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Stable isotope ratio profiling of testosterone preparations

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Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is the preferred method of confirming the administration of exogenous testosterone by athletes. This relies on synthetic testosterone preparations being depleted in ^{13}C compared to natural testosterone. There is concern, however, about the existence of synthetic testosterone products that are unexpectedly ^{13}C-enriched and which may allow athletes to circumvent the current GC-C-IRMS test. Further to the reported studies of legitimate pharmaceutical-grade testosterone products, a detailed analysis of seized materials from border-level seizures was required to obtain intelligence concerning trends in 'black market' testosterone manufacture and distribution. The sample set collected for this study between 2006 and 2009 inclusive provided a $\delta^{13}C$ range (n=266) of −22.9‰ to −32.6‰ with mean and median values of −28.4‰ and −28.6‰, respectively. Within this distribution there were 24 samples (9%) confirmed to have $\delta^{13}C$ values in the range reported for endogenous urinary steroid metabolites (≥−25.8‰). The benefit of $\delta^{13}C$ profiling for testosterone preparations was demonstrated by the ability to identify specific seized products that can be target tested for future intelligence purposes.

In addition, the potential of stable hydrogen isotope ratio ($^2H/^1H$; δ^2H) discrimination to complement $\delta^{13}C$ analysis was investigated. Methodologies for the determination of δ^2H values by gas chromatography-thermal conversion-isotope ratio mass spectrometry (GC-TC-IRMS) were developed to provide a δ^2H range (n=173) of −177‰ to −268‰ with mean and median values of −231‰ and −234‰, respectively.

Keywords: testosterone (T); stable carbon isotope ratio ($\delta^{13}C$) analysis; gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS); stable hydrogen isotope ratio (δ^2H) analysis; gas chromatography-thermal conversion-isotope ratio mass spectrometry (GC-TC-IRMS)

Introduction

Despite the large number of synthetic steroids that are abused by athletes in their quest to enhance sporting performance, testosterone (androst-4-ene-17β-ol-3-one; Figure 1) remains a potential performance-enhancing drug within the world athletic community.[1] Synthetic testosterone has been commercially manufactured and legitimately marketed for more than 50 years for medical use.[2] Widespread production of testosterone has made it a simple matter for substantial amounts to be diverted for use as a performance-enhancing drug. Sophisticated analytical methods centred on gas chromatography-mass spectroscopy (GC-MS) have been most successful at identifying and quantifying testosterone together with its key metabolites in an athlete's urine samples. However, a frequently used defense by the athlete is that the testosterone levels detected are natural variations in endogenous testosterone production.

It has been known for some time that the stable isotope ratios of carbon ($^{13}C/^{12}C$; $\delta^{13}C$) in human endogenous testosterone differ from those in commercial synthetic testosterone products. The first demonstration of the differences between the ^{13}C content in gonadal testosterone and pharmaceutical testosterone preparations was presented by Southan *et al*.[3] The first use of this to detect testosterone abuse was published by Becchi *et al*.[4] The use of commercial gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) commenced after 1990 and was first used in Olympic doping control for the Atlanta Summer (1996) and the Nagano Winter (1998) games. The basis for the success of GC-C-IRMS in distinguishing endogenous from exogenous testosterone rests on the assumption that the steroid synthesized by a mammalian testis has a different range of stable carbon isotope values than synthetic analogues derived from *stigmasterol* and *sitosterol*; plant sterols obtained from ^{13}C depleted soy (*Glycine spp.*).[5] Work by Ueki and Okano[6] to validate their GC-C-IRMS method prior to the Nagano Winter Olympics examined 12 testosterone preparations from a variety of manufacturers. They found a $\delta^{13}C$ range of −26.2‰ to −32.8‰ with a mean value of −28.9‰. A similar study by de la Torre *et al*.[7] on 10 testosterone preparations gave a range of −25.9‰ to −32.8‰ with a mean of −28.9‰.

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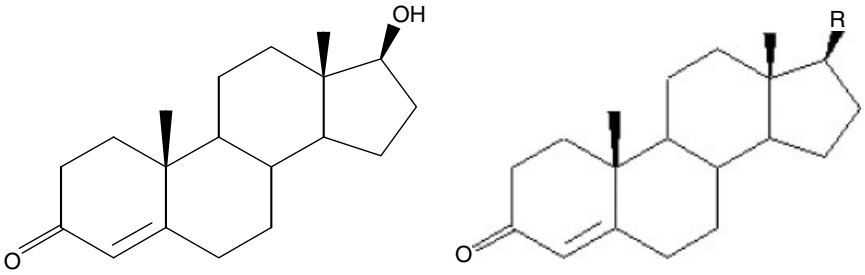
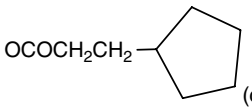


Figure 1. Structure of testosterone (left; androst-4-en-17β-ol-3-one) and testosterone esters (right). **R** = **$OCO(CH_2)_5CH_3$** (enanthate)
$OCO(CH_2)CH_3$ (propionate)
$OCO(CH_2)_8CH_3$ (decanoate)
$OCOCH_2CH_2CH(CH_3)_2$ (isocaproate).



(cypionate).

Today, laboratories accredited by the World Anti-Doping Agency (WADA) measure a number of parameters when determining whether or not testosterone abuse has occurred. These comprise the: (1) testosterone glucuronide to epitestosterone glucuronide ratio (T/E); (2) androsterone and etiocholanolone levels; (3) the dehydroepiandrosterone glucuronide level; (4) the $\delta^{13}C$ values of androsterone, etiocholanolone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol; and (5) the respective $\Delta\delta^{13}C$ values to endogenous reference compounds.[8] Since 2004, WADA has set the limit for $\delta^{13}C$ values of target steroids at less than -28.0‰ and the $\Delta\delta^{13}C$ limit at >3.0‰. These values have been set largely based on the earlier work by Ueki and Okano[6] and de la Torre *et al.*[7] These isotopic profiling surveys of testosterone preparations are now 10 years old and no more-recent studies have been reported. During this time there has been the release of testosterone gel products in the USA (2000) and Europe (2003) for replacement therapy in men with conditions of low endogenous testosterone production. This accompanied evidence of testosterone gel application increasing lean body mass, muscle strength, and haemoglobin concentrations.[9] Results of a study with hypogonadal men showed the potential difficulty in detecting misuse of testosterone gel products with the available methods of doping control.[10] Moreover, any recent changes in chemical and pharmaceutical industries, such as new source materials for steroid manufacture which change the $\delta^{13}C$ values of pharmaceutical testosterone would have profound consequences for the use of GC-C-IRMS as a confirmatory technique for testosterone abuse in athletes.

This article will first describe the updated measurement of $\delta^{13}C$ values for a selection of legitimate testosterone products with comparison to previous reports. Second, seized testosterone preparations submitted to the National Measurement Institute by the Australian Customs and Border Protection Service during 2006–2009 inclusive were profiled for $\delta^{13}C$ values. In addition, hydrogen is another element for which the potential of isotopic signatures ($^2H/^1H$; δ^2H) to further discriminate endogenous and synthetic testosterone preparations are being investigated by doping control laboratories.[11] As the relative mass difference between ^2H and ^1H is larger than between ^{13}C and ^{12}C it can be expected that the signature will be more pronounced.[12–14] Methodology for the determination of δ^2H values by GC-Thermal Conversion (TC)-IRMS will be presented for the profiling of legitimate and seized testosterone preparations.

Experimental

Reagents and chemicals

CU/USADA 30-1 (5α-androstane-3β-ol, $\delta^{13}C = -29.7 \pm 0.1$‰) was provided by Thomas Brenna (Department of Nutritional Sciences, Cornell University, Ithica, NY, USA). Testosterone reference materials were obtained from Steraloids (Newport, RI, USA) and the National Measurement Institute Chemical Reference Materials Collection (Sydney, NSW, Australia). Testosterone cypionate, testosterone decanoate, testosterone enanthate, testosterone propionate and methyltestosterone were obtained from the National Measurement Institute Chemical Reference Materials Collection (Sydney, NSW, Australia). Potassium carbonate and potassium hydrogen carbonate were purchased from BDH chemicals (Poole, UK). Trimethylchlorosilane (TMCS) was purchased from Grace (Deerfield, PA, USA). Carbon dioxide (>99.9%), ultra high-purity helium (>99.999%), high-purity oxygen (>99.5%) and hydrogen (>99.5%) were purchased from BOC gases (Sydney, NSW, Australia). HPLC grade acetonitrile, hexane and methanol were purchased from Merck (Darmstadt, Germany). Water was obtained from a Millipore filtration system capable of 10 MΩ/cm^3 or better.

Pharmaceutical and veterinary testosterone preparations

Fourteen commercial pharmaceutical testosterone preparations were analyzed as part of this study (Table 1). *Sustanon 250*® ($\delta^{13}C = -29.9 \pm 0.3$‰; n=59) produced by Organon in the Netherlands (Batch #612 603, Expiry 09–2008) containing a mix of testosterone isocaproate, propionate, decanoate and phenylpropionate esters was analyzed as a quality control (QC) to verify precision of the acid hydrolysis method. Six legitimate veterinary testosterone preparations (Table 2) were collected by the Australian Sports Drug Testing Laboratory.

High performance liquid chromatography (HPLC) purity determination of testosterone preparations

The method, adapted from Walters *et al.*,[15] homogenized the sample by careful shaking prior to sub-sampling. An accurate

Table 1. GC-C-IRMS results (mean from n=4, S.D = 0.1‰ to 0.2‰) for legitimate pharmaceutical testosterone products surveyed. 5α-androstane-3β-ol (INSTD) analyzed with each injection provided a mean $\delta^{13}C$ value of −29.8‰ with a SD of 0.2‰ (n=56)

Product	Ester *Dose*	Form	Manufacturer	Exp. date	$\delta^{13}C_{VPDB}$ T ±0.5 (‰)
Andriol	Undecanoate *40 mg*	Oral oil capsules	Organon (the Netherlands)	09-2011	−27.6
Reandron® 1000	Undecanoate *1000 mg*	I.M. ampoule	Bayer Schering (Germany)	10-2013	−28.0
Primoteston®Depot	Enanthate *250 mg*	I.M. syringe	Bayer Schering (Germany)	03-2010	−27.7
Sustanon® 250	Mix *250 mg*	I.M. ampoule	Organon (the Netherlands)	09-2008	−29.9
				11-2013	−29.9
Testopel	–	Implant	Slate Pharma (USA)	09-2003	−30.4
Monument	–	Implant	Monument (USA)	03-2004	−31.8
College	–	Implant	College (USA)	12-2004	−31.4
Organon	–	Implant	Organon (Ireland)	11-2003	−30.5
				01-2011	−30.4
TESTOGEL™	– *10 mg*	Transdermal gel	Bayer Schering (Belgium)	12-2011	−28.3
Androderm®	– *5 mg/24 h*	Transdermal patch	Watson Laboratories Inc. (USA)	10-2002	−27.4
				12-2010	−27.6
Andromen Forte	– *5% (wt/wt)*	Transdermal cream	Lawley (Australia)	06-2011	−27.8

Table 2. GC-C-IRMS results (n=4, S.D=0.1‰ to 0.2‰) for six intramuscular veterinary testosterone preparations surveyed. 5α-androstane-3β-ol (INSTD) analyzed with each injection provided a mean $\delta^{13}C$ value of −29.7‰ with a SD of 0.2‰ (n=24).

Product	Ester *Dose*	Form	Manufacturer	Exp. date	$\delta^{13}C_{VPDB}$ T ±0.5 (‰)
VR®TESTOPROP	Propionate *50 mg/mL*	I.M. vial	JUROX Pty. Ltd. (Australia)	10-1999	−28.3
VR®TESTO L.A.	Cypionate *100 mg/mL*	I.M. vial	JUROX Pty. Ltd. (Australia)	01-2000	−29.4
ROPEL	Enanthate *75 mg/mL*	I.M. bottle	Dover (Australia)	03-2010	−29.2
TEPRO Hormone	Propionate *100 mg/mL*	I.M. vial	International Animal Health Products (Australia)	03-1997	−29.5
AVP Supertest	Propionate *100 mg/mL*	I.M. vial	Vetsearch (Australia)	11-1999	−28.5
TESTOSUS	– *100 mg*	I.M. vial	JUROX Pty. Ltd. (Australia)	03-2000	−26.6

weight (5 mg to 25 mg) was dissolved in chloroform (25 mL) to make 0.2 mg/mL to 1.0 mg/mL sample solutions. An aliquot (100 µL) was added to methyltestosterone (1 mg/mL, 50 µL) internal standard in an LC vial and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in acetonitrile/water (60 : 40, 1 mL). HPLC analysis was performed using an Agilent 1200 series (Sao Paulo, CA, USA) with an injection volume of 20 µL. Separation was achieved using an Agilent Eclipse XDB–C18 (3.0 × 150 mm, 3.5 µm) column maintained at 25 °C. The mobile phase flow was 1.0 mL/min with an acetonitrile/water gradient started at 60 : 40 for 2 min, then 80 : 20 at 2.5 min, 90 : 10 at 12 min, 100 : 0 at 13 min, and held until 18 min. Diode array detection of testosterone esters was performed at λ = 229 nm. The response factor for each testosterone ester was determined relative to a 5-point reference standard calibration (0.02 mg/mL to 0.10 mg/mL), from which the purity (% m/m) was determined from the internal standard quantification method.

Hydrolysis of testosterone esters for GC-C/TC-IRMS analysis

Testosterone preparations were homogenized with careful shaking before sub-sampling of 1 mg equivalent free testosterone into clean, screw-cap test tubes. With each batch of 18 testosterone preparations, the QC and a reagent blank were included. 5α-androstane-3β-ol (100 µg/mL, 100 µL) was added as a surrogate. Methanolic-HCl was prepared fresh by the dropwise addition of trimethylchlorosilane (2 mL) to methanol (18 mL) cooled in an ice bath.[16] An aliquot (1 mL) of this solution was added to the testosterone ester sample and the tube capped before hydrolysis for 90 min using a hot block operated at 60 °C.

Isolation of free testosterone

The sample was cooled to room temperature before water (2 mL) was added to quench the hydrolysis reaction. Carbonate buffer (pH 9.8) was prepared by dissolving potassium carbonate (40 g)

and potassium hydrogen carbonate (40 g) in water (200 mL). An aliquot (1 mL) of this buffer was added to the hydrolysate and the sample gently shaken. Testosterone was extracted with hexane (3 × 5 mL) using a rotary shaker followed by centrifugation (2000 rpm), with the combined organic extracts transferred to a clean, dry, test tube and dried under nitrogen. The residue was reconstituted with cyclohexane (300 µL) before being transferred to a GC vial for GC-MS and GC-C/TC-IRMS analysis.

Gas chromatography-mass spectrometry (GC-MS)

An Agilent 6890 GC coupled to an Agilent 5973 MSD (Sao Paulo, CA, USA) was used. The carrier gas was helium with a flow rate of 1.8 mL/min for a pressure of 17.3 psi. The injection volume was 2 µL (10 : 1 split) at 280 °C. The GC column (0.25 mm I.D × 30 m) was a J&W DB17-MS cross-linked 50% phenyl-methyl siloxane (0.25 µm film thickness). The column temperature was programmed from 180 °C (1 min) to 250 °C at 12 °C/min, to 280 °C at 3 °C/min, then to 300 °C at 15 °C/min and held for 4 min. The MSD acquired data in scan mode from *m/z* 40 to *m/z* 450 using HP Chemstation® software (Sao Paulo, CA, USA).

Each sample extract was analyzed by full scan GC-MS for relative retention determination of testosterone to 5α-androstane-3β-ol (INSTD) with electron impact spectral comparison of testosterone to the NMI reference material according to WADA criteria.[17] The purity of liberated testosterone was verified by spectral deconvolution of peaks at the leading edge, apex, and tailing edge to identify extraneous ion contributions from coeluting species.

GC-C/TC-IRMS analysis of free testosterone

An Agilent 6890 GC (Sao Paulo, CA, USA) coupled to a Thermo ConFlo IV interface and Thermo Delta V Plus IRMS (Bremen, Germany) was used for compound specific isotopic analysis of testosterone. The GC conditions were equivalent to those described for full-scan GC-MS except for injection being carried out in splitless (0.8 min) mode. Data was acquired using ISODAT® NT 2.8 software (ThermoScientific, Bremen, Germany).

$\delta^{13}C$ analysis by GC-C-IRMS

The oxidation reactor for combustion was operated at 940 °C. High-purity oxygen gas was flushed through the furnace for 3 h prior to a sequence of 25 injections. The reduction reactor temperature was 620 °C. The $\delta^{13}C$ values, reported as per mille (‰) differences[18] from the Vienna Pee Dee Belemnite (VPDB) international standard, were measured relative to $\delta^{13}C = -6.8‰$ for the CO_2 reference gas, determined from NBS-22 via NBS-19 by dual-inlet IRMS analysis (Environmental Isotopes, Sydney, NSW, Australia).[19] The GC-C-IRMS instrument was calibrated to the 5α-androstane-3β-ol certified reference standard ($\delta^{13}C = -29.7 \pm 0.1‰$) that was added to each sample as an internal standard. Bias (0.1‰) was determined from the mean difference observed for this standard relative to the certified $\delta^{13}C$ value. The testosterone standard was analyzed every five injections for comparison to its assigned value ($\delta^{13}C = -30.0 \pm 0.4‰$) determined by Elemental Analyser (EA)-IRMS. Figure 2 shows a GC-C-IRMS chromatogram with the retention times of 5α-androstane-3β-ol (INSTD; 500 s) and testosterone (939 s) from which relative-retention-based (rR = 1.88) identification was made with comparison to the full scan GC-MS analysis. The presence of androst-4-en-3,17-dione (androstenedione; ADIONE) starting material, observed in the majority of testosterone preparations, is seen as a small unresolved peak on the tail of testosterone. Fractionation was avoided by integrating both peaks together. Precision (0.2‰) was determined from the pooled standard deviation of the testosterone standard (n=35). An estimation of measurement uncertainty was performed by combining the contributions of bias and precision using the square root of the sum of squares.[20] The standard uncertainty (u) for $\delta^{13}C$ measurements was calculated to be ±0.22‰. At a 95% confidence interval (with a coverage factor of 2) an expanded uncertainty (U) for $\delta^{13}C$ measurements was estimated to be ±0.5‰. This estimate was

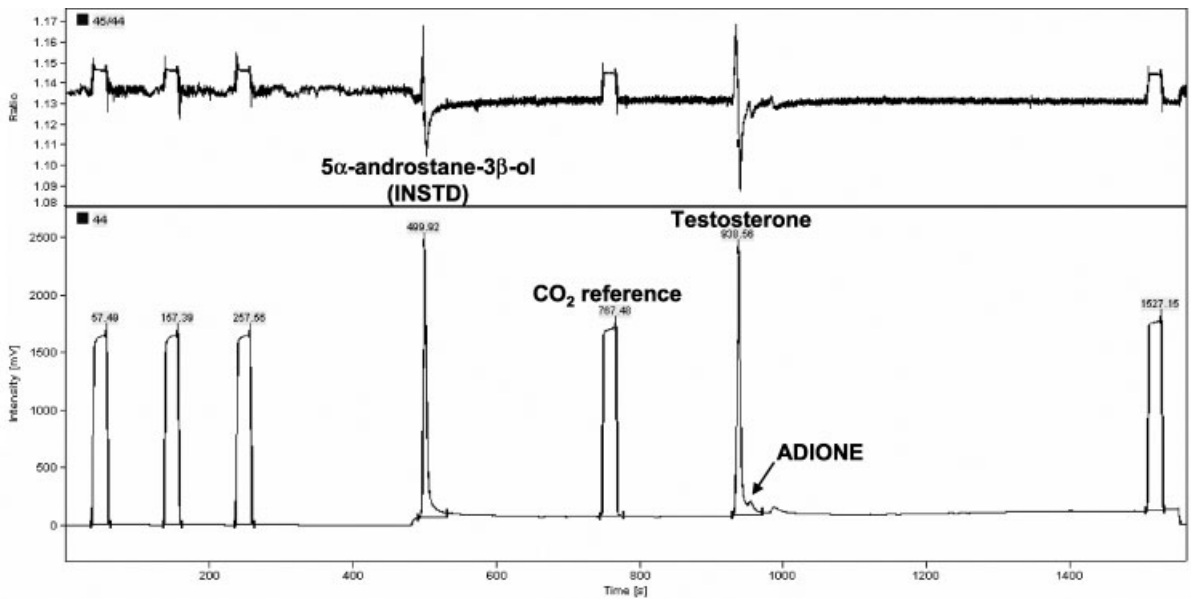


Figure 2. GC-C-IRMS chromatogram of the testosterone reference standard. The top trace shows the ratio of *m/z* 45 to *m/z* 44. The bottom trace (*m/z* 44) shows the retention times of 5α-androstane-3β-ol (INSTD; 500 seconds) and testosterone (939 seconds) to provide a relative retention of 1.88. Testosterone $\delta^{13}C$ values were determined relative to the CO_2 reference pulse ($\delta^{13}C_{VPDB} = -6.8‰$) at 767 seconds.

verified as being fit-for-purpose in comparison to the range of values recorded for the QC ester sample.

δ^2H analysis by GC-TC-IRMS

To enable efficient instrument reconfiguration for δ^2H analysis to be performed a post-column modification incorporating two 4-port micro switching valves (Valco, Houston, Texas USA) was made as illustrated by Figure 3. The high temperature thermal conversion reactor was operated at 1440 °C. Isooctane (2 × 0.2 µL, 20 : 1 split injection) was used to condition the reactor tube prior to a sequence of 50 injections. The GC-TC-IRMS instrument was calibrated relative to Vienna Standard Mean Ocean Water (VSMOW) via the 5α-androstane certified reference standard (δ^2H = −297 ± 2‰) purchased from Indiana University by a series of five initial measurements and injections between every five testosterone samples. Single-point anchoring[21] of δ^2H values was achieved by using 5α-androstane as the common standard from which the H_2 reference gas was used to determine the δ^2H values of testosterone samples. The associated correction was verified as fit-for-purpose by comparison to the testosterone QC sample, analyzed at least twice in each sequence, with its assigned value (δ^2H = −250 ± 4‰) determined by TC-IRMS. Precision (2‰) was determined from the pooled standard deviation of the testosterone QC (n=35) to provide an estimate of measurement uncertainty (±≥4‰). System stability was established by daily determinations of the H_3^+ factor (2.4 ± 0.2 ppm/nA) prior to each sequence of samples. Figure 4 shows a GC-TC-IRMS chromatogram with the retention times of 5α-androstane-3β-ol (INSTD; 641 s) and testosterone

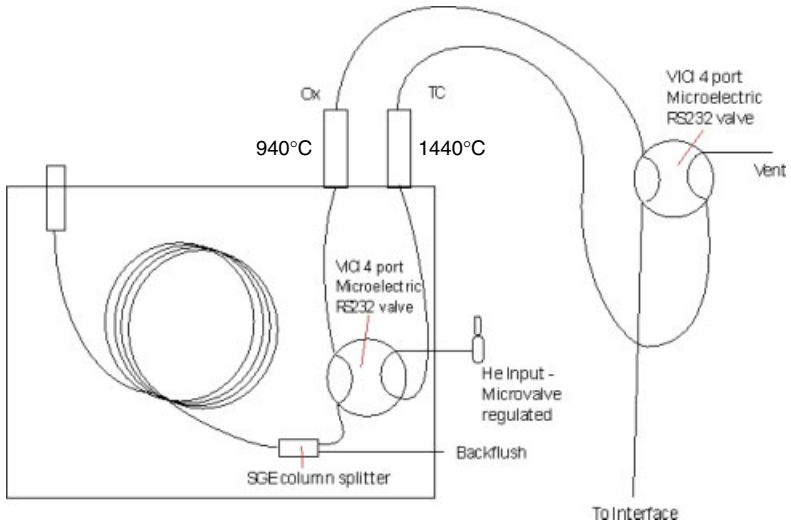


Figure 3. Schematic of GC-C/TC-IRMS system with post-column modifications (Courtesy of Brian Jones, ThermoScientific, Sydney, Australia).

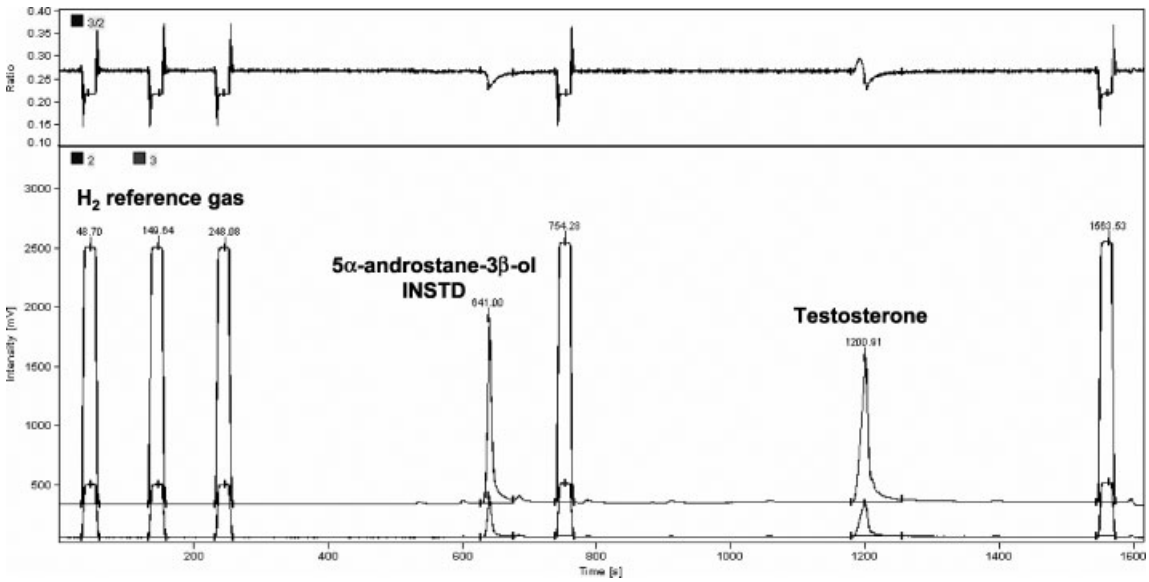


Figure 4. GC-TC-IRMS chromatogram of the testosterone reference standard. The top trace shows the ratio of *m/z* 3 to *m/z* 2. The bottom trace (*m/z* 2 and *m/z* 3) shows the retention times of 5α-androstane-3β-ol (INSTD; 641 seconds) and testosterone (1201 seconds) to provide a relative retention of 1.87. Testosterone δ^2H values were determined relative to the H_2 reference pulse (δ^2H_{VSMOW} = −340‰) at 754 seconds. The presence of androst-4-ene-3,17-dione (androstenedione; ADIONE) starting material, observed in the majority of testosterone preparations, is seen as a small shoulder on the tail of the testosterone peak.

(1201 s) from which relative-retention-based (rR = 1.87) identification was made with comparison to the full scan GC-MS analysis.

EA/TC-IRMS of high-purity testosterone esters

Seized samples of ≥95% purity were subjected to $\delta^{13}C$ and $\delta^{2}H$ profiling by bulk stable isotope analysis techniques. A ThermoScientific Flash Elemental Analyser EA 1112 (Delft, the Netherlands) with thermal conversion (TC) capability connected to a ConFlo IV interface and Delta V Plus Mass Spectrometer was used to determine $\delta^{13}C$ and $\delta^{2}H$ values by continuous flow. Data was acquired using ISODAT® NT 2.8 software (ThermoScientific, Bremen, Germany). Prior to sequence acquisitions, zero enrichment pulses were added using each of the two reference gases. The standard deviation of nine 20-second gas pulses was determined to be less than 0.1‰ for CO_2 and 1‰ for H_2. Sample sequences for $\delta^{13}C$ and $\delta^{2}H$ analysis were bracketed by triplicates of C36 *n*-alkane certified reference standard ($\delta^{13}C = -30.0 \pm 0.1$‰, $\delta^{2}H = -247 \pm 3$‰) purchased from Indiana University. System performance was verified by analysis of a high-purity testosterone (+98%) quality control every five samples.

$\delta^{13}C$ analysis by EA-IRMS

Crimped tin capsules containing 0.5 ± 0.1 mg sample material were introduced into a Thermo Scientific *No Blank* chamber and pressurized with high-purity oxygen gas. The sample entered the combustion furnace operated at 980 °C where it burnt exothermically in a stream (250 mL/min, 3 s) of high-purity oxygen. The oxidized sample was reduced *in situ* in the presence of copper before water was removed from the resultant gas stream using a magnesium perchlorate filled trap. A postreactor GC column, operated at 35 °C separated evolved N_2 and CO_2. Helium pressure was set to 100 kPa to enable a run time of 650 s. Measured $\delta^{13}C$ values were determined relative to CO_2 gas ($\delta^{13}C = -6.8$‰) that was calibrated against NBS 19 (Environmental Isotopes Pty. Ltd, Sydney, NSW, Australia). The precision of $\delta^{13}C$ measurements was determined to be 0.2‰ contributing to an expanded uncertainty of ±0.4‰.

$\delta^{2}H$ analysis by TC-IRMS

Silver foil capsules containing 0.15 ± 0.05 mg sample material were introduced into the TC reactor consisting of an Alumina ceramic outer containing a glassy carbon tube with glassy carbon granulate and silver wool packing. The thermal conversion furnace was operated at 1440 °C and the postreactor GC column at 85 °C. Helium pressure was set to 330 kPa to enable a run time of 300 s. Measured $\delta^{2}H$ values were determined relative to H_2 gas ($\delta^{2}H = -340$‰) calibrated against certified reference materials, namely; C36 *n*-alkane ($\delta^{2}H = -247 \pm 2$‰), phenanthrene ($\delta^{2}H = -84 \pm 3$‰) and Icosanoic acid methyl ester ($\delta^{2}H = +76 \pm 2$‰) purchased from Indiana University. Subsequent repeat analysis (n=21) of the high-purity testosterone quality control assigned the $\delta^{2}H$ value of this sample as −250 ± 4‰. The H_3^+ factor (2.4 ± 0.2 ppm/nA) was determined from reference H_2 gas pulses with signal size linearly incremented. Precision of $\delta^{2}H$ measurements as monitored by standards and laboratory controls was determined to be 2‰ contributing to an estimated expanded uncertainty of ±4‰.

Confirmation of $\delta^{13}C$ values for suspect testosterone preparations

Samples recording $\delta^{13}C$ values for testosterone greater (i.e. less negative) than −26.0‰ were subject to a confirmation procedure. The hydrolysis, isolation and GC-C-IRMS analysis was repeated on triplicate sub-samples and the $\delta^{13}C$ values of the four measurements compared and accepted within U = ±0.5‰. To eliminate method fractionation as a cause for ^{13}C-enrichment, the same sample extract was subject to purity analysis by monitoring the presence of original esters. 17α-Methyltestosterone (1 mg/mL, 50 µL) internal standard was added before the sample was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in acetonitrile/water (60 : 40, 1 mL) and submitted for HPLC analysis according to the method described earlier. The efficiency of the acid hydrolysis reaction performed on testosterone esters was observed to be greater than 99% in all confirmation samples.

Statistical analysis

Mean, median, standard deviations, *t*-tests and single-factor ANOVA were determined using Microsoft® Office Excel 2003 (11.8169.8221) SP3.

Results and Discussion

$\delta^{13}C$ analysis of testosterone preparations by GC-C-IRMS

Legitimate pharmaceutical and veterinary testosterone products

The 14 products profiled for this study recorded $\delta^{13}C$ values in good agreement with previous reports of pharmaceutical testosterone with a range −26.5‰ to −31.8‰ (Table 1).[6,7] Importantly for doping control, no product surveyed had a $\delta^{13}C$ value higher (i.e. more ^{13}C-enriched) than reported endogenous urinary steroid metabolite reference intervals.[22–25] Of particular interest were transdermal testosterone gel products for which there is limited information pertaining to their $\delta^{13}C$ values. The four non-esterified testosterone preparations in this category had a $\delta^{13}C$ range of −27.4‰ to −28.3‰. Notably there was good agreement between the current result for *Androderm*® (−27.6‰) and a previous report (−27.4‰).[26] In addition, $\delta^{13}C$ values for this preparation were consistent across a 10-year interval between batches surveyed in the current study. Excellent comparability was also observed between a recently acquired *Sustanon 250*® (−29.9‰) preparation and the QC collected in 2004 (−29.9‰). The four subcutaneous products were the most ^{13}C-depleted ($\delta^{13}C = -30.4$‰ to −31.8‰) and therefore represent the most detectable category of testosterone preparation from the perspective of doping control. Consideration was given to the availability of veterinary products for abuse by athletes. To this end, six preparations were surveyed from a sample collection performed during the year 2000 (Table 2). The $\delta^{13}C$ range recorded was −26.6‰ to −29.5‰, with the sample corresponding to the high value belonging to a free testosterone isolated from an aqueous solution. From an anti-doping perspective, no testosterone preparation surveyed from the medical or veterinary pharmaceutical products was found to have unexpectedly high ^{13}C content.

Seized testosterone products

The collection of seized testosterone preparations for this study displayed a ramped longitudinal trend from 2006 (n=6),

continuing with 2007 (n=31) and 2008 (n=50), to the end of 2009 (n=179). Notable points regarding the sample population include:

- The testosterone content of collected preparations ranged from 3 ± 2% m/m to 100 ± 1% m/m.
- The ratio of low purity oils to high-purity solids was 3 : 1.
- The ratio of cypionate/enanthate/propionate/mixed ester types was 1 : 8:4 : 4, with long-acting enanthate esters (n=120) being most prevalent.
- There were 39 high-purity (i.e. ≥95%) testosterone ester samples amenable to EA/TC-IRMS analysis.

The δ^{13}C distribution for seized testosterone preparations (n=266) measured by GC-C-IRMS displayed a left-skewed distribution with mean of −28.4‰, median of −28.6‰, minimum of −32.6‰ and maximum of −22.9‰ (Figure 5). Importantly for doping control, 24 testosterone samples (9%) were found to display δ^{13}C values within reference intervals (−17.3‰ to −25.8‰) reported for endogenous urinary androgen metabolites.[22–25] Interestingly, these were all found for samples collected between July 2009 and December 2009 inclusive, before which the maximum (i.e. most ^{13}C-enriched) value recorded from the 93 samples analyzed up until that time was −26.9‰. An almost equal δ^{13}C distribution between high-purity solids (n=14) and low purity oils (n=10) was found for these samples. Their submission was found to represent six clusters based on the date received and packaging details. A feature common to these sample groups was the apparently deliberate mislabelling, with respect to product description and/or company details, employed by distributors to elude border controls. Most likely, the ^{13}C content of these samples reflects new source materials being used to supply the demand of counterfeit testosterone products.[27] The statistical effect of the left-skewed distribution for seized samples was to increase the mean δ^{13}C value, which was −28.7‰ when these 24 samples are excluded. There was no significant difference between the means of the seized and pharmaceutical sample sets within the synthetic testosterone population (*t*-test, P=0.08). As expected, these sample sets became more similar with the exclusion of the ^{13}C-enriched samples (*t*-test, P=0.65). A further 24 samples (9%) recorded δ^{13}C values in the range −25.9‰ to −27.0‰, which if administered, would presumably make the determination of their synthetic origin difficult due to the dilution effect from endogenous steroid precursors *de novo* and the potential for ^{13}C-depleted urinary steroids in some individuals.[28] No patterns with respect to the submission details of this sample group were identified.

Comparison of the ester types revealed a significant difference between the means of the enanthate (n=120) and propionate (n=68) sample groups (*t*-test, P<0.01). Figure 6 shows the median, inter-quartile range and ^{13}C-enriched spread for the

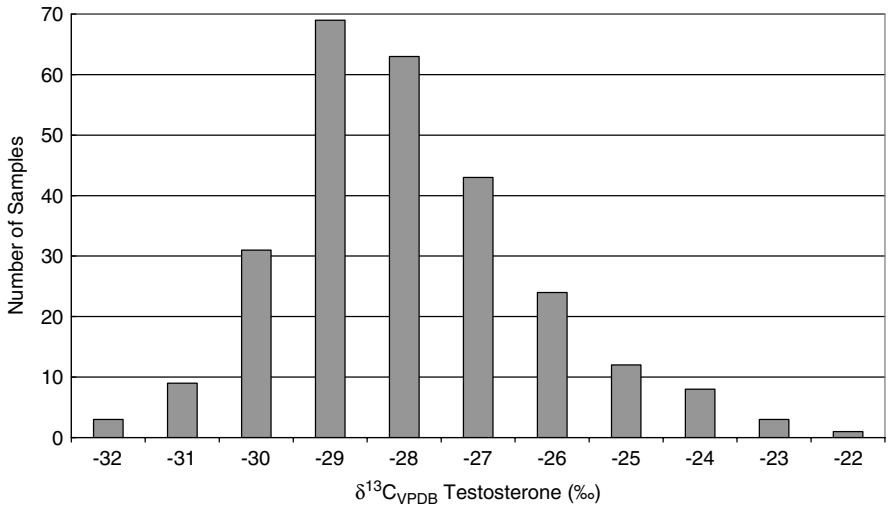


Figure 5. Frequency distribution of δ^{13}C values recorded for seized testosterone preparations (n=266) collected between 2006 and 2009 inclusive.

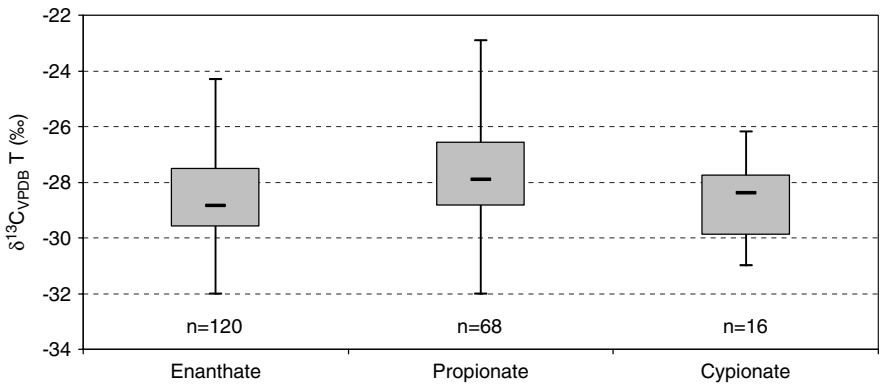


Figure 6. Box-plot analysis of δ^{13}C values recorded from the three individual ester types in the seized testosterone population.

Table 3. GC-TC-IRMS results (mean from n=4, S.D = 2‰ to 3‰) for legitimate pharmaceutical testosterone products surveyed. 5α-androstane-3β-ol (INSTD) analyzed with each injection provided a mean δ^{13}C value of −324‰ with a SD of 3‰ (n=44)

Product	Ester *Dose*	Form	Manufacturer	Exp. date	δ^2H_{VSMOW} T ±4 (‰)
Andriol	Undecanoate *40 mg*	Oral oil capsules	Organon (the Netherlands)	09-2011	−238
Reandron® 1000	Undecanoate *1000 mg*	I.M. ampoule	Bayer Schering (Germany)	10-2013	−222
Primoteston®Depot	Enanthate *250 mg*	I.M. syringe	Bayer Schering (Germany)	03-2010	−213
Sustanon® 250	Mix *250 mg*	I.M. ampoule	Organon (the Netherlands)	09-2008 11-2013	−240 −241
Organon	–	Implant	Organon (Ireland)	01-2011	−298
TESTOGEL™	– *10 mg*	Transdermal gel	Bayer Schering (Belgium)	12-2011	−275
Androderm®	– *5 mg/24 h*	Transdermal patch	Watson Laboratories Inc. (USA)	10-2002 12-2010	−293 −290
Andromen Forte	– *5% (wt/wt)*	Transdermal cream	Lawley (Australia)	06-2011	−240

Table 4. GC-TC-IRMS results (n=4, S.D=2‰ to 3‰) for six intramuscular veterinary testosterone preparations surveyed. 5α-androstane-3β-ol (INSTD) analyzed with each injection provided a mean δ^2H value of −326‰ with a SD of 3‰ (n=24).

Product	Ester *Dose*	Form	Manufacturer	Exp. date	δ^2H_{VSMOW} T ±4 (‰)
VR®TESTOPROP	Propionate *50 mg/mL*	I.M. vial	JUROX Pty. Ltd. (Australia)	10-1999	−246
VR®TESTO L.A.	Cypionate *100 mg/mL*	I.M. vial	JUROX Pty. Ltd. (Australia)	01-2000	−271
ROPEL	Enanthate *75 mg/mL*	I.M. bottle	Dover (Australia)	03-2010	−267
TEPRO Hormone	Propionate *100 mg/mL*	I.M. vial	International Animal Health Products (Australia)	03-1997	−263
AVP Supertest	Propionate *100 mg/mL*	I.M. vial	Vetsearch (Australia)	11-1999	−247
TESTOSUS	– *100 mg*	I.M. vial	JUROX Pty. Ltd. (Australia)	03-2000	−267

propionate sample group to be shifted towards higher δ^{13}C values. This raises the potential of ^{13}C fractionation present in the esterification procedure being greater for longer chain ester groups. There was no difference observed in the means (*t*-test, P>0.1), median, or inter-quartile range for the cypionate sample group (n=16) in relation to the enanthate and propionate groups.

δ^{13}C analysis of high-purity testosterone esters by EA-IRMS

The δ^{13}C values of high-purity (≥95%) testosterone ester samples (n=39) provided a range from −24.5‰ to −31.4‰. Single factor ANOVA demonstrated no significant difference between the liberated free testosterone measured by GC-C-IRMS and intact esters measured by EA-IRMS for these samples (P=0.41). High ^{13}C content in 14 high-purity samples of the 24 confirmed seizures was verified by EA-IRMS recording δ^{13}C values greater than −26.5‰, providing further evidence that any unidentified fractionation in the method was not responsible for the GC-C-IRMS results obtained.

δ^2H analysis of testosterone preparations by GC-TC-IRMS

Legitimate pharmaceutical and veterinary testosterone products

The 10 products profiled for this study recorded a mean δ^2H value of −255‰ and the range −213‰ to −293‰ (Table 3). The transdermal testosterone gel products displayed negative δ^2H values (−275‰, −290‰ and −293‰) in relation to the mean but reflected a δ^2H value reported previously (−283‰).[11] Conversely, two of the intra-muscular products; *Primoteston®Depot* (−213‰) and *Reandron® 1000* (−222‰) were relatively ^{2}H-enriched. There was good agreement between batches of the *Androderm®* (−290‰ and −293‰) product collected over a 10-year period. Excellent comparability was also observed between a recently acquired *Sustanon 250®* (−241‰) preparation and the QC ester (−240‰) collected in 2004. Only one subcutaneous product was collected recently and available for GC-TC-IRMS analysis. It represented the most ^{2}H-depleted (δ^2H = −298‰) but with only limited urinary steroid δ^2H profiling available[11] it is difficult to discuss the relevance for doping control. Profiling of the six veterinary products for δ^2H values revealed a mean of −261‰ and a more narrow range from −246‰ to −271‰ (Table 4).

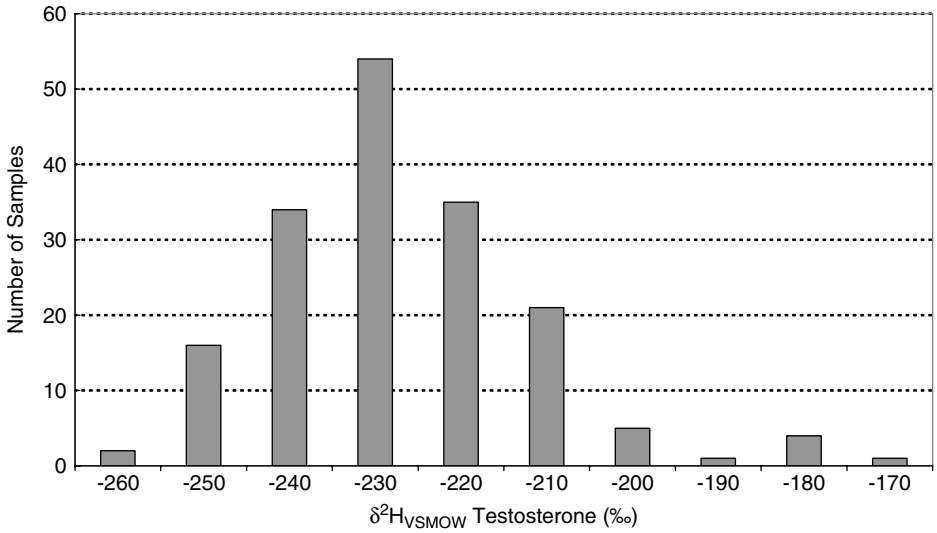


Figure 7. Frequency distribution of δ^2H values recorded for seized testosterone preparations (n=173).

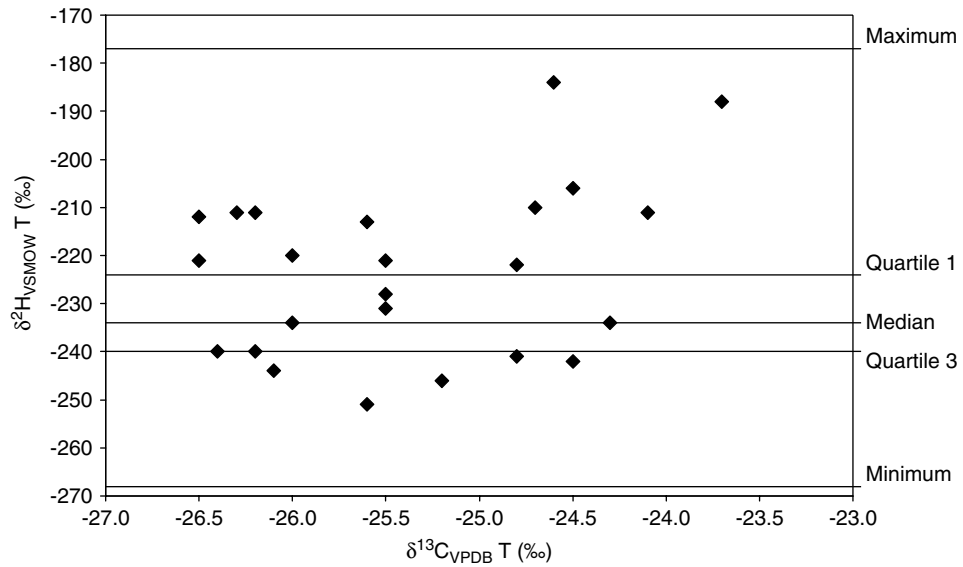


Figure 8. Bivariate analysis of δ^2H and δ^{13}C values for ^{13}C-enriched sample group (n=24) recorded for the seized testosterone population with respect to the δ^2H maximum, median, minimum and inter-quartile range.

Seized testosterone products

The δ^2H distribution for seized testosterone preparations (n=173) measured by GC-TC-IRMS displayed a left-skewed distribution with mean of −231‰, median of −234‰, maximum of −177‰ and minimum of −268‰ (Figure 7). Potential correlation between the δ^{13}C and δ^2H values for the ^{13}C-enriched sample group was investigated but none was found from a random distribution obtained from bivariate analysis. This was also the case for the entire bivariate distribution of δ^2H/δ^{13}C values. Further inspection revealed that more than half of the ^{13}C-enriched sample group (n=13) recorded δ^2H values in the first quartile of the distribution in Figure 7, indicating a preference for higher ^{2}H content (Figure 8). Further profiling of seized testosterone preparations will be required to verify this finding for doping control.

Unlike the δ^{13}C distribution, comparison of the ester types revealed no significant difference between the means of the enanthate (n=66) and propionate (n=54) sample groups (*t*-test, P>0.20). There was also no difference observed between the median or inter-quartile range for the three ester groups (Figure 9). Hence ^{2}H fractionation does not appear to be significant in the esterification of groups containing different numbers of hydrogen atoms.

δ^2H analysis of high-purity testosterone esters by TC-IRMS

The δ^2H values of high-purity (≥95%) testosterone ester samples (n=32) provided a range from −182‰ to −262‰. Single factor ANOVA demonstrated no significant difference between the liberated free testosterone measured by GC-TC-IRMS and the intact ester measured by TC-IRMS for these samples (P=0.53). Eight of the 14 high-purity samples from the 24 confirmed ^{13}C-enriched sample group recorded δ^2H values in the first quartile, thereby supporting the results presented in Figure 8.

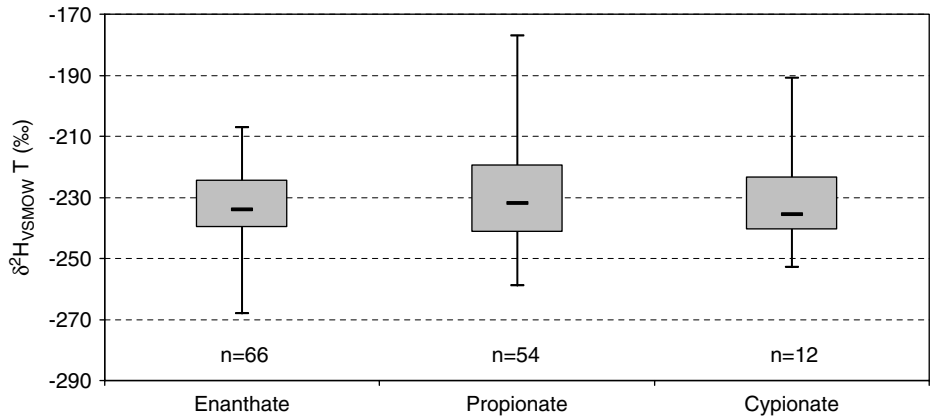


Figure 9. Box-plot analysis of δ^2H values recorded from the three individual ester types in the seized testosterone population.

Table 5. Summary statistics from the stable isotope ratio (δ^{13}C and δ^2H) profiling of seized testosterone preparations

Parameter	$\delta^{13}C_{VPDB}$ T (‰)	δ^2H_{VSMOW} T (‰)
n	266	173
Mean	−28.4	−231
SD	1.7	15
Maximum	−22.9	−177
IQR 1 (25% interval)	−27.3	−224
Median	−28.6	−234
IQR 3 (75% interval)	−29.6	−240
Minimum	−32.6	−268
U (at k=2)	±0.5	±4

Conclusions

The results from this study, summarized in Table 5, provide intelligence to anti-doping laboratories concerning the ^{13}C and ^{2}H content of synthetic testosterone preparations. The finding that 9% of seized materials analyzed were found to have δ^{13}C values of testosterone that lie in the endogenous urinary steroid range has raised the possibility of false-negative results being reported from current GC-C-IRMS methodologies. Most likely, the ^{13}C content of these samples reflects new source materials being used to supply the demand of counterfeit testosterone products. Information about such products has been communicated to doping control laboratories and it is envisaged that this profiling programme will be expanded to monitor the δ^{13}C value of legitimate and seized testosterone preparations obtained from an international sample population. This study represents the first comprehensive profiling of synthetic testosterone δ^2H values. There is evidence to suggest that ^{13}C-enriched testosterone preparations may have a preference for higher ^{2}H content. This can not be substantiated, however, until urinary steroid profiling is conducted to establish reference intervals for δ^2H and $\Delta\delta^2$H values with respect to suitable endogenous reference compounds.

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