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Two-dimensional gas chromatography with heart-cutting for isotope ratio mass spectrometry analysis of steroids in doping control

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The accuracy and precision of gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) measurements are highly dependent on analyte purity. Reliable analysis of urinary steroids for doping control therefore requires extensive and time-consuming sample preparation (i.e. liquid chromatography fraction collection) prior to GC-C-IRMS analysis. The use of two-dimensional GC (GC-GC) with heart-cutting (Deans Switch) as a possible approach to reduce the sample purification required for IRMS analysis is described herein. The system uses a low thermal mass oven (LTM) incorporated into an existing GC-C-IRMS system. GC-GC allowed the use of a cyanopropyl/phenyl column in the first dimension to optimize the separation of underivatized steroids, while a phenyl-methylpolysiloxane column in the second dimension focuses the selectively cut analytes into narrower peaks for more sensitive and reliable MS analysis. In addition, to confirm analyte identity, eluent from the second GC was split, with 20 % entering a scanning MS, and 80 % flowing to the IRMS. As a proof concept, the developed method was then used to analyze a single spot urine (5 ml) from an individual receiving T therapy (2 × 50 mg sachets of Testogel®). The T delta value (−27.8 ‰, [T] = 38 ng/ml) was clearly distinct from 11-ketoetiocholanolone (−22.5 ‰) (used as an endogenous reference compound (ERC)), indicating T as being of exogenous origin. The simultaneous analysis by the scanning MS yielded a full scan mass spectrum of the same chromatographic peak, thus confirming the peak to be T. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: IRMS; Steroids; GC-GC; Heart-cutting; Testosterone; Multidimentional GC

Introduction

The potential abuse of testosterone (T) in sports remains a significant problem, with 1884 'atypical findings' reported by the World Anti-Doping Agency (WADA) in 2010.[1,2] The popularity of T use in this context is most likely a consequence of multiple factors, including the wide range of formulations available, its anabolic properties (though other anabolic steroids display increased potency), and perhaps the difficulty in proving T administration.[3] This difficulty arises from the endogenous nature of T, which means it is present in all doping control samples. Criteria based on concentration thresholds such as T (as glucuronide) at 200 ng/ml (adjusted to a specific gravity 1.020), and the peak area or height ratio of T to epitestosterone (EpiT) (threshold of 4:1) are currently applied under WADA documentation to select samples for additional analysis.[2] These thresholds though do not provide definitive proof of T administration. Natural outliers outside these thresholds are known to occur (i.e. 9:1), while conversely, in some instances the administration of T may not result in these thresholds being exceeded.[4,5] An alternative ratio based on T to luteinizing hormone (LH), which is suppressed following T administration, can also prove useful in possible cases of T administration.[6,7] A superior approach to population derived reference limits may be to monitor an individual athletes (intra-individual) steroid profile over time, allowing athletes to act as their own reference.[8]

Gas chromatography isotope ratio mass spectrometry (GC-C-IRMS) has been employed over the last 15 years to confirm the administration of synthetically derived endogenous steroids.[9–11] The technique relies on the fact that the isotopic composition of elements within a substance are not fixed, but influenced by isotopic fractionation.[12,13] As such, the isotopic profile of a compound is dependent on the source of materials used and the processes involved in its synthesis. IRMS was first developed in the 1940s and 1950s as a technique that can precisely measure these small differences in the isotopic ratio of an element (usually carbon for steroid analysis).[14,15] The technique can therefore distinguish between the same molecule derived from different sources and/or produced by differing methods. Isotope ratio data for non-isotopically enriched compounds is usually reported as a delta value, which represents the difference between the carbon isotope ratio of the analyte of interest and that of the given reference standard (Equation 1).[16]

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$$\delta^{13}C = \frac{\left(^{13}C/^{12}C\right)sample - \left(^{13}C/^{12}C\right)standard}{\left(^{13}C/^{12}C\right)standard} \times 1000 \quad (1)$$

For carbon, the recognized international standard is Pee Dee Belemnite, a source of carbon rich in the ^{13}C isotope. Therefore, most terrestrial sources of carbon display a negative delta value.

In doping control, rather than relying solely on the use of absolute delta values for a reporting threshold, the difference in the delta value between that of T (and/or its metabolites) can be compared to steroids from a distinct metabolic pathway. These latter steroids, frequently termed endogenous reference compounds (ERCs), are not influenced by the exogenous steroid administration and thus reflect the basal isotopic signature of endogenously produced molecules within an individual. This approach therefore relies on the relative difference in isotope ratios of the marker and ERC and is often referred to as 'δ delta'. A significant difference (currently 3 ‰ (plus associated uncertainty) in the delta value of the steroid administration marker and the ERC indicates exogenous administration under current WADA standards [2].

Much of the early work in this area focused on the analysis of T metabolites such as 5α- and 5β-androstane-3α,17β-diols (5αDiol and 5β-Diol), androsterone (A) and etiocholanolone (E), due to their relative abundance in urine compared to T.[10,17–21] The time window for confirming T administration using these analytes rather than T itself is decreased as they do not solely derive from T, and so any perturbation in the isotope ratio caused by the administration of T is diluted.[22] While successful methods have been developed for T analysis, the desire to achieve baseline separation for the analytes of interest in GC-C-IRMS analysis means these methods typically rely on multiple extensive and time consuming sample preparation steps such as liquid chromatography (LC) fraction collection.[23,24]

A further consideration for IRMS is derivatization, which improves chromatography and has therefore proved popular for steroid analysis using GC.[10,17–21] Derivatization though results in the addition of extra carbons to the analyte which alters the carbon isotope ratio of the target compound. While this effect can be accounted for, extra uncertainty will be added to the reported delta value.[24] Also careful interpretation of mono- and di-acetate data is required. Thus the analysis of underivatized steroids may be preferred provided that adequate chromatography can be achieved.[23]

The GC-C-IRMS approach does have some limitations as a consequence of the requirement to convert analytes to CO_2 prior to analysis. IRMS analysis lacks molecular confirmatory power, as useful structural information for the analyte is no longer available.[22] Peak identification in conventional GC-C-IRMS analysis therefore relies on retention time matching to known standards.

This paper describes the evaluation of a modified Isoprime GC-C-IRMS system which was hoped to offer improved sensitivity and confirmatory power for doping control purposes, while simultaneously reducing the need for extensive sample preparation. Of particular importance was to focus on the analysis of underivatized steroids to remove the need for acetate correction (correcting for influence of carbons added during derivatization), and to target T directly rather than a metabolite, to eliminate metabolic isotope dilution and therefore extend the time window for the confirmation of T administration by isotopic analysis. A large volume injector or PTV (programmable temperature vaporization) was used to increase the amount of analyte on column and thus sensitivity. To increase the confirmatory power, microfluidic technology was employed to split column eluate between the IRMS and a conventional quadrupole mass spectrometer (>5:1), allowing the simultaneous acquisition of IRMS data and full scan mass spectra of eluting analytes. Microfluidics were further employed in conjunction with an Agilent LTM oven to facilitate GC-GC analysis with the aim of improving the chromatographic power of the instrument. Previously published work on the analysis of steroids in urine utilizing multidimensional gas chromatography (MDGC) has focused on a differing technique termed comprehensive GC-GC.[25,26] MDGC analysis of urinary steroids in both the published work and this manuscript was investigated to facilitate the baseline separation of steroid markers of synthetic endogenous steroid administration from interfering urinary components without the use of HPLC clean-up. The approach outlined here differs from the previously published work as it relies on heart-cutting desired regions of the chromatogram from the first column onto the second, here using a Deans Switch. As such heart-cutting can be described as a targeted approach, with only pre-selected regions of the primary chromatogram containing targeted peaks being transferred onto the second column. Comprehensive GC-GC can be described as untargeted, with all analytes from the primary column being transferred to the secondary column. Transfer between the two GCs is regulated by a modulator which allows a plug of components eluting from the primary GC to be rapidly trapped and continuously transferred to the second column, thus generating secondary chromatograms every 2–8 s. MDGC with IRMS first reported in 1990s for the analysis of complex plant extracts in flavour analysis, with later work on environmental pollutants and non-methane hydrocarbons produced by burning. A primary consideration for such work has been to maintain adequate chromatographic peak shape, with many different types of flow connections used.[27–31]

This paper evaluated the use of microfluidics as a means to facilitate a simpler GC-GC system. In the 1st dimension, a polar 60 m primary column (VF-624 ms, 6% cyanopropyl/phenyl, 60 m 0.32 mm × 1.8 μm) was used to maximize the separation of underivatized steroids, while a more conventional (for steroid analysis) column, (DB-5 (5 %-phenyl)-methylpolysiloxane, 25 m 0.32 mm × 0.5 μm) was used in the second dimension, to focus peaks prior to analysis (while maintaining peak separation of the selectively cut analytes) by both the IRMS and quadrupole mass spectrometer.

Materials and methods

Reagents

Androsterone (A), etiocholanolone (E), testosterone (T), epitestosterone (EpiT), pregnanediol (PD) and 11-ketoetiocholanolone (11-K), octacosane, dotriacontane and *E. coli* were purchased from Sigma-Aldrich Company Ltd (Poole, UK). Certified delta $^{13}C/^{12}C$ isotopic standards for octacosane, dotriacontane were purchased from Chiron (Trondheim, Norway). Methanol, ethyl acetate, cyclohexane, potassium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Fisher Scientific (Loughbourgh, UK). All water was purified to 18 MΩ, using an Elga Maxima coupled to an Elga Purelab Option - R15, Waters (Manchester, UK). BondElut® Certify (C8/SCX) cartridges were purchased from Phenomenex (Macclesfield, UK).

Standards

Individual hydrocarbon standards were initially prepared as a concentrated stock solution in cyclohexane 300 ng/μl. All subsequent standards were made from re-constitutions of these initial stocks in ethyl acetate prior to injection.

Individual steroid standards were initially prepared as a concentrated stock solution in methanol 200 ng/μl. All subsequent standards were made from re-constitutions of these initial stocks in ethyl acetate prior to injection.

All injections were performed in ethyl acetate as solvent venting parameters within the utilized method were optimized for this solvent.

Extraction method

Urine (5.0 ml) was placed in a 20 ml glass tube together with 500 μl of phosphate buffer (pH 6.7) and 500 μL of *E. coli* solution (5000 Fishman units/ml in phosphate buffer pH 6.7). Tubes were then capped, vortexed, and placed on a heating block set to 65 ±5 °C for 1 h to hydrolyze the steroid glucuronides. After cooling, samples were centrifuged for 5 min (1900 *g*). The hydrolyzed urine was then loaded onto BondElut® Certify (C8/SCX) cartridges conditioned with 2 ml methanol and 2 ml water. [32] Cartridges were then washed with 2 ml water and 2 ml methanol/water (90:10 *v/v*) and vacuum dried for 10 min. Following further washing with 2 ml hexane, cartridges were again dried under vacuum for 10 min. Elution was performed using 2 mL methanol/ethyl acetate (5:95 *v/v*). The eluate was dried under nitrogen at 60 ±5 °C. Samples were then evaporated to dryness and reconstituted in 55 μl of ethyl acetate for analysis by GC-GC-MS/IRMS.

GC-GC-MS/IRMS - Instrument description

Figure 1 shows a simplified schematic of the instrument, which consists of a Gerstel MPS-2 autosampler in conjunction with a PTV: Gerstel CIS (cooled injection system) 4 fitted with a septumless head and a baffled liner (Gerstel p/n 011711-010-00). Chromatographic separation was achieved in the first dimension by a VF-624 ms (60 m × 0.32 mm × 1.8 μm) column installed in an Agilent 6890 N GC, with the eluate from this column being diverted either to a flame ionization detector (FID) or second GC column (DB-5, 25 m × 0.32 mm × 0.5 μm) by an Agilent microfluidic Deans Switch. The second column was situated in an Agilent LTM oven residing outside of the primary oven and under individual thermal control. Eluate from this second column was diverted by a purged microfluidic splitter between a 5973 N MSD and an Isoprime IRMS via a GC-IV furnace and Nafion® membrane.

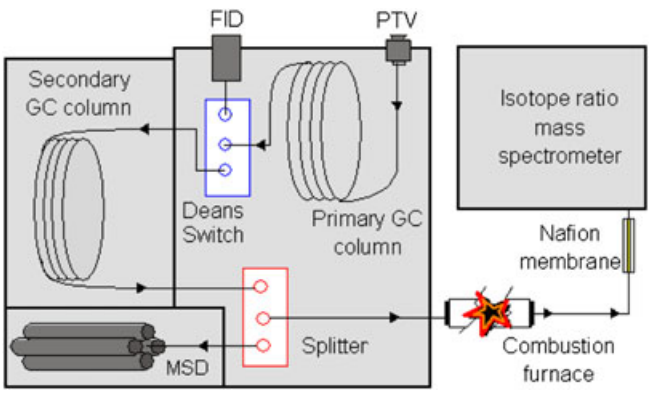

Figure 1. Simplified schematic of the developed GC-GC-MS/IRMS instrument.

Instrument conditions – Traceability and reproducibility of standards

Sample (40 μl) was injected at 3.2 μl/s into the cooled CIS (70 °C for 0.3 min). The CIS temperature was increased at 12 °C/s to 280 °C (3 min) with the pressure constant at 54.37 psi. The vent flow was 100 ml/min, the vent time 0.3 min and the purge time 2.3 min. The primary GC conditions were 200 °C (1 min), 35 °C/min to 280 °C (109 min). Secondary GC conditions were 170 °C (68 min), 5 °C/min to 180 °C (30 min), 35 °C/min to 260 °C (0 min), 5 °C/min to 300 °C (5 min). Pressure was kept constant at the Deans Switch (30.6 psi), and at the microfluidic splitter (6.0 psi).

Instrument conditions– Urine analysis

Sample extract (40 μl) was injected at 3.2 μl/s into the cooled CIS (70 °C for 0.3 min, with a vent flow of 100 mL/min, vent time 0.3 min). The CIS temperature at 12 °C/s to 280 °C (3 min) and the column pressure was constant at 38.3 psi. The primary GC conditions were 80 °C (1 min), 35 °C/min to 280 °C (158 min). Secondary GC conditions were 75 °C (68 min), 5 °C/min to 80 °C (68 min), 35 °C/min to 260 °C (0 min), 2 °C/min to 300 °C (1 min). Pressure was kept constant at the Deans Switch (24.9 psi) and microfluidic splitter (13.0 psi). "Cut" times from the primary GC to the secondary GC were 96.0–98.5 min for A, 118.5–121.5 min for 11-K and 132.2–135.5 min for T.

Results and discussion

Method parameters

The developed method utilizes a 40 μl injection volume to increase sensitivity by maximizing the amount of analyte on column. This was considered particularly important for the detection of T concentrations below 50 ng/ml without the need to extract larger volumes of urine. The injection speed (3.2 μl/s) and initial inlet temperature (70 °C) were used to facilitate optimal conditions for solvent (ethyl acetate) evaporation. The initial CIS hold time matched the vent time (0.3 min, vent flow was 100 ml/min) to allow the solvent to be vented prior to the transfer of steroids onto the head of the GC column. Comparison of recoveries following splitless injection (1 μl) and solvent venting injections up to 100 μl showed no noticeable reduction in recovery provided that adequate vent times were used (data not shown for brevity). The inlet, Deans Switch and splitter pressures were calculated based on the temperatures quoted in the materials and methods section.

Chromatographic separation of the underivatized steroids (focusing on the separation of T and EpiT) was initially optimized using a conventional GC-MS with a range of GC columns phases. Maximum peak resolution between T and EpiT was found using a 60 m 6% cyanopropyl column. This column was then selected for the GC-GC-MS/IRMS method development. While shorter run times could be achieved using an alternative primary column such as a HP-50+, decreased resolution between T and EpiT would be observed. This would have a detrimental effect on the isotope ratio due to increased amounts of EpiT being concurrently cut with T. An alternative approach utilizing a shorter, narrower VF-624 ms

column (30 m × 0.15 mm × 0.84 μm) displayed a considerable degree of peak overloading due to the amount of analyte needed to get a reliable signal for IRMS data on our instrument. Furthermore, the relationship between the pressures within the system (inlet, Deans Switch, splitter) and the capillary restrictions (GC columns and connecting capillaries) can be considered a limitation of the system as not all combinations of column dimensions and pressures will provide a system with suitable flow conditions for Deans Switching, flow splitting and dual MS analysis.

Traceability and reproducibility of standards

The process of 'cutting' peaks from the first column to the second could potentially result in isotopic fractionation. To demonstrate that fractionation was not a problem, if appropriate cut times were selected, standards characterized by a validated conventional GC-C-IRMS method were analyzed by the developed GC-GC-MS/IRMS approach with the results displayed in Table 1. Six injections of octacosane and dotriacontane were analyzed, with octacosane being cut onto the second column for subsequent mass spectral analysis. The two sets of data compared well, with mean delta values differing by less than 0.3‰ for the two approaches and both standards deviations being below 0.2, which is comparable to previously published data.[27,28] The octacosane analyzed (sourced from Sigma-Aldrich) is used as a within batch system suitability for our validated GC-C-IRMS method and has previously been compared to a certified octacosane purchased from Chiron, Norway. Data for the analysis of this certified standard which was used in our laboratory cylinder delta value calibration is also presented in Table 1 for comparison. A further comparison of a T standard also produced good data with the difference in mean delta values being 0.2‰, with standard deviations of 0.16 and 0.04. The correlation in data was achieved despite the retention time increasing from less than 21 min to over 100 min and the two sets of analysis taking place over a year apart. It should be noted our validated GC-C-IRMS method for T analysis involves acetylation prior to GC-C-IRMS analysis. For comparative purposes with the GC-GC method, the previous data has been corrected for the influence of derivatization using a mass balance approach.

Development of urinary analysis method

For further method development a standard mix of E, A, T, EpiT, 11-K and PD was used. These compounds were chosen as they represent both potential markers of synthetic endogenous steroid administration (T, and its metabolites A and E) and two steroids which are commonly used as ERCs (PD and 11-K). [10,20,22,23] In addition, EpiT was included as the separation of this compound from T when analyzed underivatized by GC was one of the aims of this research.

Figure 2 shows a FID trace obtained from the steroid mix (100 ng injected) on the developed GC-GC-MS/IRMS after separation on the primary GC column. The elution order of the steroids is known from the preliminary GC-MS work. Excellent separation was achieved for A, E and 11-K, and T was almost completely resolved from the co-eluting PD and EpiT, although the retention times for the steroids are much longer (between 90 and 140 min) than conventional methods. The peaks were approximately 2 mins wide (baseline), the peak shapes being highly Gaussian with little observable peak tailing. The lack of peak tailing is considered crucial as the entire peak must be cut from the first to the second column to avoid isotopic fractionation. [27,28]

Based on the retention data from the FID trace, 'cut-times' were used to divert column eluate from the primary chromatogram, which contained peaks of interest, onto the secondary GC column. Based on the chromatography achieved (Figure 2), T and A were cut as markers of endogenous steroid administration while 11-K was selected as an ERC. E, EpiT and PD were not cut and so were still detectable on the FID trace. As the purpose

Table 1. A comparison of an octacosane (C28) and testosterone standard analyzed by a conventional GC-C-IRMS and the developed GC-GC-MS/IRMS. [i] certified delta value. [ii] Analyzed as a acetate, presented delta values corrected using a mass balance approach

Compound (source)	N	Configuration GC or GC-GC	Amount injected (ng)	Mean Retention time (s)	Mean Peak Height (nA)	Mean Delta value (SD)
C28 (Chiron −29.85 ‰[i])	10	GC	400	744.8	3.7	−29.7 (0.09)
C28 (Sigma)	10	GC	400	744.3	3.3	−29.7 (0.12)
C28 (Sigma)	6	GC-GC	100	6402.8	2.0	−30.0 (0.12)
Testosterone [ii] (Sigma)	3	GC	200	1234.4	1.8	−28.7 (0.04)
Testosterone (Sigma)	3	GC-GC	100	6349.2	1.4	−28.9 (0.16)

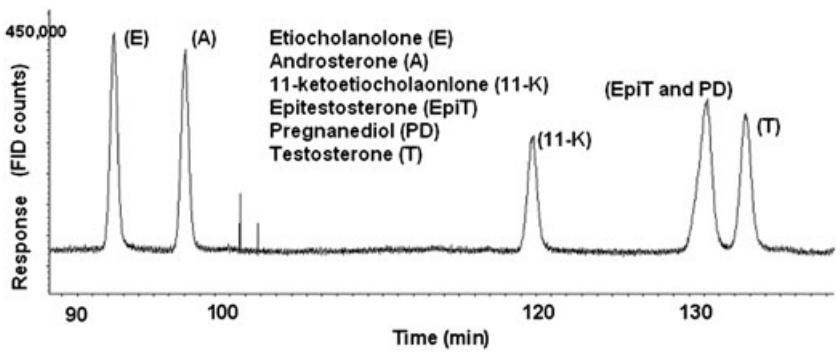

Figure 2. FID trace (primary column chromatography) of A, E, 11-K, PD, T and EpiT (100 ng injected into the GC-GC-MS/IRMS).

of this initial work was to establish the relative potential of the system and not develop a finalized method the three analytes chosen here for MS analysis were considered sufficient. In addition, as increased regions of the primary chromatogram are cut, the likelihood of peaks co-eluting in the second dimension is increased, thus cut times were limited.

Figure 3 displays the FID signal following the implementation of heart-cutting. The regions of the chromatogram cut towards the second GC can clearly be seen by the decrease in signal (A, 11-K and T). The high baseline signal on the FID is most likely due to column bleed from the polar cyanopropyl/phenyl column which is operated at its maximum hold temperature.

Following heart-cutting, peaks were further separated by the secondary GC column prior to mass spectral analysis. The eluate from the second GC was split between a quadrupole mass spectrometer and the IRMS thus giving simultaneous isotopic and structural information for each peak. Figure 4 shows the MSD chromatogram for the peaks selectively cut from the primary column. The three steroids (A, T and 11-K) are all clearly resolved with minimal peak tailing. Peaks displayed on the MSD trace were around 12 s wide. The chromatography was considered particularly good as, at the MSD, each peak had gone through a total of 85 m of GC column, the Deans Switch and the microfluidic splitter. The spectrum in Figure 4 is for T. [33]

A comparison of the chromatograms recorded on the MSD and IRMS is shown in Figure 5. A small increase in retention time can be observed on the IRMS trace but a comparison of the two traces allows clear identification of peaks recorded on the IRMS.

Urine extracts

As a proof of concept, urine (5.0 ml) containing exogenous T (from administration of T) at a concentration of 38 ng/ml (typical concentration for a Caucasian male) was extracted and analyzed by the developed GC-GC-MS/IRMS method (Figure 6).[34] Sample extraction prior to the analysis consisted of deconjugation and solid phase extraction, which took about 2 h. As samples were reconstituted in 55 μl prior to a 40 μl injection only one injection was possible. As with steroid standard analysis A, T and 11-K were selected for mass spectral analysis by heart-cutting with the delta values for the 3 peaks being −27.8 ‰ for T, -25.6 ‰ for A and −22.5 ‰ for 11-K. Of particular interest was that the δ delta

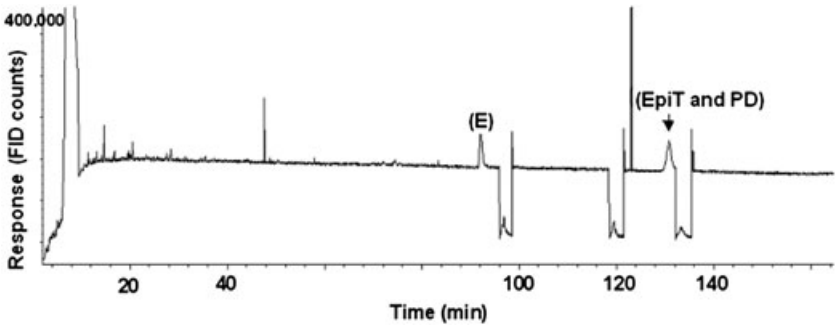

Figure 3. FID trace (compounds not sent to the MS/IRMS) of an A, E, 11-K, PD, T and EpiT (40 ng each injected) standard analyzed by GC-GC-MS/IRMS. A (~98 mins), 11-K (~119 min) and T (~132 min) have been heart-cut onto the second GC column, with the cut times clearly demonstrated by the decrease in signal response on the FID.

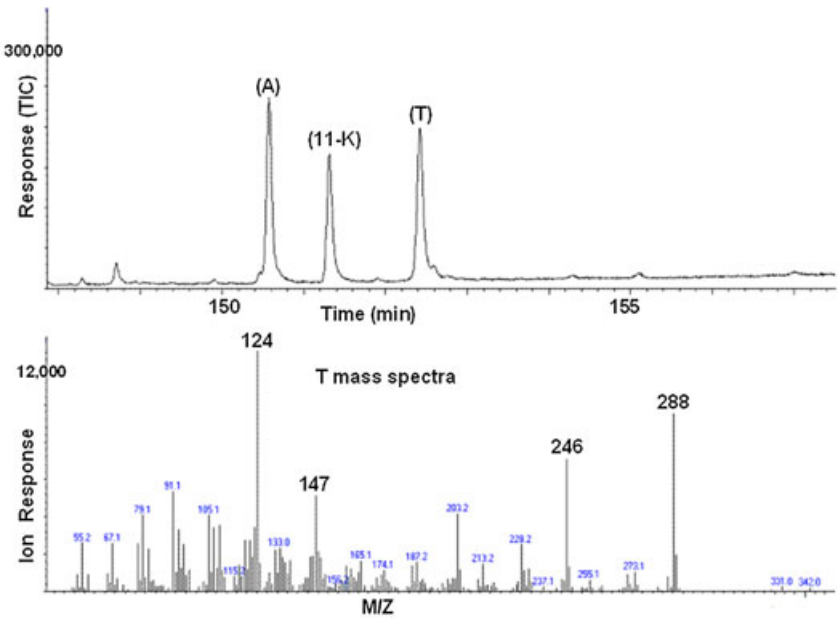

Figure 4. MSD data (total ion chromatogram – top and T spectrum – bottom). Analysis of A, E, 11-K, PD, T and EpiT (40 ng injected) by GC-GC-MS/IRMS. A, T and 11-K have been heart-cut onto the second GC column, and then analyzed by the quadrupole and IRMS mass spectrometers.

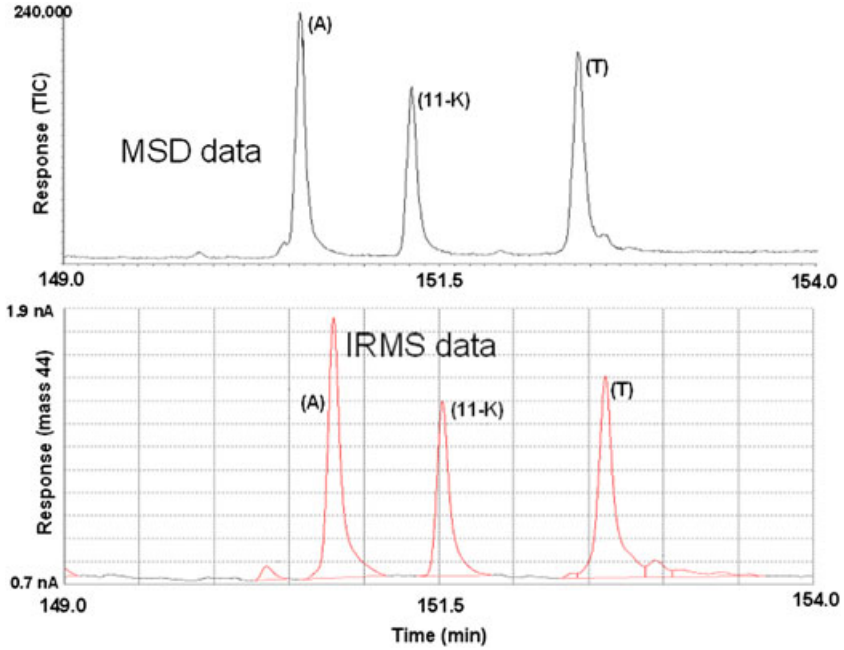

Figure 5. MSD data (top) and IRMS data (bottom). Analysis of A, E, 11-K, PD, T and EpiT (40 ng injected) by GC-GC-MS/IRMS. A, T and 11-K have been heart-cut onto the second GC column, and are subsequently analyzed by the quadrupole and IRMS mass spectrometers (offset by approximately 12 s).

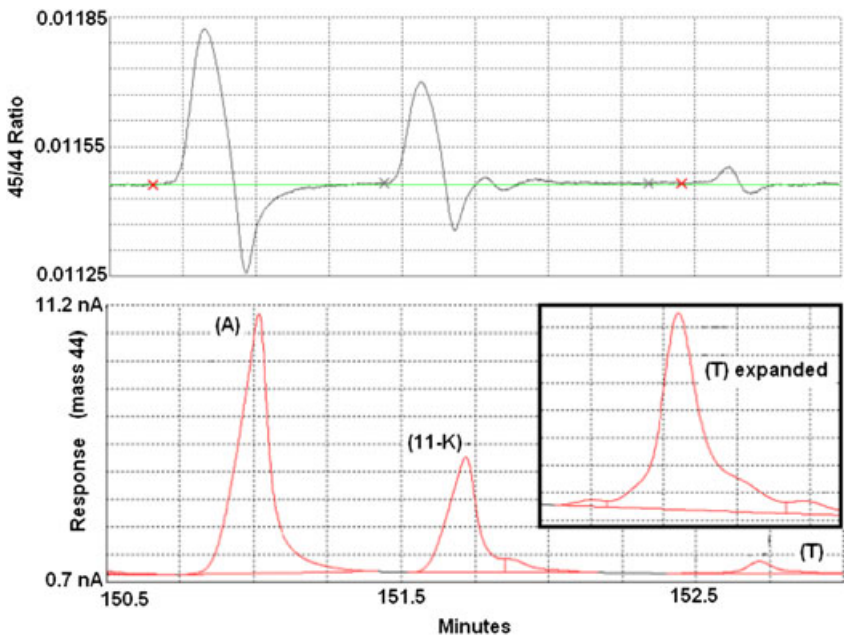

Figure 6. IRMS ratio trace (*m/z* 45/44, top) and chromatogram (*m/z* = 44, bottom) following analysis by GC-GC-MS/IRMS, of an extracted T administration urine (5.0 ml), with a T concentration of 38 ng/ml (expanded T peak displayed in the inset).

difference between A and 11-K was 3.1‰ close to the limit given by WADA for the determination of exogenous steroid administration.[2] The difference between T and 11-K was however much greater (5.3 ‰) and therefore is much more strongly indicative of the T administration. This difference in the δ delta values for T and A versus 11-K is a consequence of around two thirds of the 17-oxo steroids E and A originating from the adrenal steroids such as androstenedione and DHEA.[35,36] The data presented here clearly suggests the improved sensitivity of directly targeting T rather than A and E, where isotopic perturbation would be diluted by the adrenal steroid contribution.

Conclusions

The developed GC-GC-MS/IRMS method may offer an alternative approach for the analysis of underivatized urinary steroids without the need for extensive sample preparation. The use of a

PTV (Gerstel CIS 4) allowed improved sensitivity by increasing the amount of analyte on column. A microfluidic flow-splitting device was used to allow simultaneous acquisition of full scan mass spectra of IRMS peaks thus enhancing the confirmatory power of the technique.

While this paper offers an initial assessment of the approach, it is acknowledged that much further work is required. Of particular interest would be assessing the robustness of the approach over time including factors such as retention time stability, the influence of column bleed, and matrix effects from different sources of urine.

Secondary to the analysis of T itself, the targeting of 5(α)-Diol and5 (β)-Diol may prove advantageous over A and E due to the decreased influence of adrenal steroid metabolism. The incorporation of another ERC in addition to 11-Keto such as the 11 β –hydroxyandrosterone would also improve the reported data by allowing the calculation of alternative δ delta values. Further optimization of the sample preparation procedure and analyte cut-times may also facilitate analyte peaks which do not exhibit the small degree of co-elution which is seen in the data presented here.

Usually a urinary sample with an atypical T/EpiT ratio is subjected to confirmatory IRMS analysis, the established approach requiring purification by LC with collected fractions being analyzed by GC-C-IRMS to determine the isotopic ratio of T and other steroids.[24] Given the large number of samples with a T/E ratio in excess of 4:1, sample clean-up by LC places a particular demand on labour within the laboratory. [34,37] The application of GC-GC may provide sufficient purification for IRMS analysis to indicate whether the T in a sample is abnormal, without first having to resort to LC for sample preparation. Samples that have not meeting the WADA criteria for an adverse finding can thus be rapidly rejected, it being of little consequence that each run is 2 h long, even more so when performed overnight.

Furthermore, any sample with an abnormal T value can then be subjected to a more conventional approach, including LC clean-up. Ideally, GC-GC would eradicate the need for LC clean-up altogether but further developmental work is required to make sure the approach is robust enough to withstand future challenges to adverse findings.

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