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Certification of steroid carbon isotope ratios in a freeze-dried human urine reference material

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An accurate method for the measurement of carbon isotope ratios of steroids in human urine has been developed at the National Measurement Institute, Australia (NMIA) for the certification of a freeze-dried human urine reference material (CRM NMIA MX005). The method measures $\delta^{13}C$ values by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) analysis following hydrolysis, solvent extraction and high performance liquid chromatography (HPLC) purification. Reference $\delta^{13}C$ values for testosterone metabolites etiocholanolone, androsterone, and endogenous reference compounds (ERCs) 11β-hydroxyandrosterone and pregnanediol were determined, as well as information $\delta^{13}C$ values for testosterone, epitestosterone, 11-oxoetiocholanolone, and a range of differences ($\Delta^{13}C$) between testosterone metabolites and ERCs. The measurement uncertainty was rigorously evaluated with expanded uncertainties for the reference $\delta^{13}C$ values between 1.1 and 1.6 ‰ at the 95% coverage level. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: certified reference material; steroids; carbon isotope ratio; GC-C-IRMS; measurement uncertainty

Introduction

Testosterone is an anabolic androgenic steroid listed by the World Anti-Doping Agency (WADA) on its Prohibited List 2011[1] as a substance the use of which is 'prohibited at all times (in- and out-of-competition)'. As testosterone is naturally present in the body, identifying instances of testosterone administration requires an ability to demonstrate whether the testosterone present in a sample has an origin outside the normal metabolic processes. In most cases (over 90%),[2] analysis of the stable carbon isotope ratios of specific urinary steroids is able to satisfy this requirement. Synthetic testosterone is generally produced from soybean (*Glycine max*), which has a significantly different carbon isotope composition compared to steroids produced in the body.[3] As a result, the administration of synthetic testosterone is detectable via a change in the carbon isotopic composition of testosterone and its metabolites (e.g. androsterone and etiocholanolone) in the subject's urine, when compared to other urinary steroids that are not derived from the administered steroid but which are produced by a different metabolic pathway (endogenous reference compounds, or ERCs). For the detection of testosterone abuse, typical ERCs include pregnanediol, 11-oxoetiocholanolone and 11β-hydroxyandrosterone.[4] In order to effectively detect the administration of endogenous steroids, and to ensure the legal defensibility of the results, it is vital that the measurements can be demonstrated to be free from bias and have a small and well-known measurement uncertainty. Certified reference materials (CRMs), particularly CRMs that are matrix matched, can provide reference values that can be used in method development and validation to demonstrate the absence of significant bias. Certified reference materials used for calibration also provide metrological traceability of the measurement results.[5]

Stable carbon isotope analysis of organic samples involves combusting the analyte and measuring the relative abundance of ^{12}C and ^{13}C isotopes in the carbon dioxide produced. Carbon isotope ratios are reported as $\delta^{13}C$ values in per mil units (‰) relative to the Vienna Pee Dee Belemnite (VPDB) scale.[6] Measurements are performed against a reference material traceable to the carbon isotope ratio embodied in the calcium carbonate reference material NBS-19, by which the VPDB scale is defined, and the $\delta^{13}C$ value calculated according to Equation 1.

$$\delta^{13}C_{\text{analyte/VPDB}} = \left[\left(\frac{^{13}R_{\text{analyte}}}{^{13}R_{\text{std}}}\right)\left(\frac{\delta^{13}C_{\text{std/VPDB}}}{1000}+1\right)-1\right] \quad (1)$$

$\delta^{13}C_{x/VPDB}$ $\delta^{13}C$ value of x relative to the VPDB scale
$^{13}R_x$ carbon isotope ratio $^{13}C/^{12}C$ for x

As a difference of 3 ‰ between a steroid metabolite and an ERC is considered by WADA to be consistent with steroid administration,[4] expanded uncertainties of the measurements at the 95% coverage probability of the order of 1.5 ‰ are required.

For the determination of carbon isotope ratios from the carbon dioxide measurements, it is assumed that chemically similar compounds which are combusted in the same oxidation furnace within a single analysis will have the same proportion of the ^{17}O-containing species contributing to the *m/z* 45 peak,[7] as described previously.[8] A similar approach was also used by Kioussi *et al.*[9] This allows for a very straightforward calculation when a chemically similar compound co-injected with the analyte is used as an internal standard, as the relative contribution to the *m/z* 45 peak by the ^{17}O-containing species cancels when a ratio of the *m/z* 45/44 of the analyte and internal standard is calculated, as in Equation 1. However, this approximation only holds when the *m/z* 45/44 ratio of the internal standard (a similar steroid added prior to analysis) is matched to

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the *m/z* 45/44 ratio of the analyte in the sample. Estimation of measurement uncertainty is simplified using this method.

The National Measurement Institute of Australia (NMIA), with support from WADA, has certified the freeze-dried human urine certified reference material (CRM) NMIA MX005 for the $\delta^{13}C$ values of etiocholanolone (Et), androsterone (A), 11β-hydroxyandrosterone (11β-HA) and pregnanediol (PD). Information values for 11-oxoetiocholanolone (11-OEt), testosterone (T) and epitestosterone (E) were also determined, as well as $\Delta^{13}C$ values between the testosterone metabolites and certified ERCs. The A-ERC $\Delta^{13}C$ values are consistent with testosterone administration, while the Et-ERC $\Delta^{13}C$ values are of typical natural variation.

Experimental

Reagents and standards

All solvents and reagents were of analytical grade. All aqueous solutions and buffers were prepared using water from a Sartorius Arium® 611UV 18.2 $M\Omega cm^{-1}$ supply (Sartorius AG, Goettingen, Germany). β-glucuronidase from *Escherichia coli* K12 was obtained from Roche Diagnostic GmbH (Mannheim, Germany) and 5% dimethyldichlorosilane (DMDCS) in toluene solution from Sigma-Aldrich (St Louis, MO, USA).

High purity standards of etiocholanolone, androsterone, testosterone, epitestosterone and gestrinone were obtained from NMIA (Chemical Reference Materials, Australia), while standards of 5α-androstanol, 11-oxoetiocholanolone, 11β-hydroxyandrosterone and pregnanediol were purchased from Steraloids (Newport, RI, USA). A 19-noretiocholanolone standard, and a testosterone standard that had been prepared with a $\delta^{13}C$ value closer to that of steroids in human urine, were obtained from NMIA (Australian Sports Drug Testing Laboratory). Two steroid mixtures with certified $\delta^{13}C$ values traceable to VPDB via NIST RM 8559 (methane and ethane), CU/USADA 33–1 and CU/USADA 34–1,[10] were obtained from the Brenna Laboratory (Cornell University, Ithaca, NY, USA). Two carbon dioxide gas mixtures were obtained from NMIA (Reference Gas Mixtures), with carbon isotope ratios similar to those of the steroids in the urine reference material.

Deactivation of glassware

The surface of all glassware used was coated with a solution of 5% DMDCS in toluene and allowed to react for 15 s. The glassware was then rinsed twice with toluene, then twice with methanol and allowed to dry.

Hydrolysis and extraction

The MX005 CRM bottles were prepared according to the instructions provided with the material, and aliquots (10 ml) of the reconstituted material were taken for use within the same day. All samples were adjusted to pH ~6 using phosphoric acid (1:10 dilution in water), 50 μL of β-glucuronidase was added and the mixture held at 40 °C for 3 h. Carbonate buffer (pH 10, 20%, 600 μl) and NaCl (1 g) was added to each sample and vortexed to dissolve. The samples were then extracted with hexane (5 ml) three times. All three extracts were combined and evaporated to dryness under nitrogen at 50 °C after addition of 50 μl of gestrinone solution (5 μg/g in methanol). The residue was redissolved in 35 μl of methanol, and 35 μl of water was added.

HPLC clean-up

High performance liquid chromatography (HPLC) purification was carried out using a Waters 2795 Separations Module (Waters, Milford, MA, USA) connected to a Waters 2487 Dual Wavelength Absorbance Detector and a Foxy Junior fraction collector (Teledyne Isco, Lincoln, NE, USA). Detection of the gestrinone retention time marker was by UV absorption at 245 nm. The reconstituted extracts were purified using an Alltech Alltima 250 × 4.6 mm 5μm C18 column at 1.0 ml/min according to the conditions in Table 1. The five collected fractions (F1–F5), and the steroids in each fraction, are provided in Table 2.

In order to directly compare the carbon isotope ratio of the testosterone metabolites to the ERCs, a proportion of the F4 fraction was spiked into both F1 (68 μl) and F5 (238 μl) fractions, matching the amount of androsterone and etiocholanolone added to the amount of ERC in the fraction. The steroid internal standards were then added to all fractions to match the concentration of the steroid in the fraction, before evaporating to dryness under nitrogen and mild heat (50°C). The combinations of analyte and internal standard (including the carbon dioxide reference gases) used are listed in Table 3.

GC-C-IRMS analysis

The fractions were reconstituted in ethyl acetate to a concentration of approximately 15–20 μg/ml and analyzed using a Thermo

Table 1. HPLC parameters for the purification of urinary steroids

Injection volume	50 μl
Column	Alltech Alltima 250 × 4.6 mm 5μm C18
Flow rate	1.00 ml/min
Solvent gradient	0.0 min: 46% acetonitrile/water →
	8.5 min: 63% acetonitrile/water →
	8.7 min: 68% acetonitrile/water →
	16.0 min: 76% acetonitrile/water →
	16.2 min: 100% acetonitrile, hold 4.8 min →
	21.5 min: 46% acetonitrile/water, hold 5.5 min
Detection	Waters 2487 Dual Wavelength Absorbance Detector
	λ = 195 nm (0.0-8.5 min, 12.0–21.5 min)
	λ = 245 nm (8.5–12.0 min)
Data Acquisition	Dionex Chromeleon (version 6.8)
Fraction collection	Dionex AFC-300 Automated Fraction Collector

Table 2. Fraction collection for urinary steroids purified by HPLC

Fraction	Time (min:s)	Analytes
F1	7:20–7:90	11-oxoetiocholanolone, 11β-hydroxyandrosterone
F2	9:20–9:95	Testosterone
F3	10:45–11:10	Epitestosterone
F4	12:40–13:80	Etiocholanolone, Androsterone
F5	14:60–15:60	Pregnanediol

Table 3. Internal standards used for analysis of the target steroids in the urine CRM

Internal Standards (approx $\delta^{13}C$)	Steroid Analytes (Measured $\delta^{13}C$ ‰)						
	A (−27.9)	Et (−24.6)	T (−30.3)	11-OE (−23.2)	11β-HA (−23.6)	PD (−23.4)	E (−24.5)
CO_2 MD8908 (δ ^{13}C ~ −29 ‰)	•	*	•	*	*		
CO_2 MD8909 (δ ^{13}C ~ −24 ‰)						•	•
CO_2 cylinder G (δ ^{13}C ~ −22 ‰)	*	*			•		
5α-androstanol (δ ^{13}C ~ −31 ‰)	*	*	•	*	*	*	*
Testosterone (δ ^{13}C ~ −24 ‰)	•	•		•	•	•	
19-noretiocholanolone (δ ^{13}C ~ −28 ‰)	•	*		*	*		
11β -hydroxyandrosterone (δ ^{13}C ~ −30 ‰)			•				

• Internal standard used to determine the reference value.
* Internal standard added but not used to determine the reference value.

Scientific Delta V isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) via a Thermo Scientific GC Combustion III interface, according to the conditions in Table 4. Every ten sample injections were bracketed by duplicate injections of the CU/USADA reference materials.

Measurement equation

The reference materials CU/USADA 33–1 and CU/USADA 34–1 were used for calibration. The $\delta^{13}C$ value of the internal standard, and the apparent $\delta^{13}C$ value of the CO_2 gas pulse (similar to technique described by Zhang *et al.*[10]), were assigned using a calibration equation defined by the linear relationship between the $\delta^{13}C$ value and the measured *m/z* 45/44 peak area ratios of the seven steroid standards in the CU/USADA reference materials. As each point is a different steroid, the linearity of the calibration curve supports the assumption that similar compounds have the same proportion of the ^{17}O-containing species contributing to the *m/z* 45 peak after chromatographic separation and combustion in the same system. The measured ion current ratios for each steroid in the reference materials were also monitored periodically to ensure that no significant changes were occurring over time during the analysis. The $\delta^{13}C$ value of the analyte in the sample was then calculated using Equation 2. Although a calibration curve is used to assign the $\delta^{13}C_{IS}$ value, this calculation is essentially a single point calibration procedure, where the measured *m/z* 45/44 ratio and instrumental intensity of the internal standard have been matched as closely as possible to the analyte of interest. This minimises the effects of many of the systematic biases involved in IRMS measurements, and also simplifies the calculations for $\delta^{13}C$ values of the steroids in the urine.

$$\delta^{13}C_{analyte} = \frac{R_{analyte}}{R_{IS}} \cdot \left(\delta^{13}C_{IS} + 1000\right) - 1000 \qquad (2)$$

$\delta^{13}C_{analyte}$	measured value for the analyte (‰) obtained by the reference method
$R_{analyte}$	peak area ratio *m/z* 45/44 of the analyte in the sample
R_{IS}	peak area ratio *m/z* 45/44 of the reference gas peak injected during the sample analysis
$\delta^{13}C_{IS}$	$\delta^{13}C$ value of the reference gas (‰) assigned using the calibration equation

Results

Measurement uncertainty

In order to determine the measurement uncertainty for the reference $\delta^{13}C$ values of the steroids in the urine, a full measurement equation was defined and a standard uncertainty for each parameter was estimated. The basic measurement equation (Equation 2) was expanded to include appropriate additional factors to allow for potential measurement bias.

Table 4. GC-C-IRMS parameters for the analysis of urinary steroids

Injection	2 µl; Splitless (20.0 psi, 0.8 min); 250 °C
Column	J&W Scientific DB-17ms 30 m × 0.25 mm × 0.25 µm film
Carrier gas	Helium; constant flow; 1.2 ml/min
Oven temperature program	130 °C → 132 °C @ 10 °C min^{-1} → 250 °C @ 30 °C min^{-1} → 262 °C @ 2 °C min^{-1}; hold 4 min → 290 °C @ 5 °C min^{-1} → 300 °C @ 30 °C min^{-1}; hold 3 min
Oxidation	940 °C
Reduction	650 °C
Water removal	Nafion™ semi-permeable membrane with He carrier gas
Data acquisition	Thermo Scientific ISODAT NT 2.0

These factors were assigned a value of unity, unless a bias introduced by the factor was detected. The expanded equation for characterisation of the reference value is given in Equation 3.

$$\delta^{13}C_{\text{analyte}} = F_M \cdot F_{IS} \cdot F_{IC} \cdot \frac{R_{\text{analyte}}}{R_{IS}} \cdot \left(\delta^{13}C_{IS} + 1000\right) - 1000 \quad (3)$$

$\delta^{13}C_{\text{analyte}}$	reference value for the analyte (‰) obtained by the reference method, including an allowance for bias
F_M	factor to allow for the effect of the matrix and extraction process on the measured value
F_{IS}	factor to allow for the effect of the choice of internal standard on the measured value
F_{IC}	factor to allow for the effect of the instrumental method and calibration procedure on the measured value

The standard measurement uncertainties of each factor in the expanded equation are combined using appropriate sensitivity coefficients to give the combined standard measurement uncertainty of the reference values.[11] This is shown in Equation 4, where u_{char} is the standard uncertainty of the measured value for $\delta^{13}C_{\text{analyte}}$.

$$u_{\text{char}}^2 = \left(\frac{\partial\left(\delta^{13}C_{\text{analyte}}\right)}{\partial(F_M)}\right)^2 u_{F_M}^2 + \left(\frac{\partial\left(\delta^{13}C_{\text{analyte}}\right)}{\partial(F_{IS})}\right)^2 u_{F_{IS}}^2 + \left(\frac{\partial\left(\delta^{13}C_{\text{analyte}}\right)}{\partial(F_{IC})}\right)^2 u_{F_{IC}}^2 + \left(\frac{\partial\left(\delta^{13}C_{\text{analyte}}\right)}{\partial\left(R_{\text{analyte}}/R_{IS}\right)}\right)^2 u_{R_{\text{analyte}}/R_{IS}}^2 + \left(\frac{\partial\left(\delta^{13}C_{\text{analyte}}\right)}{\partial\left(\delta^{13}C_{IS}\right)}\right)^2 u_{\delta^{13}C_{IS}}^2 \quad (4)$$

According to ISO Guide 35,[12] the uncertainty in the assigned values for the CRM also requires an allowance for possible variation in the measurand due to heterogeneity between bottles and instability of the material over its storage lifetime and during transport. This has been incorporated into the overall uncertainty associated with each reference value as shown in Equation 5.

$$u_{\text{CRM}}^2 = u_{\text{char}}^2 + u_{\text{homogeneity}}^2 + u_{\text{LTS}}^2 + u_{\text{STS}}^2 \quad (5)$$

u_{CRM}	combined uncertainty in the reference value for an analyte in the CRM
u_{char}	uncertainty in the measured values obtained by the reference method for the analyte, including an allowance for bias
$u_{\text{homogeneity}}$	uncertainty in the reference values arising from possible between-bottle variability (in homogeneity of the batch)
u_{LTS}	uncertainty related to the long term storage stability of the δ^{13}C values in the reference material (at −20 °C until the expiry of certification)
u_{STS}	uncertainty in the short term stability of the δ^{13}C values at elevated temperature in transport (at ambient temperature up to a month)

Uncertainty of $R_{\text{analyte}}/R_{IS}$

The standard uncertainty from random errors in the measured values of R_{analyte} and R_{IS} was estimated by examining the variance of results in sets of data where these variables were repeatedly measured while other potential sources of variability were controlled. Estimates of the potential magnitude of systematic errors that could lead to bias in the measured values of $R_{\text{analyte}}/R_{IS}$ are introduced in the factor F_M. The repeatability of the results for replicate analyses of a given unit of the CRM in a single batch of analyses is used as an estimate of the standard uncertainty of the ratio R_{sample}/R_{IS}. This factor includes the uncertainty due to small variations in the instrument response, such as fluctuations in the combustion reactor conditions that may occur over the course of a batch of analyses.

Uncertainty of $\delta^{13}C_{IS}$ ‰

The calibration relation of the measured peak area ratios and the certified δ^{13}C values of the seven steroids in the CU/USADA reference materials were used to assign the value of $\delta^{13}C_{IS}$ from the calculated peak area ratio. Equation 6 gives the standard uncertainty of this assignment.[13] As the standard uncertainty of the reference materials were significantly smaller than the uncertainty of $\delta^{13}C_{IS}$ estimated using Equation 6, it was assumed the uncertainty of the reference materials were captured using this equation. The CU/USADA mixed steroid standards were analysed repeatedly throughout each batch, and each individual analysis in a single batch was included in the estimation.

$$s_{\hat{x}_0} = \frac{s_{y/x}}{b}\sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}} \quad (6)$$

$s_{\hat{x}_0}$	uncertainty of $\delta^{13}C_{IS}$
$s_{y/x}$	standard deviation of the regression
b	slope of the calibration line
m	number of replicate observations of the response (mean y_0)
n	number of points in the calibration line
$\bar{y}$ and $\bar{x}$	means of the calibration data ($\bar{y}$ is average measured *m/z* 45/44 ratio for the calibration materials, $\bar{x}$is the average of the certified δ^{13}C values)
x_i	*i*th *x* value (certified δ^{13}C value) from the calibration set

Value and uncertainty of F_M

The potential bias introduced by urine matrix effects, and the extraction procedure, was estimated by comparing the δ^{13}C

values of a mixed free steroid standard solution with those of the same standard solution after extraction from the unhydrolysed urine CRM. In the absence of interfering matrix-coextractives, or isotopic fractionation during the extraction process, the delta values of the steroid standards would remain unchanged. The potential for additional interferences after hydrolysis of the glucuronides in the urine could not be assessed by this approach, as the analytes are all endogenous, and a blank urine matrix cannot be obtained. However, no additional interferences were revealed when urine extracts containing androsterone, etiocholanolone, epitestosterone and pregnanediol were run on both DB-5ms and DB-17ms columns. A Student *t*-test was used to compare the results with and without extraction, and only for 11-oxoetiocholanolone and epitestosterone was there found to be a statistically significant difference at the 95% confidence level. In these cases it was decided to provide only information values rather than reference values. For the other analytes, F_M was assigned a value of unity and the uncertainty of F_M was estimated as the standard deviation of the mean of the ratio of the $\delta^{13}C$ values for a given analyte with and without extraction.

Uncertainty of F_{IS}

Multiple internal standards were used in measurement of the $\delta^{13}C$ reference values of the steroids in the urine CRM. No significant differences were found using an Analysis of Variance (ANOVA) between results obtained for any analyte using internal standards with well-matched $\delta^{13}C$ values ($\delta^{13}C_{IS}$ within 10% of $\delta^{13}C_{analyte}$). The CRM reference value was calculated from all results processed with well-matched internal standards, therefore the uncertainty of this factor is incorporated in the precision uncertainty estimate.

Uncertainty of F_{IC}

Extracts from nine units of the urine CRM were pooled to form a composite sample, and subdivided to give a set of identical samples. These were analyzed by two different laboratories, with instruments calibrated using reference materials with independent traceability to the carbon isotope ratio embodied in NBS-19, by which the VPDB scale is defined. A Student *t*-test for groups of unequal variance was applied to the results. Only the results for testosterone were found to be significantly different at the 95% confidence level, because of its very low concentration, therefore it is not possible to provide a reference value for testosterone in the urine CRM at present. For the other analytes, the uncertainty of this factor was estimated as the pooled standard deviation of the results from both laboratories.

Homogeneity

The homogeneity of all steroid $\delta^{13}C$ values except epitestosterone was assessed using a one-way ANOVA of the results of duplicate samples from seven bottles of the urine CRM. An ANOVA was not possible for epitestosterone as the concentration in the CRM is very low (10 ng/ml) and the duplicate extracts were combined in order to achieve the necessary detection limit. At the 95% level of confidence, only the results for testosterone and pregnanediol suggested a small but statistically significant difference between results from different bottles. The standard uncertainty of this factor, $u_{homogeneity}$, for testosterone and pregnanediol was estimated from the between-bottle variance given by the ANOVA. For epitestosterone a combined precision and homogeneity term was used. For the other analytes where no evidence of inhomogeneity was found, this factor was not included in the total measurement uncertainty.

Stability

An estimate of the standard uncertainty due to long term storage (long-term stability, u_{LTS}) was determined by comparing the $\delta^{13}C$ values of the steroids in the urine CRM after storage at −20 °C for various times over a period of 35 months. No statistically significant trend in the results was detected at the 95% confidence level, and u_{LTS} was estimated from the uncertainty associated with the slope, multiplied by the length of the certification period in accordance to Guide 35.[12]

The standard uncertainty due to potential instability during transport (short-term stability, u_{STS}) was similarly estimated by comparing $\delta^{13}C$ values of the steroids in the urine CRM after at storage room temperature over a month. None of the analytes demonstrated a statistically significant trend to indicate a lack of stability, and u_{STS} was estimated from the uncertainty associated with the slope.

Combining uncertainties

For each of the resulting standard uncertainties, the degrees of freedom were determined and the standard uncertainties were combined using appropriate sensitivity coefficients for each input factor.[11] Sensitivity coefficients mathematically describe the dependence of the result $\delta^{13}C_{CRM}$ on each input factor. Figure 1 and Table 5 show the contributions to the combined

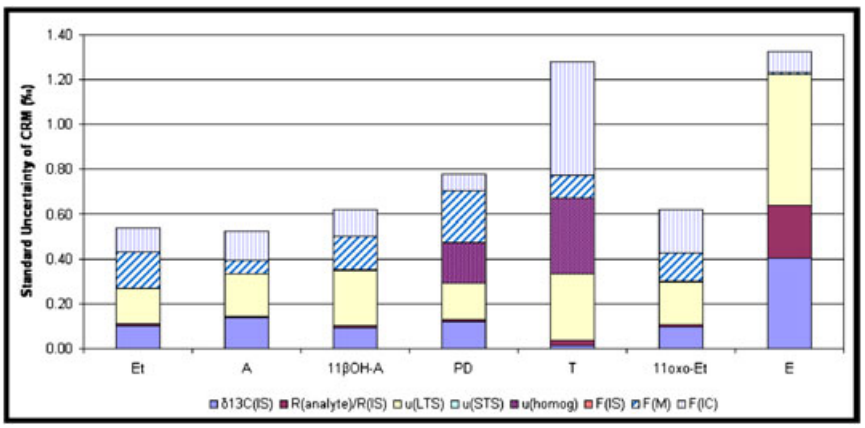

Figure 1. Contributions of measurement uncertainty components to the combined standard uncertainty of each analyte.

Table 5. Contributions of measurement uncertainty components to the combined standard uncertainty of each analyte

	Standard uncertainty (‰)						
Source of Uncertainty	Et	A	T	11-OEt	11β-HA	PD	E
$R_{analyte}$, R_{IS} (repeatability)	0.12	0.06	0.17	0.08	0.07	0.10	0.26
$\delta^{13}C_{IS}$	0.25	0.25	0.13	0.45	0.25	0.34	0.63
F_{IS}	0.10	n/a	n/a	0.15	0.20	n/a	0.52
F_M	0.30	0.18	0.36	0.26	0.29	0.42	0.11
F_{IC}	0.26	0.27	0.79	0.33	0.25	0.25	0.32
u_{LTS}	0.29	0.32	0.62	0.32	0.37	0.35	0.81
u_{STS}	0.01	0.01	0.03	0.05	0.05	0.01	0.04
$u_{homogeneity}$	n/a	n/a	0.66	n/a	n/a	0.38	Captured in precision
Combined standard uncertainty	0.57 ‰	0.52 ‰	1.28 ‰	0.65 ‰	0.61 ‰	0.78 ‰	1.25 ‰

standard uncertainty of $\delta^{13}C_{CRM}$ for each analyte. The resulting combined uncertainty was then converted to an expanded uncertainty at the 95% level of confidence using the appropriate coverage factor (k), determined using the total effective degrees of freedom.

Reference and information values

Reference values and expanded uncertainties at the 95% coverage interval were determined for etiocholanolone, androsterone, 11β-hydroxyandrosterone and 5β-pregnanediol: Et −24.6 ± 1.2 ‰; A −27.9 ± 1.1 ‰; 11β-HA −23.6 ± 1.3 ‰; PD −23.4 ± 1.6 ‰. Information values and expanded uncertainties (95% coverage interval) were determined for testosterone, 11-oxoetiocholanolone and epitestosterone: T −30.3 ± 2.6 ‰; 11-OEt −23.2 ± 1.3 ‰; E −24.5 ± 2.7 ‰.

Information values and expanded uncertainties (95% confidence level) were also determined for the differences between the testosterone metabolites and the certified ERCs ($\Delta^{13}C$ values): 11β-HA – A, 4.3 ± 0.9 ‰; 11β-HA – Et, 1.1 ± 1.1 ‰; PD – A, 4.0 ± 1.1 ‰.

Conclusions

An accurate gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) method for the analysis of steroid $\delta^{13}C$ values in urine was developed. This method was used to certify the $\delta^{13}C$ values of etiocholanolone, androsterone, 11β-hydroxyandrosterone and pregnanediol in the freeze dried human urine matrix reference material NMIA MX005. Information values for testosterone, 11-oxoetiocholanolone and epitestosterone, as well as a range of $\Delta^{13}C$ values, were also determined. The certified reference values have an expanded uncertainty of between 1.1 and 1.6 ‰. These low uncertainties were achieved by considerable optimisation of the method, although it should be noted that all potential variance and biases were rigorously investigated and included in the uncertainty budget. While reference values for 11-oxoetiocholanolone and testosterone are not provided at present, reference values for these analytes may be released in the future after further investigation.

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