 23 did not declare any ethnicity. These two groups were combined and listed as "unidentified" in Table 1. Table 1 also shows the percentile distribution of ng epitestosterone/mg creatinine (ng-E/mg-cr) for all individuals. The distribution of urinary epitestosterone was not gaussian (Fig. 1, top panel), but as shown in the bottom panel of Fig. 1, the distribution of the natural log of epitestosterone concentrations (ln[E]) was gaussian ($A^2 = 0.52$; $P = 0.19$). The mean urinary ln[E] was 3.30 (SD, 0.706). The geometric mean was 27.0 μg/L (93.6 nmol/L). Compared with this distribution, the IOC cutoff for reporting epitestosterone cases [200 μg/L (693 nmol/L)] is 2.8 *z*-scores greater than the mean. There were no differences in the ln[E] distributions of the five ethnic groups shown in Table 1 ($P < 0.19$). The distributions of ng-E/mg-cr and ln[ng-E/mg-

Table 1. Distribution of urinary epitestosterone concentrations (μg/L) by ethnicity.[a]

Percentile	All	Asian	Black	Caucasian	Hispanic	Unidentified[b]
10	11 (10)	11 (9)	8 (7)	11 (10)	13 (11)	9 (9)
25	17 (14)	16 (14)	17 (10)	17 (16)	18 (14)	17 (12)
50	29 (21)	27 (19)	25 (19)	29 (24)	29 (23)	26 (21)
75	45 (32)	41 (28)	52 (25)	45 (35)	47 (31)	43 (30)
90	68 (44)	61 (36)	77 (44)	65 (46)	69 (45)	68 (42)
Minimum	3 (3)	3 (4)	5 (5)	5 (5)	4 (3)	6 (6)
Maximum	162 (91)	116 (91)	108 (74)	126 (82)	162 (73)	152 (56)
n	456	99	34	166	88	69

[a] Values in parentheses are ng urinary epitestosterone/mg creatinine.
[b] Unidentified, unknown + other.

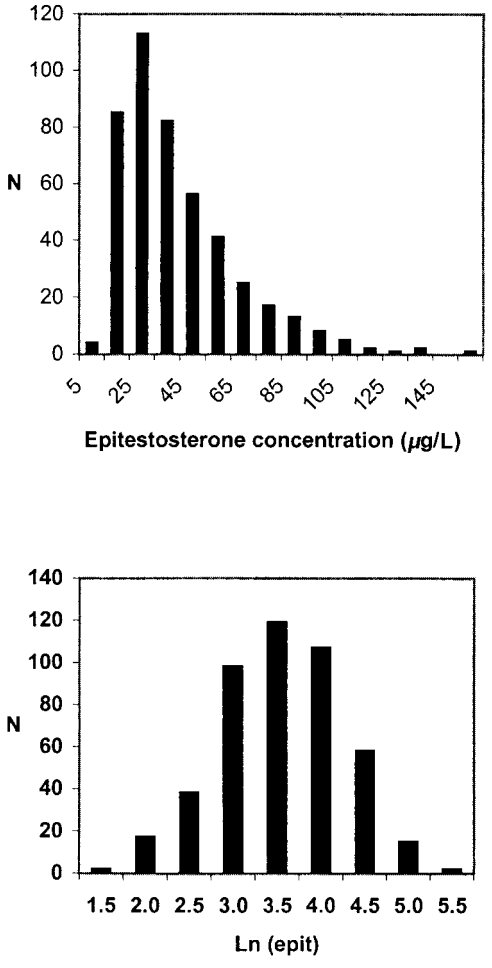

Fig. 1. Distribution of urine epitestosterone concentrations (*top*) and natural log of urine epitestosterone concentrations (*bottom*) for 456 male medical students.

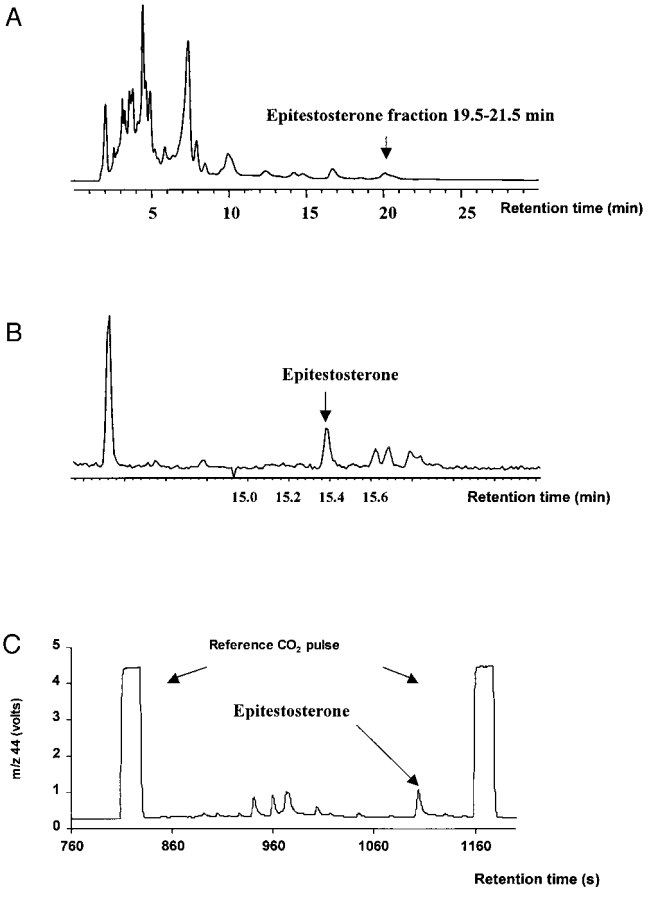

Fig. 2. Chromatograms of urine extract.

(*A*), HPLC chromatogram of a representative urine extract (diode array/ultraviolet detection) after semipreparative clean-up. (*B*), GC/MS total-ion chromatogram of the urine extract HPLC fraction shown in *A*. (*C*), GC/C/IRMS chromatogram of the urine extract HPLC fraction shown in *A*.

cr] were not gaussian, but the log-power transformation of ng-E/mg-cr normalized the data ($A^2 = 0.20$; $P = 0.25$). The center of the distribution ($z = 0$) corresponded to 21.6 ng-E/mg-cr, and the log-power distribution parameters *(19)* were $x_t = 3.234$; $S_{xt} = 0.478$; $C = 3.790$; and $\kappa = 1.053$.

GC/MS identification and GC/C/IRMS analysis of urinary epitestosterone

Shown in Fig. 2A is a HPLC chromatogram of a representative urine extract; the GC/MS total-ion chromatogram of the corresponding HPLC fraction is shown in Fig. 2B. Urinary epitestosterone was identified by matching retention time (± 2%) and mass spectrum (ion ratios, ± 20%) with those of a calibrator. The chromatogram shows a symmetrical peak and no evidence of coeluting compounds. Fig. 2C shows the GC/C/IRMS chromatogram of the HPLC fraction. The carbon dioxide peak corresponding to the combusted epitestosterone peak eluted at 1101 s. Its shape was symmetric with no tailing.

$\delta^{13}C$ values for synthetic epitestosterone

As shown in Table 2, the $\delta^{13}C$ values obtained for epitestosterone from four different chemical suppliers ranged from −30.3‰ to −34.9‰. The epitestosterone $\delta^{13}C$ value was higher for the compound obtained from vendor A than for those obtained from the other three suppliers ($P < 0.0001$). There were no significant differences in the $\delta^{13}C$ values for epitestosterone from vendors B, C, and D ($P > 0.08$).

Table 2. Mean $\delta^{13}C$, SD, and CV for synthetic epitestosterones obtained from four chemical vendors.

	Vendor			
	A	B	C	D
Mean,[a] ‰	−30.3	−34.4	−34.5	−34.9
SD, ‰	0.27	0.29	0.18	0.45
CV, %	0.89	0.82	0.53	1.3

[a] Mean of five samples of each epitestosterone.

Table 3. Within- and between-assay precision of the negative and positive QC samples.

Within-assay precision			Between-assay precision		
	δ^{13}C, ‰			δ^{13} C, ‰	
Aliquot	Neg-QC	Pos-QC	Day	Neg-QC	Pos-QC
1	−23.0	−33.1	1	−21.1	−33.5
2	−23.3	−33.9	2	−22.7	−33.8
3	−22.4	−33.9	3	−22.4	−34.3
			4	−22.6	−33.1
Mean	−22.9	−33.6		−22.2	−33.7
SD	0.44	0.46		0.73	0.52
CV, %	1.9	1.4		3.3	1.6
Max-Min[a]	0.87	0.81		1.55	1.25

[a] Maximum − minimum.

QC and precision

The descriptive statistics for the within- and between-assay precision experiment using Neg-QC and Pos-QC are shown in Table 3. The within-assay SDs for Neg-QC and Pos-QC were ± 0.44‰ (CV = 1.9%) and ± 0.46‰ (CV = 1.4%), respectively. The between-assay SDs for Neg-QC and Pos-QC were ± 0.73‰ (CV = 3.3%) and ± 0.52‰ (CV = 1.6%), respectively. For the within- and between-assay precision, the mean δ^{13}C values for epitestosterone in the Neg-QC were −22.9‰ and −22.2‰, respectively. For the Pos-QC, the corresponding values were −33.6‰ and −33.7‰, respectively.

Control group δ^{13}C values and epitestosterone concentrations

In the urines obtained from the 43 healthy students with epitestosterone concentrations ≥40 μg/L (139 nmol/L), the mean δ^{13}C value of epitestosterone for all was −23.8‰ (range, −21.8‰ to −25.6‰; SD, 0.93; Table 4), and these data were normally distributed ($A^2 = 0.42$; $P = 0.25$). In addition, Table 4 shows the means, SDs, and CVs for the students as a function of their declared ethnicity. The mean δ^{13}C values for the four ethnic groups ranged from −23.2‰ (blacks) to −24.2‰ (Caucasian) and did not differ significantly ($P = 0.27$). The range of epitestosterone concentrations was 43–138 μg/L (149–478 nmol/L; *z*-scores, 0.7–2.3). The median urinary T/E ratio was 0.58 (range, 0.06–2.04).

δ^{13}C values in athletes' urines with high epitestosterone concentrations

Shown in Table 5 are summaries of the epitestosterone and testosterone concentrations, T/E ratios, and δ^{13}C values for urines from the 10 male athletes with urinary epitestosterone concentrations >180 μg/L (>624 nmol/L). The median concentration of epitestosterone in these urines was 249 μg/L (863 nmol/L) [range, 180-1176 μg/L (624–4077 nmol/L)]. The median testosterone concentration and T/E ratio were 71 μg/L (range, 8–1183 μg/L) and 0.30 (range, 0.04–1.11), respectively. Except for athlete 10, the epitestosterone δ^{13}C values were similar to those of the control group and within the range of ± 3 SD (−26.6‰ to −21.0‰). The epitestosterone δ^{13}C value for athlete urine 10 was very low (−32.6‰) and 9.4 *z*-score units below the mean of the control group.

Table 4. Urinary epitestosterone δ^{13}C values for 43 healthy male controls.

	All	Hispanic	Caucasian	Black	Asian
Mean, ‰	−23.8	−23.6	−24.2	−23.2	−23.8
SD, ‰	0.93	0.98	0.74	0.68	1.43
CV, %	3.9	4.1	3.1	2.9	6.0
n[a]	43	16	15	5	4
Minimum	−25.6	−25.6	−25.2	−24.2	−25.3
Maximum	−21.8	−22.3	−22.4	−22.6	−21.8
Range	3.8	3.4	2.8	1.6	3.5

[a] The ethnicity of three individuals was not declared.

δ^{13}C values for acetylated androsterone and etiocholanolone from athletes' urines with high epitestosterone concentrations

The urinary androsterone and etiocholanolone δ^{13}C values for the athletes were measured to determine the likelihood that testosterone or a related steroid was administered *(13)*. Table 5 shows the δ^{13}C values for 7 of these 10 samples. There was insufficient volume to perform the IRMS analysis on the other three urines. Except for athlete 10, the δ^{13}C values for acetylated etiocholanolone (range, −20.7‰ to −23.3‰) and androsterone (range, −20.2‰ to −21.9‰) were within the reference interval *(13)*. For athlete 10, the δ^{13}C values for etiocholanolone acetate (−30.7‰) and androsterone acetate (−31.4‰) were in the range found in testosterone users *(13)*.

Discussion

The quality of GC/C/IRMS measurements depends first on chromatographic peak purity. In this assay, the epitestosterone peak was separated from all other peaks, and its shape was symmetric. Peak purity was achieved by optimizing an extraction method that included semi-preparative HPLC fractionation, and peak purity was checked by GC/MS analysis. The within- and between-assay imprecision of the two QC samples was very low (CV <4%) and in excellent agreement with imprecision data for our diol and androsterone/etiocholanolone IRMS studies *(12, 13)*. All samples tested produced a sufficient IRMS response with only 5 mL of urine; thus, the method is suitable for doping control where a limited volume of urine is available. It takes ~2 days to process one batch of samples, but the turnaround time can be shortened to one 18-h day. Most IRMS assays for steroids determine the δ^{13}C value of derivatized steroids *(12–15, 21)*. In this assay, we were able to obtain the δ^{13}C values on underivatized epitestosterone. Derivatization was not necessary to improve peak symmetry and resolution.

Table 5. Concentrations of epitestosterone and testosterone, T/E ratios, and δ^{13} values for epitestosterone, etiocholanolone acetate, and androsterone acetate in 10 urine samples obtained from athletes whose drug-taking behavior was unknown.

	Epitestosterone				δ^{13}C values		
Athlete	μg/L[a]	ng/mg-cr	T/E	Testosterone, μg/L	E[b] (*z*-score[c])	Etio	Andro
1	180	42	0.31	53	−23.6 (−0.1)	−22.0	−21.1
2	203	64	0.04	8	−23.3 (−0.5)	−23.3	−21.9
3	218	55	0.43	89	−21.3 (−2.6)	−20.8	−20.4
4	219	72	0.10	17	−23.4 (−0.4)	−20.7	−20.4
5	248	67	0.20	45	−23.3 (−0.5)	−21.9	−21.1
6	251	NA[d]	0.60	139	−26.1 (2.5)	NA	NA
7	324	NA	0.15	43	−23.0 (−0.8)	NA	NA
8	328	97	0.61	155	−22.2 (−1.7)	−21.4	−20.2
9	405	NA	0.32	116	−22.3 (−1.5)	NA	NA
10	1176	426	1.11	1183	−32.6 (9.4)[e]	−30.7	−31.4

[a] (μg/L)/(288.42/1000) = SI units (nmol/L).
[b] E, epitestosterone; Etio, etiocholanone acetate; Andro, androsterone acetate.
[c] *z*-score for Eδ^{13}C value: [control group mean (−23.8‰) − sample value]/SD of control group.
[d] NA, no urine available for analysis.
[e] Value for athlete 10 was very low.

The δ^{13}C values for samples of epitestosterone obtained from four different chemical companies ranged from −30.3‰ to −34.9‰. These values were similar to those in previous reports on synthetic steroids *(14, 21–23)*. It is possible that the epitestosterone obtained from vendors B, C, and D came from a common source. The mean epitestosterone δ^{13}C value for the control group of 43 students was −23.8‰ (SD, 0.93‰), which is similar to the value reported (−23.3‰) for underivatized urinary testosterone *(22)*. The low SD yields a relatively narrow ± 3 SD range (−21.0‰ to −26.6‰). In sport drug testing, it is typical to require a positive case to be at least +3 SD from the mean. Thus, in testing for potential epitestosterone administration, the critical value is the difference between the control group mean −3 SD and the δ^{13}C values for the epitestosterone administered. This difference, which ranges from 3.7‰ to 8.3‰, is relatively large, indicating that the assay will be useful for detecting doping with epitestosterone.

The optimal approach for establishing the clinical validity of an epitestosterone detection scheme is to administer epitestosterone to healthy volunteers and analyze urine samples collected at various times. Because epitestosterone is not available in the US as an approved pharmaceutical, it is not possible to perform such a study unless an investigational new drug permit is obtained from the Food and Drug Administration. Currently, obtaining an investigational new drug permit is not an option. We therefore determined the δ^{13}C values for epitestosterone in urine samples obtained from selected athletes. These samples come to us for routine sport testing, and we have no information on the histories of the athletes who provide them. Because the steroid screening analysis estimates the concentration of epitestosterone, we selected samples for IRMS analysis that had high epitestosterone concentrations.

The concentration of epitestosterone in the urine of athlete 10 [1176 μg/L (4077 nmol/L)] is the highest we have encountered in the latest 45 000 samples from athletes tested. In addition, the epitestosterone δ^{13}C value of −32.6‰, the lowest observed in this study, is 9.4 *z*-score units above the mean of the control group. These data strongly suggest that athlete 10 recently administered synthetic epitestosterone. If an athlete takes synthetic testosterone or a steroid that is metabolized to androsterone and etiocholanolone, the δ^{13}C values for androsterone and etiocholanolone acetate are −30.0‰ or lower *(13)*; thus the values in Table 4 (−31.4‰ and −30.7‰) strongly suggest that athlete 10 was also taking testosterone. Only 2% of administered epitestosterone is recovered in urine as androsterone and etiocholanolone *(4)*. Thus, the low δ^{13}C values are not likely to be attributable to metabolism of epitestosterone to androsterone and etiocholanolone. In addition, this athlete's T/E ratio (1.1) was the same as the median of 3710 male athletes *(9)*; thus, his very high epitestosterone (1176 μg/L) was balanced by an equally high testosterone (1183 μg/L), which is a additional indication that he was taking testosterone. There is no evidence that testosterone is metabolized to epitestosterone *(6, 24)*; thus it is most unlikely that the epitestosterone δ^{13}C values are influenced by testosterone administration.

The epitestosterone concentrations in the urine of the remaining nine athletes (athletes 1–9 in Table 5) were all >180 μg/L (624 nmol/L), but for eight of the nine, the epitestosterone δ^{13}C value *z*-scores were negative. In other words, the values were greater than the mean of the control group. Athlete 6 was suspicious for exogenous

epitestosterone use because his epitestosterone concentration was 251 μg/L (870 nmol/L) and his δ^{13}C value was −26.1‰. The latter, however, was within ± 3 SD (−21.0‰ to −26.6‰) of the control group mean. Thus, the only evidence that any of the other athletes (athletes 1–5 and 7–9) used epitestosterone is the high concentration of epitestosterone. This indicates that the IOC epitestosterone cutoff flags samples that might not represent epitestosterone abuse. Use of an epitestosterone/creatinine cutoff might offer an improvement. The IOC requires laboratories to report cases with epitestosterone >200 μg/L (693 nmol/L) so that further investigation may be conducted. This situation is satisfactory provided that sanctions are not automatically taken in cases in which the epitestosterone concentration is >200 μg/L (693 nmol/L) and no δ^{13}C value is available.

There are no other publications on the detection of epitestosterone administration by determining epitestosterone δ^{13}C values. In a study involving one participant who received 50 mg of epitestosterone, the δ^{13}C value of a diol metabolite (measured as the diacetate) of epitestosterone was highly negative *(15)*. In addition, after the administration of testosterone enanthate to cattle, the δ^{13}C value of diacetates of urine epitestosterone fell from approximately −25‰ to −30‰ and remained low for more than 16 days (unlike humans, cattle metabolize testosterone to epitestosterone) *(25)*. The time course of urinary epitestosterone δ^{13}C values after epitestosterone administration in humans has not been reported.

This IRMS method for determining the δ^{13}C values for urinary epitestosterone was developed to provide much needed additional support for the detection of doping with epitestosterone. Epitestosterone has no clinical use (other than to lower the urine T/E ratio) and is not available as a pharmaceutical; therefore, a urine sample with an epitestosterone concentration >200 μg/L (693 nmol/L) and a δ^{13}C value lower than −26.6‰ (control group mean − 3 SD) provides convincing evidence of epitestosterone doping. Such cases are likely to also have low δ^{13}C values for urinary testosterone metabolites, which reflect doping with testosterone or another steroid that is metabolized to testosterone. In addition, we have provided a detailed description of the distribution of urine epitestosterone concentrations in healthy controls, which is useful for interpreting high concentrations of urinary epitestosterone.

We thank Kathleen Schramm for superb technical assistance and Boro Starcevic, Gary Green, Andreas Breidbach, and Brian Ahrens for scientific advice. This study was supported in part by a grant from the sport consortium consisting of the National Collegiate Athletic Association, the National Football League, and the United States Olympic Committee.

References

1. Korenman SG, Wilson H, Lipsett MB. Isolation of 17-α-hydroxyandrost-4-en-3-one (epitestosterone) from human urine. J Biol Chem 1964;239:1004–6.
2. Dehennin L. Secretion by the human testis of epitestosterone, with its sulfoconjugate and precursor androgen 5-androstene-3β,17α-diol. J Steroid Biochem Mol Biol 1993;44:171–7.
3. Acevedo HF, Corral-Gallardo J. Epitestosterone: an in vitro metabolite of delta-4-androstenedione in a sclerocystic ovary. J Clin Endocrinol Metab 1965;25:1675–7.
4. Wilson H, Lipsett MB. Metabolism of epitestosterone in man. J Clin Endocrinol Metab 1966;26:902–14.
5. Dorfman RI, Shipley RA. Androgens. New York: John Wiley & Sons, 1956:124pp.
6. Dehennin L, Matsumoto AM. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. J Steroid Biochem Mol Biol 1993;44:179–89.
7. Uralets VP, Gillette PA. Over-the-counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3β,17β-diol; and 19-nor-4-androsten-3,17-dione: excretion studies in men. J Anal Toxicol 1999; 23:357–66.
8. Bowers LD. Oral dehydroepiandrosterone supplementation can increase the testosterone/epitestosterone ratio. Clin Chem 1999;45:295–7.
9. Catlin DH, Hatton CK, Starcevic SH. Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athletes' urine. Clin Chem 1997;43:1280–8.
10. Dehennin L. Detection of simultaneous self-administration of testosterone and epitestosterone in healthy men. Clin Chem 1994;40:106–9.
11. Brooks RV, Jeremiah G, Webb WA, Wheeler M. Detection of anabolic steroid administration to athletes. J Steroid Biochem 1979;11:913–7.
12. Aguilera R, Chapman TE, Starcevic B, Hatton CK, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with testosterone based on urine diols: controls and athletes with increased testosterone/epitestosterone ratios. Clin Chem 2001;47:292–300.
13. Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary androsterone and etiocholanolone δ^{13}C (‰) values by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 2000;14: 2294–9.
14. Shackleton CH, Phillips A, Chang T, Li Y. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids 1997;62:379–87.
15. Shackleton CH, Roitman E, Phillips A, Chang T. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: potential use in gas chromatography isotope ratio mass spectrometry. Steroids 1997;62:665–73.
16. Catlin DH, Starcevic B. HPLC method for the assay of creatinine in urine. J Liq Chromatogr 1991;14:2399–408.
17. Catlin DH, Kammerer RC, Hatton CK, Sekera MH, Merdink JM. Analytical chemistry at the Games of the XXIIIrd Olympiad in Los Angeles 1984. Clin Chem 1987;33:319–27.
18. Catlin DH, Leder BZ, Ahrens B, Hatton CK, Finklestein JS. Epitestosterone: precursor, metabolites, and effect of androstenedione administration. Steroids 2002:in press.
19. SAS/Stat guide for personal computers, Ver. 8.1. Cary, NC: SAS Institute, 1992.
20. Boyd JC, Lacher DA. A multi-stage gaussian transformation algorithm for clinical chemistry data. Clin Chem 1982;28:1735–41.

21. Ueki M, Okano M. Analysis of exogenous dehydroepiandrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 1999; 13:2237–43.

22. Horning S, Geyer H, Machnic M, Hilkert A, Oebelman J. Detection of exogenous testosterone by $^{13}C/^{12}C$ analysis. In: Donike M, Geyer H, Gotzmann, eds. Proceedings of the 14th Cologne workshop on dope analysis (4). Köln, Germany: Sport und Buch Strauss, 1996:275–84.

23. de la Torre X, Gonzalez JC, Pichini S, Pascual JA, Segura J. $^{13}C/^{12}C$ isotope ratio MS analysis of testosterone, in chemicals and pharmaceutical preparations. J Pharm Biomed Anal 2001;24: 645–50.

24. Donike M, Barwald KR, Klostermann K, Schänzer W, Zimmermann J. Nachweis von exogenem Testosteron. In: Heck H, Hollmann W, Liesen H, eds. Sport: Leistung und Gesundheit, Köln, Germany: Kongressbd. Dtsch Sportarztekongress, Deutscher Ärzte-Verlag, 1983:293–8.

25. Ferchaud V, Le Bizec B, Monteau F, Andre F. Characterization of exogenous testosterone in livestock by gas chromatography/ combustion/isotope ratio mass spectrometry: influence of feeding and age. Rapid Commun Mass Spectrom 2000;14:652–6.