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Carbon isotope ratio analysis of endogenous glucocorticoid urinary metabolites after cortisone acetate and adrenosterone administration for doping control

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Glucocorticoids are listed on the World Anti-Doping Agency (WADA) Prohibited List of substances. The detection of the administration of hydrocortisone and cortisone is complicated by the fact that the human body also produces these steroids naturally. Gas chromatography-combustion-isotope ratio mass spectrometry can be utilized to determine the use of endogenous glucocorticoids by measuring the carbon isotope ratio (CIR) of their resulting metabolites in human urine samples. A comprehensive sample preparation protocol for the analysis of endogenous glucocorticoid urinary metabolites was developed and validated, incorporating the use of high performance liquid chromatography (HPLC) for purification and chemical oxidation for derivatisation. Target compounds were tetrahydrocortisol and tetrahydrocortisone, and 11β-hydroxyetiocholanolone, 11-oxoetiocholanolone and 11β-hydroxyandrosterone, while pregnanediol functioned as the endogenous reference compound. Urine samples from a population of 50 volunteers were analyzed to determine CIR reference limits. Excretion studies of the endogenous glucocorticoid preparation cortisone acetate (25 mg oral) and the dietary supplement adrenosterone (75 mg oral) were conducted with six male individuals. Variable changes in steroid metabolite isotopic composition were found across subjects after administration. The study also revealed that CIR analysis of the major glucocorticoid metabolites tetrahydrocortisol and tetrahydrocortisone is necessary to unambiguously distinguish administration of cortisone and adrenosterone, the former officially restricted to out-of-competition use by athletes, the latter not being restricted at the current time. Moreover, this study reaffirms that CIR methods for the doping control of endogenous steroids should not rely upon a single ERC, as the administration of an appropriate precursor to that ERC could cause complications during analysis. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: doping control; steroids; isotope ratio mass spectrometry; glucocorticoids

Introduction

Glucocorticoids (corticosteroids, GCS) are an important class of therapeutic drug due to their potent anti-inflammatory properties. They may also be used as doping agents and their abuse can produce adverse health effects.[1] Therefore they are listed on the World Anti-Doping Agency (WADA) Prohibited List.[2,3] The urinary metabolites of exogenous GCS such as budesonide[4] or triamcinolone acetonide[5] are routinely screened by liquid chromatography-tandem mass spectrometry (LC-MS/MS) or gas chromatography–mass spectrometry (GC-MS) for anti-doping purposes.[6–8] However, the detection of the administration of pharmaceutical preparations of cortisol (hydrocortisone, 11β,17α,21-trihydroxypregnan-4-ene-3,20-dione, F) and cortisone (17α,21-dihydroxypregnan-4-ene-3,11,20-trione, E) is complicated by the fact that both compounds are also produced naturally by the human body.[9] Measurement of the ratio of tetrahydrocortisol (3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one, THF) to tetrahydrodeoxycortisol (3α,17α,21-trihydroxy-5β-pregnan-20-one, THS) has been proposed as a screening marker for identifying suspicious urine samples in doping control.[10] Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)[11,12] of the oxidation products of the major metabolites THF and tetrahydrocortisone (3α,17α,21-trihydroxy-5β-pregnan-11,20-dione, THE) has been shown to be effective in confirming the exogenous administration of endogenous GCS preparations.[13] In contrast, analysis of the oxidation products of the minor GCS metabolites 11β-hydroxyetiocholanolone (3α,11β-dihydroxy-5β-androstan-17-one, 11β-OHEt), 11-oxoetiocholanolone (3α-hydroxy-5β-androstane-11,17-dione, 11-oxoEt) and 11β-hydroxyandrosterone (3α,11β-dihydroxy-5α-androstan-17-one, 11β-OHA) was less impressive.[13] Recent collaborative work has demonstrated that these 11-oxygenated-C_{19} compounds (11-oxy-C_{19}) are also metabolites of adrenosterone (androst-4-ene-3,11,17-trione), a naturally occurring adrenal steroid occasionally found in nutritional supplements.[14] They are also extensively used as 'endogenous reference compounds' (ERC)[15,16] in routine GC-C-IRMS methods for the confirmation of endogenous anabolic androgenic steroid administration in doping control.[17–20]

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This work aims to utilize GC-C-IRMS and the oxidation approach to sample preparation to further investigate the carbon isotope ratios (CIR) of the major and minor endogenous GCS metabolites in human urine samples with and without the administration of endogenous GCS metabolite precursors. Urine samples from a reference population of 50 drug-free subjects and six male subjects separately administered cortisone acetate and adrenosterone were analysed by GC-C-IRMS. To prepare the method for potential doping control usage, the pregnenolone metabolite pregnanediol (5β-pregnane-3α,20α-diol, PD) was incorporated to act as an 'independent' ERC for comparison to selected target compounds (TC).[21–23] PD is derived from a separate biosynthetic pathway to the androgens and is hence free from the theoretical interference of testosterone co-administration which may occur if androsterone or etiocholanolone were utilized as ERC.[13]

Experimental

Reference materials

Steroid certified reference materials were obtained from Chemical Reference Materials, National Measurement Institute (North Ryde, Australia). Other steroid standards were obtained from Steraloids (Newport, RI, USA) and Sigma Chemical Co. (St Louis, MO, USA). 5α-Androstan-3β-ol (5α-ol, CU/USADA 30–1) was a gift from the Division of Nutritional Sciences, Cornell University (Ithaca, NY, USA).

Chemicals and reagents

β-Glucuronidase enzyme from *Escherichia coli* K12 was obtained from Roche Diagnostics (Mannheim, Germany). BondElut C18® solid phase extraction cartridges (500 mg, 3 mL, #1210 2028) were purchased from Varian (Palo Alto, CA, USA). All general laboratory chemicals and reagents were of analytical grade. Potassium dichromate ($K_2Cr_2O_7$, ≥ 99.5 %, SigmaUltra) was obtained from Sigma Aldrich (Castle Hill, Australia). All organic solvents were of HPLC grade and were obtained from Merck (Darmstadt, Germany). Water was obtained using a Millipore filtration system (Bedford, MA, USA).

Reference population

The reference population consisted of urine samples collected from 50 elite athlete volunteers, with written informed consent and ethics approval.[19] Samples originated from Australia (AUS, $n = 14$: 14 male) and New Zealand (NWZ, $n = 36$: 32 male, 4 female). The average age was 21. After collection, samples were measured for pH and specific gravity, aliquoted and stored at −20 °C until analysis.

Administration studies

Six healthy male subjects (Subjects A–F, 21–34 years old) completed two separate administration studies with informed consent. Appropriate ethics approval was obtained from Southern Cross University (Lismore, NSW, Australia, ECN-05-24) and no special diet or exercise regime was maintained during the study periods. An appropriate equilibration interval between studies was taken (> 4 weeks). In Study 1, a single tablet of Cortate™ containing 25 mg cortisone acetate was ingested. For Study 2, one capsule of the 11-OXO™ supplement (containing 75 mg adrenosterone) was consumed. Two pooled baseline urine samples (AM and PM) were collected the day before administration. Spot urine samples were collected for 24 h directly after administration. Two further pooled samples (AM and PM) were collected on the second day after administration. Urine samples were measured for pH and specific gravity, aliquoted and stored at −20 °C until analysis.

Sample preparation and analysis

Urinary GCS metabolites were prepared for GC-C-IRMS analysis using the oxidation approach.[24–27] The major endogenous GCS metabolites THF and THE contain a C_{20}-C_{21} corticosteroid side-chain which is not typically amenable to direct GC analysis. Derivatization is normally required, for example the formation of methyloxime trimethylsilyl ether derivatives[28] – which can be time-consuming and may lead to a loss of sensitivity if incomplete reaction occurs and multiple products are formed. Chemical oxidation on the other hand, removes the polar side-chain and converts all secondary hydroxyl groups to ketones, producing a derivative suitable for GC analysis. Furthermore, no additional carbon atoms are added during the derivative formation negating the requirement to correct the raw $\delta^{13}C$ values obtained from the GC-C-IRMS.[29,30] The disadvantage with the approach is that chemical information can be reduced. As outlined in Figure 1, both THF and THE and also 11β-OHEt and 11-oxoEt, can combine to form the same derivative after oxidation – 5β-androstanetrione (5β-androstane-3,11,17-trione, 5β-AT). Likewise, the other major GCS metabolite 5α-tetrahydrocortisol (5α-THF) and 11β-OHA can form the isomeric derivative 5α-AT, while PD is converted to 5β-pregnanedione (5β-pregnan-3,20-dione, Pdione). The implemented sample preparation protocol is based upon the work of Buisson, Mongongu *et al.*[13] where high performance liquid chromatography (HPLC) purification enabled isolation of these potentially interfering analyte classes (major and minor) into separate fractions before oxidation to androstanetrione derivatives. Although time consuming, the process ensures clean, interference free fractions for reliable GC-C-IRMS determination of both the major and minor GCS metabolite classes.

Urine samples were processed in batches of up to 20 samples, including blank (purified water), negative (blank urine) and positive (pooled urine made from cortisone acetate and adrenosterone administration urines from previous studies[13,14]) quality control samples. Steroid glucuronide metabolites were initially isolated from urine (4–30 mL) and purified as described in a previous publication.[31] Briefly, solid phase extraction was completed with previously conditioned BondElut C18 SPE cartridges (4.5 mL H_2O wash, 6 mL methanol elution). After enzymatic hydrolysis of the dried and reconstituted extract (1.5 mL of 0.2 M phosphate buffer, 60 μL β-glucuronidase, 1.5 h at 50 °C), the deconjugated steroids were isolated by liquid-liquid extraction (K_2CO_3 buffer, pH 9, *tert*-butyl-methyl ether, 2 x 4 mL). The combined organic extract was fortified with β-trenbolone retention time marker, dried and reconstituted in acetonitrile/water for purification by HPLC (Waters 2695 HPLC with a Waters 996 photodiode array detector) on a reverse-phase C18 column (Phenomenex Gemini C18 column, 250 mm x 4.6 mm, 3 μm) using a water/acetonitrile mobile phase gradient (10–100 % acetonitrile over 18 min) with automated fraction collection (Gilson PrepFC). For this initial HPLC (termed HPLC A), three fractions were collected: Fraction A1 8.6–12.0 min (THF and THE); Fraction A2 12.9–14.5 min (11-oxy-C_{19} metabolites); Fraction A3

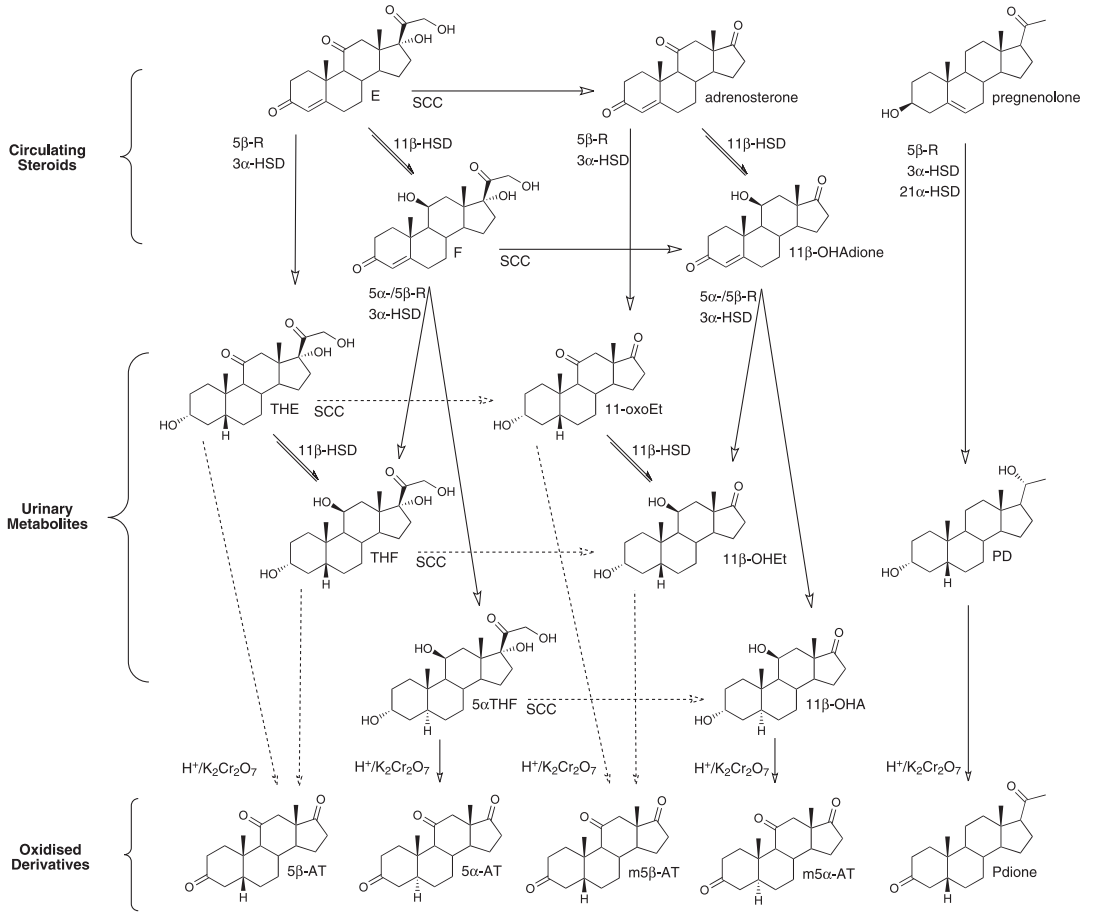

Figure 1. Endogenous GCS urinary metabolism and oxidised derivative formation with potassium dichromate. SCC = Side chain cleaving enzyme. 5α-/5β-R = 5α-/5β-reductase enzyme 11β-HSD = 11β-hydroxysteroid dehydrogenase enzyme. 3α-HSD = 3α-hydroxysteroid dehydrogenase enzyme. 21α-HSD = 21α-hydroxysteroid dehydrogenase enzyme.

21.7–24.1 min (PD), relative to β-trenbolone at 12.1 min. A high concentration standard of these analytes was run at the beginning of each batch to check retention times and collection windows. Additionally, chromatography was monitored at 345 nm (λ_{max} of β-trenbolone) to ensure adequate fraction collection for each extract injected. For selected urines originating from the adrenosterone administration study, Fractions A1 and A2 were collected together: 8.6–14.5 min (THF and THE and 11-oxy-C_{19} metabolites). The combined fraction was subsequently dried and reconstituted in acetonitrile/water for purification by HPLC using the same analytical hardware as described above except that a different HPLC gradient elution profile was used (10–100% acetonitrile over 21 min). Relative to β-trenbolone at 15.1 min, THF and THE were collected together between 10.8 and 13.3 min (A'1), with the 11-oxy-C_{19} metabolites at 15.9–18.8 min (A'2).

The collected fractions were separately evaporated to dryness before reconstitution in acetonitrile (120 μL). Oxidation reagent (200 μL, 5% *w/v* $K_2Cr_2O_7/H_2SO_4/H_2O$) was added and then the fractions were warmed on a heat block (30 °C, 20 min). Following oxidation, fractions were cooled in a water bath (< 20 °C) and the reaction was quenched by addition of 10% $NaHCO_3$ solution (300 μL). The oxidized steroids were subsequently isolated from the reaction medium with liquid-liquid extraction (2 x 4 mL, 1:2 dichloromethane/hexane). The combined organic extract was fortified with gestrinone retention time marker, dried and reconstituted in acetonitrile/water for HPLC using the same analytical parameters as described above. For the second HPLC purification (termed HPLC B), two separate fractions were collected depending on which oxidized fractions from HPLC A were to be run: Fraction B1 13.1–15.5 min (5β-AT derived from THF and THE and 5α-AT derived from 5α-THF; or 5β-AT and 5α-AT derived from the minor 11-oxy-C_{19} GCS metabolites 11-oxoEt, 11β-OH-Et and 11β-OHA, herein abbreviated as m5β-AT or m5α-AT to avoid confusion as to their origin) and Fraction B2 21.6–23.9 min (Pdione), relative to gestrinone at 15.6 min. As above, a high concentration standard of the relevant analytes was run at the beginning of each batch to check retention times and collection windows. Additionally, chromatography was monitored at 345 nm (λ_{max} of gestrinone) to ensure adequate fraction collection for each oxidized fraction injected. After collection, each fraction was supplemented with internal standard (5α-ol), thoroughly dried and reconstituted in an appropriate volume of solvent (ethyl acetate). As previously reported, each fraction was screened by full scan GC-MS (*m/z* 50–400) prior to GC-C-IRMS analysis to confirm analyte identity, establish peak purity and to estimate the analyte concentration.[19] After appropriate adjustment of the extract volume and the internal standard concentration, the extract fractions were analyzed by GC-C-IRMS employing instrumental conditions and carbon dioxide reference gas calibration as previously described.[19]

Results

Method validation

Linearity and stability

The linearity of the instrumental response for the analytes of interest was tested across a range of 25–150 ng of reference material injected on column (6 levels, 3 injections each, Figure 2). The linearity (calculated as the slope of the line of best fit and expressed as permill per volt, ‰/V) was 0.15, 0.26 and −0.06 for the analytes 5β-AT, 5α-AT and Pdione respectively. Correspondingly, chromatographic peaks with intensity within the range of 0.4–4.0 volts (V) were deemed acceptable. The internal standard 5α-ol was analyzed in every extract fraction prepared and the results obtained are summarized in Figure 3 ($n = 617$). For this analysis, no values were excluded due to peak height (i.e. < 0.4 V or > 4.0 V). The mean and median $\delta^{13}C$ were both −29.1 ‰ (± 0.5 ‰ standard deviation) (certified value −29.7 ‰ [32]). If restricted to within 0.4 V and 4.0 V, the mean and median $\delta^{13}C$ did not change, however the standard deviation improved (± 0.4 ‰, $n = 515$). This can be attributed to the larger variability observed for chromatographic peaks with amplitudes below 0.4 V ($n = 35$, $\delta^{13}C = -29.1$ ‰ ± 0.9 ‰, Figure 3). Although there was an observed bias relative to the certified value (−0.6 ‰, statistically significant difference, *t*-test, *p*-value < 0.0001), the measurement was stable over the 6 month period of the project.

Recovery/limit of detection

Overall extraction recovery was estimated by preparation and analysis of 10 mL water blanks spiked at 1000 ng/mL with each investigated steroid analyte. GC-MS quantification of oxidized extracts was estimated by comparison to reference materials. Calculated recovery for the ERC Pdione and the major GCS target compounds (TCs) was especially low relative to the 11-oxy-C_{19} metabolites (Table 1). Further experiments attributed this difference to low recovery at the oxidation step (~ 40%, data not shown). Taking into account the linearity of the instrument discussed above and assuming a urine volume of 10 mL to be extracted with a final extract volume of 10 μL and an injection volume of 2 μL, the limit of detection for each analyte was estimated and deemed fit-for-purpose for the analysis of reference population and administration study urines (Table 1).

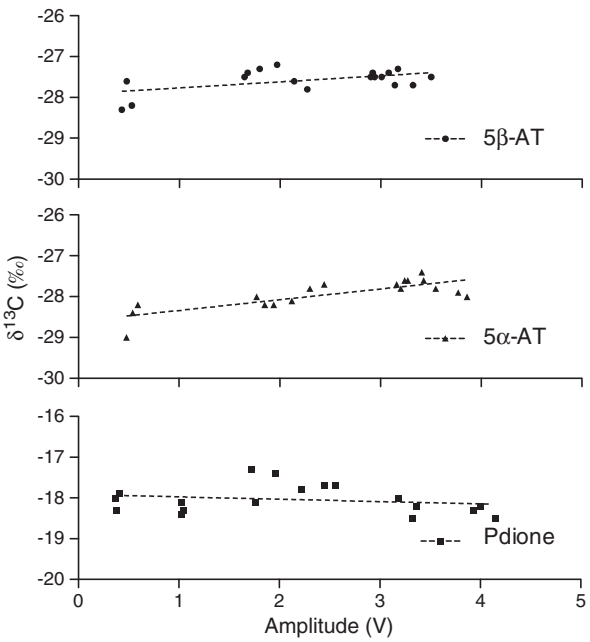

Figure 2. Linearity assessment for the three major analytes of interest.

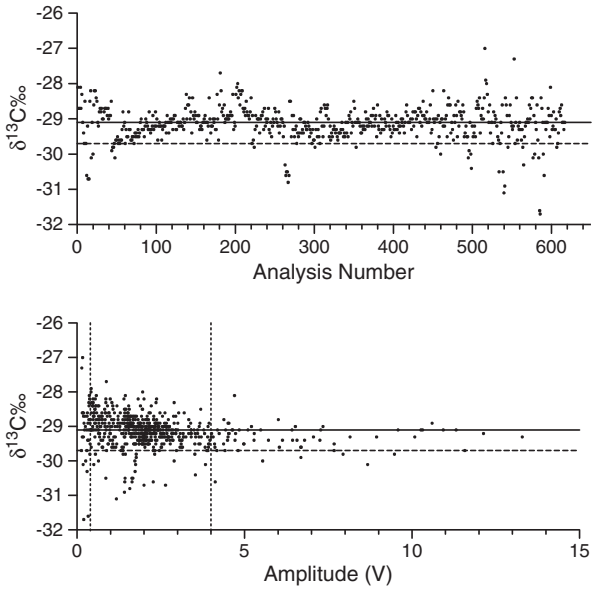

Figure 3. 5α-androstanol internal standard results over six months (n = 617). Solid line is mean $\delta^{13}C$ value. Dashed line is certified $\delta^{13}C$ value. Vertical dotted line is 0.4 V – 4.0 V linearity acceptance range.

Selectivity/specificity

As extensive clean-up using solid-phase extraction (SPE), liquid-liquid extraction and two-fold HPLC purification was used in the preparation of oxidized urine extracts, chromatographic interferences were largely avoided. Furthermore, extracts prepared for GC-C-IRMS were pre-screened by full-scan GC-MS for confirmation of peak purity and analyte identity. Example GC-C-IRMS chromatograms for the negative quality control urine fractions A1-A3 after oxidation are shown in Figure 4.

Due to the unequal concentration of the 5β- and 5α-reduced major GCS metabolites typically found in urine samples, the relative abundance of 5β-AT and 5α-AT was often unfavourable making the analysis of the smaller component 5α-AT difficult.[33] Often the much larger 5β-AT would display unacceptable

Table 1. Estimated recovery and limit of detection (LOD) using 1000 ng/mL spiked water blanks

	GCS (A1)	11-oxy-C_{19} (A2)		PD (A3)
1000 ng/mL	5β-AT	m5β-AT	m5α-AT	Pdione
Recovery (%)	12.1	64.1	60.8	14.7
St Dev (%)	1.8	10.8	11.3	4.2
LOD (ng/mL)	207	39	41	170

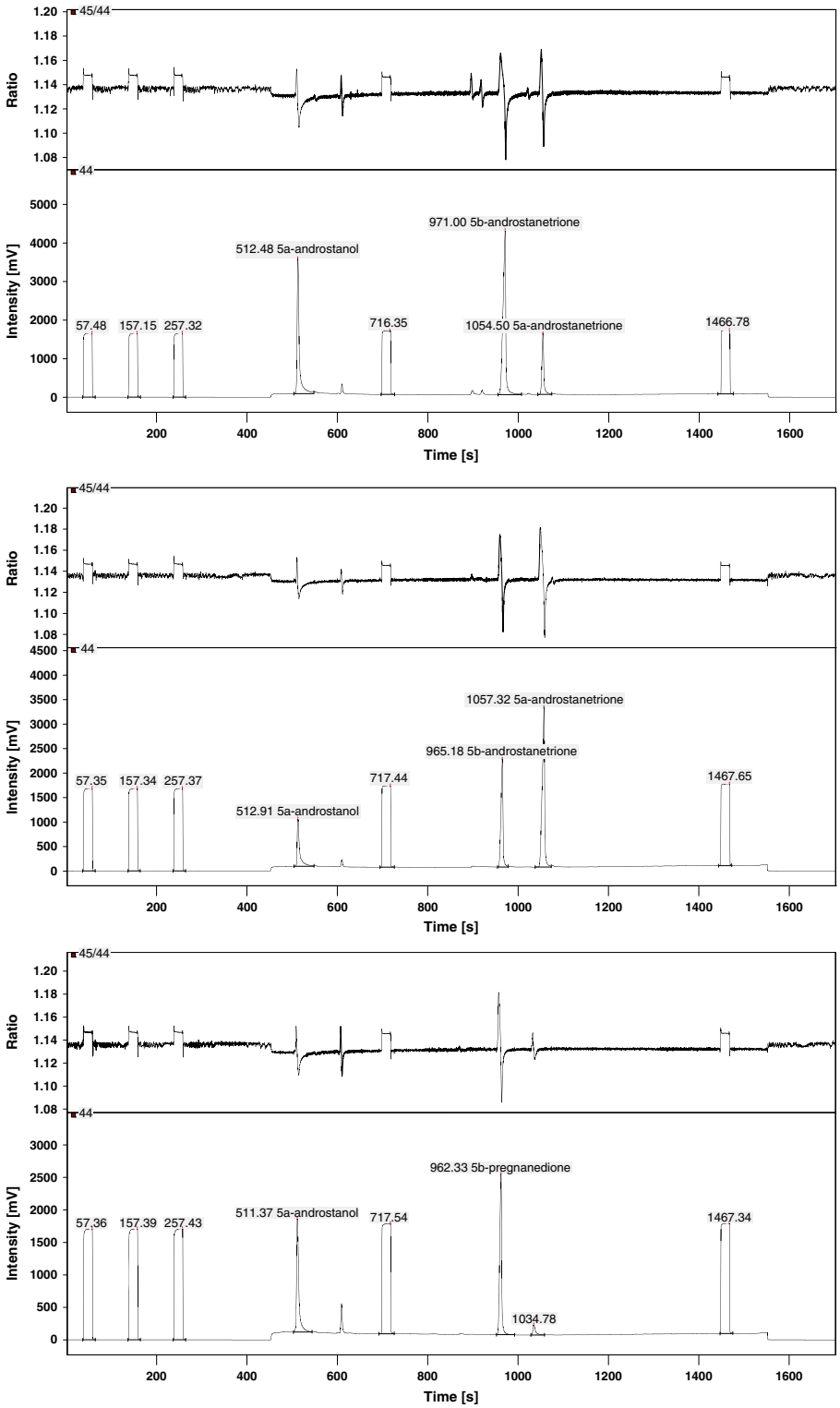

Figure 4. GC-C-IRMS chromatograms of Fraction A1 - 5b-AT, 5a-AT (top), A2 - m5b-AT, m5a-AT (middle) and A3 - Pdione (bottom).

chromatographic properties when the extract volume was optimized for the analysis of 5α-AT (Figure 4). Conversely, if the extract volume was optimized for 5β-AT, 5α-AT would be below the 0.4 V linearity limit. Therefore for the ease of this investigation, only data for 5β-AT has been collected and reported below.

Repeatability/reproducibility

Repeatability of the method was estimated by analysis of a batch of seven replicates of each quality control urine sample (Table 2). For the positive control urine, variability in the measurement was reduced with increasing concentration of the analyte. When

target compounds were elevated (as the sample originated from pooled administration study samples), the calculated standard deviation was relatively small (0.2–0.3 ‰, Table 2). In contrast, when the ERC PD was low in concentration (production suppressed due to negative feedback after exogenous steroid administration), a larger than expected variability for the oxidized derivative Pdione was shown (0.8 ‰). Reproducibility was demonstrated by the analysis of the same control urines over an extended time period ($n = 20$, one aliquot per batch of analysis, over 6 months). Table 3 summarizes the obtained data for comparison. As expected, batch-to-batch variability is increased relative to within-batch, but all calculated standard deviations were less than 1.1 ‰ indicating that the method was fit-for-purpose for the analysis of reference population and administration study urine samples for this study.

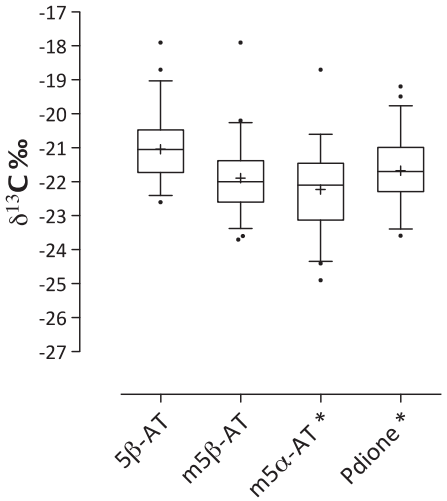

Figure 5. Reference Population ($n = 50$) - $\delta^{13}C$. * normally distributed – D'Agostino - Pearson Omnibus Normality Test

Reference population

For the 50 reference population samples tested, the mean $\delta^{13}C$ values obtained were −21.0, −21.9, −22.2 and −21.7 ‰ for the derivatives 5β-AT (THF and THE), m5β-AT (11β-OHEt and 11-oxoEt), m5α-AT (11β-OHA) and Pdione (PD) respectively. Boxplots are displayed in Figure 5. The results for these steroid metabolites compare favourably with previous profiling studies completed using the same oxidation approach[13] as well as for others using different sample preparation techniques (in free form[19] or acetylated[20]). Differences between ERC (Pdione) and TC (major and minor GCS metabolites) were also calculated (defined as $\Delta^{13}C = \delta^{13}C_{ERC} - \delta^{13}C_{TC}$). The mean $\Delta^{13}C$ for Pdione - 5β-AT, Pdione - m5β-AT and Pdione - m5α-AT were −0.6, 0.2 and 0.6 ‰ respectively (Figure 6). It should be noted that the maximum $\Delta^{13}C$ for Pdione - m5α-AT observed was 3.0 ‰ - directly at the WADA specified criterion limit for positivity of 3 ‰.[16] For this analyte, a more 'conservative'[34] limit of 3.9 ‰ (calculated as the reference limit, i.e. population mean + 3SD) would be advised to be applied if this method of analysis was to be used for doping control purposes.[18] For comparison, the corresponding reference limits calculated for the other ERC-TC pairs were 1.7 ‰ (Pdione – 5β-AT) and 3.0 ‰ (Pdione – m5β-AT).

Administration studies

Administered substrates

The cortisone acetate and adrenosterone substrates were analyzed prior to commencement of the study as previously described.[14] However to enable GC analysis, the extracted cortisone acetate was additionally converted to its oxidized derivative adrenosterone and purified by HPLC B using the protocol described for urine extracts. The mean $\delta^{13}C$ value for the active steroid in the cortisone

Table 2. Repeatability (within-batch variability) - $\delta^{13}C$ and $\Delta^{13}C$

	GCS (A1)	11-oxy-C_{19} (A2)		PD (A3)	Pdione -	Pdione -	Pdione -
$n = 7$	5β-AT	m5β-AT	m5α-AT	Pdione	5β-AT	m5β-AT	m5α-AT
Negative Control Urine							
Mean (‰)	−22.1	−22.9	−22.7	−21.8	0.3	1.1	0.9
St Dev (‰)	0.6	0.6	0.6	0.4	0.4	0.3	0.5
Positive Control Urine							
Mean (‰)	−26.4	−29.3	−30.2	−22.3	4.1	7.0	7.9
St Dev (‰)	0.3	0.2	0.3	0.8	0.9	0.8	0.8

Table 3. Reproducibility (batch-to-batch variability) over six months - $\delta^{13}C$ and $\Delta^{13}C$

	GCS (A1)	11-oxy-C_{19} (A2)		PD (A3)	Pdione -	Pdione -	Pdione -
$n = 20$	5β-AT	m5β-AT	m5α-AT	Pdione	5β-AT	m5β-AT	m5α-AT
Negative Control Urine							
Mean (‰)	−21.3	−21.9	−21.3	−21.8	−0.5	0.1	−0.5
St Dev (‰)	0.7	0.7	0.8	0.5	1.1	0.8	0.9
Positive Control Urine							
Mean (‰)	−25.9	−29.5	−29.8	−21.8	4.0	7.6	7.9
St Dev (‰)	0.7	0.6	0.6	0.8	1.0	1.0	0.9

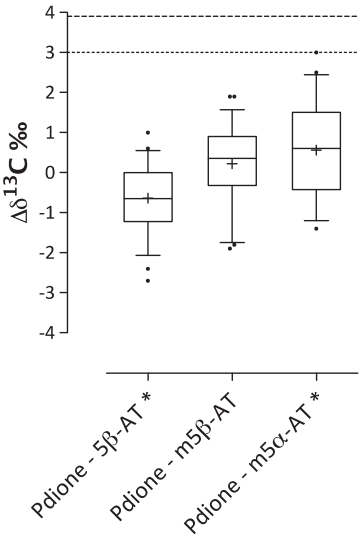

Figure 6. Reference Population ($n = 50$) - $\Delta^{13}C$. * significantly different to 0 ‰ – One sample t-test. Dotted line represents the WADA specified positivity criterion of 3 ‰. Dashed line represents the population derived reference limit for Pdione – m5α-AT of 3.9 ‰.

acetate tablet was −26.9 ‰ ± 0.5 ‰ ($n = 7$) and in the adrenosterone containing capsule was −30.4 ‰ ± 0.5 ‰ ($n = 7$).[14]

Cortisone acetate

THF and THE are known as the major metabolites of endogenous glucocorticoids such as cortisone. As expected, a pronounced change in the $\delta^{13}C$ value of the 5β-AT oxidized derivative was observed for all six subjects after oral cortisone acetate administration. The maximum depletion occurred for all subjects within 12 h of administration. The lowest $\delta^{13}C_{5\beta\text{-AT}}$ value observed was −26.6 ‰ for Subject D, 4 h after administration. The average of the minimum $\delta^{13}C_{5\beta\text{-AT}}$ recorded for each subject was −25.5 ‰ ± 0.8 ‰ as outlined in Table 4.

Additionally, the minor 11-oxy-C_{19} endogenous glucocorticoid metabolites 11β-OHEt and 11-oxoEt and also 11β-OHA were analyzed, represented by the results for the oxidized derivatives m5β-AT and m5α-AT respectively. Significantly, similar depletion to 5β-AT was observed for m5β-AT, but not for 5α-AT (Table 4).

This contrast was further demonstrated in the plotted excretion profiles of the $\Delta^{13}C$ pairs of Pdione-5β-AT, Pdione-m5β-AT and Pdione-m5α-AT for all six subjects (Figure 7). Only for Subject E was the $\Delta^{13}C_{\text{Pdione-m5}\alpha\text{-AT}}$ significantly altered after administration. Some disturbance was noted for Subjects A and F, but for Subjects B-D no change in $\Delta^{13}C_{\text{Pdione-m5}\alpha\text{-AT}}$ was apparent. Basal values for all $\Delta^{13}C$ were re-established after 36 h for the majority of subjects, though prolonged excretion was noted for Subject D.

Adrenosterone

As demonstrated in our previous research, the administration of adrenosterone leads to an increased excretion of the 11-oxy-C_{19} endogenous glucocorticoid metabolites and their concomitant depletion in ^{13}C as measured by GC-C-IRMS.[14] Adrenosterone is metabolically 'downstream' from F and E as it lacks the C_{20}-C_{21} corticosteroid side-chain. Therefore administration of adrenosterone should not alter the $\delta^{13}C$ of the major GCS metabolites. Preliminary analysis of high concentration adrenosterone administration urines using the oxidation protocol indicated that this was not the case. The cause of the observed ^{13}C depletion in 5β-AT however, was in fact the presence of minor reduced adrenosterone metabolites (e.g. 3α,17β-dihydroxy-5β-androstan-11-one) found to be eluting within the A1 fraction, which upon oxidation was capable of combining to form 5β-AT. For those small number of urines with very large concentrations of adrenosterone metabolites, thorough HPLC fraction collection was required to exclude 3α,17β-dihydroxy-5β-androstan-11-one from Fraction A1. Therefore, Fraction A1 and A2 were collected together (8.6 – 14.5 min) and subsequently re-chromatographed with a slower HPLC gradient and optimized fraction collection program allowing the isolation of THF and THE (A1`), 3α,17β-dihydroxy-5β-androstan-11-one (A1``) and the 11-oxy-C_{19} metabolites (A2) into three separate fractions. Subsequent CIR analysis of these purified fractions demonstrated that THF and THE (5β-AT) were indeed unaffected by the administration of adrenosterone (Table 4).

As expected, both 5β- and 5α-reduced 11-oxy-C_{19} metabolite derivatives displayed significant ^{13}C depletion in post-administration samples, approaching that of the administered substrate (mean value for all subjects was −30.6 ‰, Table 4). The peak of excretion appeared rapidly within 12 h, with all subjects registering $\Delta^{13}C$ values in excess of 8 ‰ (Figure 8). For the majority of subjects, prolonged depletion of m5β-AT was observed, with the $\Delta^{13}C_{\text{Pdione-m5}\beta\text{-AT}}$ still greater than 4 ‰ 24 h after administration. In contrast, m5α-AT displayed a rather faster return to basal levels, falling below the calculated reference limit for $\Delta^{13}C_{\text{Pdione-m5}\alpha-\text{AT}}$ of 3.9 ‰ by 24 h post-administration.

Table 4. Minimum $\delta^{13}C$ values (‰) recorded during cortisone acetate and adrenosterone administration studies.

$\delta^{13}C$ (‰)	Cortisone Acetate				Adrenosterone			
Subject	5β-AT	m5β-AT	m5α-AT	Pdione	5β-AT	m5β-AT	m5α-AT	Pdione
A	−25.5	−26.7	−22.7	−22.0	−22.2	−31.2	−31.2	−21.5
B	−24.2	−25.1	−20.7	−21.9	−23.0	−30.1	−30.1	−21.7
C	−25.7	−25.1	−22.1	−20.6	−23.3	−30.3	−30.3	−22.6
D	−26.6	−25.1	−21.6	−21.7	−22.3	−30.9	−30.9	−22.0
E	−25.5	−24.9	−24.1	−21.2	−22.4	−30.9	−30.9	−21.1
F	−25.2	−25.4	−21.7	−21.4	−22.8	−30.1	−30.1	−21.1
Average (‰)	**−25.5**	**−25.4**	**−22.1**	**−21.5**	**−22.7**	**−30.6**	**−30.6**	**−21.7**
St Dev (‰)	**0.8**	**0.7**	**1.1**	**0.5**	**0.4**	**0.5**	**0.5**	**0.6**

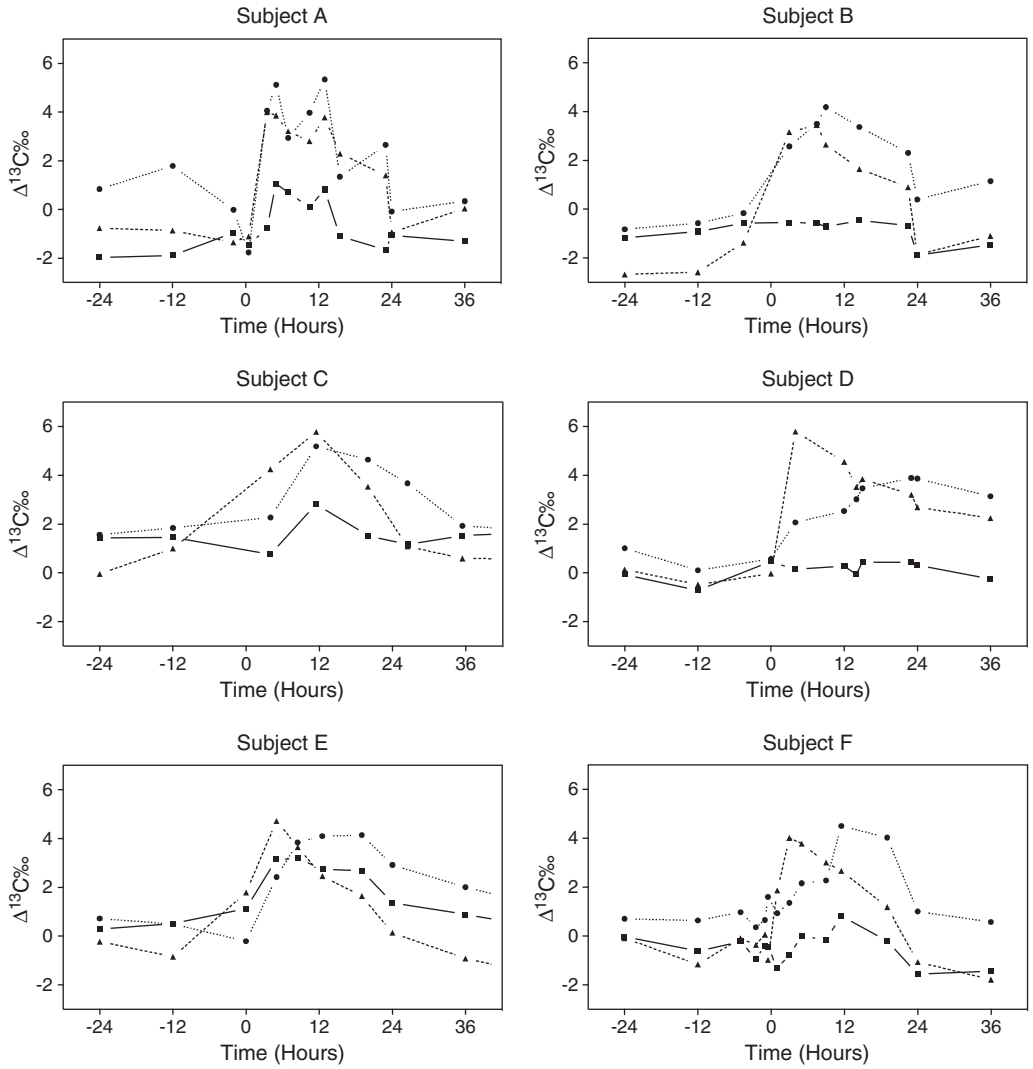

Figure 7. Cortisone acetate administration study $\Delta^{13}C$ results for Subject A – F. Triangles (Pdione – 5β-AT), Circles (Pdione – m5β-AT) and Squares (Pdione – m5α-AT).

Discussion

Doping control

The oxidation GC-C-IRMS methodology outlined has reaffirmed that substantial changes in CIR for both the major and minor endogenous GCS metabolites are observed after the administration of preparations such as the medication cortisone acetate and the dietary supplement adrenosterone. Isotopic differences between the nominated ERC and specified TCs exceeded both the WADA-defined positivity criterion of 3‰ and the experimentally derived population reference limits. Obtained method validation data has demonstrated that with appropriate improvements to extraction recovery and analysis precision, this approach would be suitable for the confirmation of the administration of endogenous GCS for doping control.[16]

Specifically, the appropriate TCs for CIR confirmation of cortisone misuse are the 5β-reduced endogenous GCS metabolite pairs THF and THE (5β-AT), and 11β-OHEt and 11-oxoEt (m5β-AT) as they display significant changes after administration relative to PD (Pdione). This can been seen in Table 5, which displays the mean $\Delta^{13}C$ for all administration study samples analyzed, separated into defined post-administration time periods. Considering the number of samples within each group, it can be calculated that 89% of samples are positive to one or more ERC-TC marker between 0 and 12 h after cortisone acetate administration. This rate falls to 75% between 12 and 24 h, and after 24 h no samples can be declared positive. When utilizing the population based reference limit to set positivity criteria (i.e. 3.9 ‰), the 5α-reduced endogenous GCS metabolite 11β-OHA (m5α-AT) was unable to identify any positive samples after cortisone acetate administration. This reinforces the findings of Buisson *et al.*, which advised that the minor GCS metabolites can give less impressive results when compared to the major metabolites.[13]

For adrenosterone, the data obtained herein confirm our previous results that both 11-oxoEt (m5β-AT) and 11β-OHA (m5α-AT) are ideal TCs for CIR doping control analysis.[14] Between 0 and 36 h, 100 % of samples were capable of being declared positive to one or more ERC-TC marker. Additionally, prolonged depletion of the m5β-AT derivative resulted in a further 50% of samples collected after 36 h also being found positive.

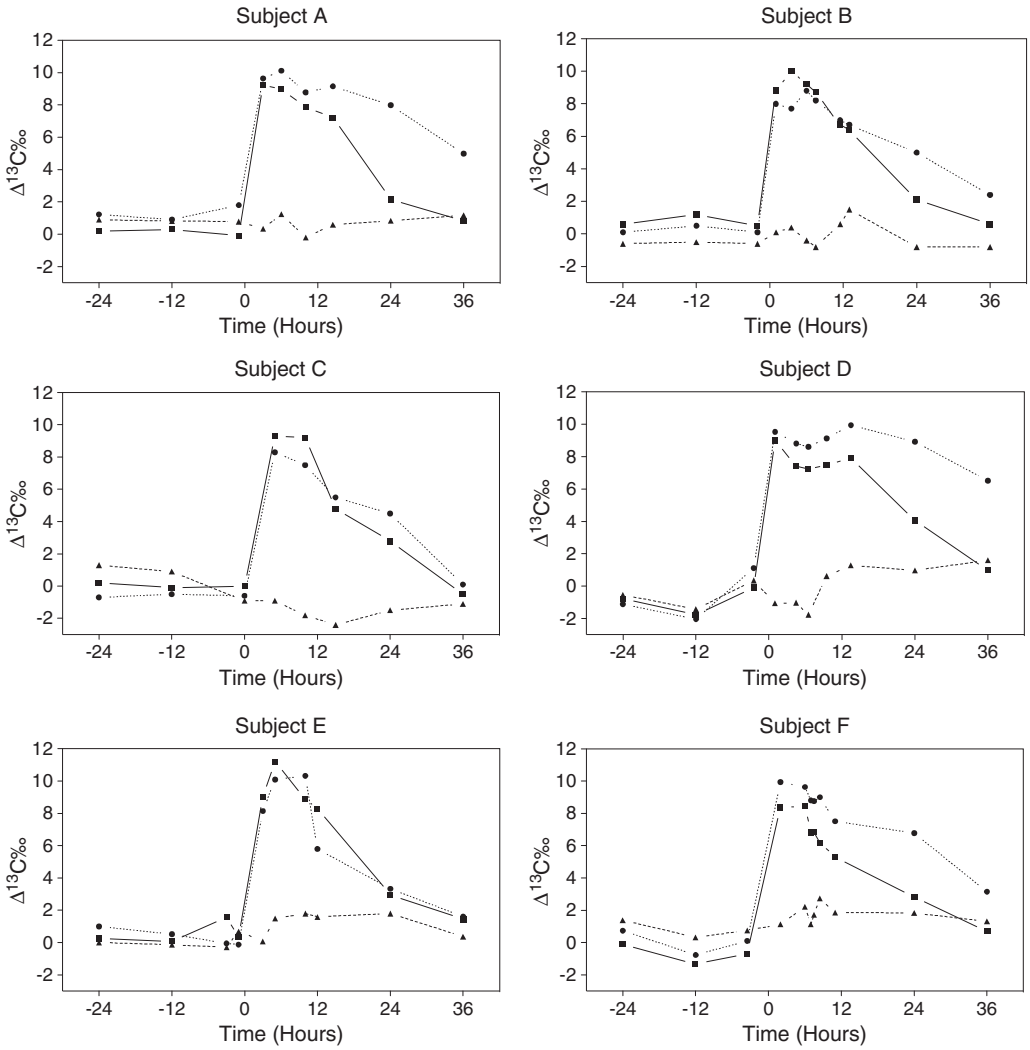

Figure 8. Adrenosterone administration study $\Delta^{13}C$ results for Subject A – F. Triangles (Pdione – 5β-AT), Circles (Pdione – m5β-AT) and Squares (Pdione – m5α-AT).

CIR results and GCS metabolism

Although generally described in anti-doping CIR literature as an endogenous GCS metabolite,[34,35] the CIR results obtained during this project suggests that 11β-OHA is primarily derived from a separate metabolic pathway, most probably adrenally produced 11β-hydroxyandrostenedione (11β-hydroxyandrost-4-ene-3,17-dione, 11β-OHAdione, Figure 1).[36] For a given steroid preparation, the resulting $\delta^{13}C$ value of its urinary metabolites after administration will be determined by:

- the $\delta^{13}C$ of the administered substrate and the size of its dosage

AND

- the basal $\delta^{13}C$ of the endogenous steroids produced by the organism and the size of the steroid metabolite pool present within (which may change due to fluctuations in production due to feedback) with which the substrate metabolites will mix

AND

- any metabolic fractionation that may occur.[37]

Table 5. Mean $\Delta^{13}C$ during specified time periods of the administration studies.

$\Delta^{13}C$ (‰)	Cortisone Acetate			Adrenosterone		
Hours	Pdione-5β-AT	Pdione-m5β-AT	Pdione-m5α-AT	Pdione-5β-AT	Pdione-m5β-AT	Pdione-m5α-AT
Pre-admin	−0.5	0.6	−0.3	0.2	0.1	0.0
0-12	3.5	2.9	0.4	0.5	8.7	8.3
12-24	2.5	3.5	0.4	0.3	7.8	6.6
24-36	0.4	2.0	0.0	0.5	6.1	2.8
36+	−0.2	1.4	−0.1	0.4	3.1	0.7

After cortisone acetate administration, little or no change to the $\delta^{13}C$ of 11β-OHA was observed for the majority of subjects. This indicates that either cortisone is not at all metabolized to 11β-OHA (i.e. 5α-THF does not undergo side-chain cleavage to 11β-OHA – unlikely as Subject E displayed a pronounced change in $\delta^{13}C$ for m5α-AT) or another endogenous steroid present in much larger concentrations (that also metabolizes to 11β-OHA) has diluted the small amount of 11β-OHA that is actually formed such that there was no discernable change in $\delta^{13}C$. Critically for the cortisone acetate study, both the low dosage (25 mg) and the $\delta^{13}C$ of the substrate (−26.9 ‰) were unfavourable for observing if small amounts of 11β-OHA were actually formed from cortisone. If the dosage was larger and the $\delta^{13}C$ of the substrate more depleted (e.g. −30 ‰), a more significant change in $\delta^{13}C$ of 11β-OHA *may* have been observed. In fact, this was the case for adrenosterone – the dosage was three times that of cortisone acetate and the substrate value was more than 3.5 ‰ more depleted. It is for this reason that a considerably more prolonged detection period of administration was observed for adrenosterone with significantly more depleted $\delta^{13}C$ values (Table 5). Regardless, an inspection of the excretion profiles for both cortisone acetate (Figure 7) and adrenosterone (Figure 8) demonstrates that there are significant individual differences in the production of endogenous GCS (i.e. the steroid pool size) and their proportion of metabolism to the major and minor metabolites.

Conclusion

Analysis of a reference population and two administration studies has demonstrated that the oxidation approach to sample preparation for GC-C-IRMS analysis of urinary steroid metabolites is an appropriate tool for anti-doping laboratories to confirm the exogenous administration of endogenous steroids such as cortisone. While the current methods of CIR analysis available for the key analyte 11-oxoEt (both free and acetylated) are simpler and less time consuming,[10,19,20] only the full analysis of the major GCS metabolites THF and THE would ensure the correct determination of the steroid used in administration. This is because the 11-oxy-C_{19} metabolites are capable of being produced by both endogenous GCS and adrenosterone, whereas THF and THE can only be derived from endogenous GCS. The 11-oxy-C_{19} metabolites have also been routinely used as ERCs for the doping control analysis of testosterone. A possible scenario exists where an athlete may consume hydrocortisone or cortisone out-of-competition or with a therapeutic use exemption (TUE, as allowed under the WADA Code[38]), or use an adrenosterone containing supplement (which is not currently on the WADA Prohibited list[3]) therefore resulting in a highly abnormal CIR test ($\Delta^{13}C_{GCS\text{-}androgen} << -3$ ‰). Or more troubling, co-administer testosterone or a testosterone prohormone with hydrocortisone, cortisone or adrenosterone resulting in depleted $\delta^{13}C$ values of steroid metabolites but a $\Delta^{13}C$ for ERC_{GCS}-$TC_{androgen}$ approaching 0 ‰. With further development to the method to improve extraction recovery and precision, the oxidation approach could be used to investigate the nature of this disturbance and elucidate its origin. This study has demonstrated that CIR methods for the doping control of testosterone should not rely upon a single ERC, as the administration of an appropriate precursor to that ERC could cause complications during analysis. Analytical methods should be developed with the capability to detect multiple ERCs, thus alleviating any interference from potential co-administrations.

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