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GC+GC with Heart-Cutting as a Simple and Rapid Sample Purification Method for GC-C-IRMS

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Abstract

This paper shows how emerging technologies such as two-dimensional gas chromatography (GC+GC) could provide a simpler approach for the analysis of steroids by isotope ratio mass spectrometry (IRMS), and furthermore enables the analysis of underivatised testosterone, rather than its metabolites in urine samples to evidence the administration of testosterone and its precursors. To facilitate analyte identification, eluent from the GC was divided, with 20 % entering a scanning MS, and 80 % flowing to the IRMS. To allow increased chromatographic separation, a low thermal mass oven (LTM) was also added, enabling a simple GC+GC system that works via selectively heart-cutting from a primary onto a secondary column. This approach allows the use of highly selective cyanopropyl/phenyl columns to optimise separation of underivatised steroids in the first dimension, while a more conventional phenyl-methylpolysiloxane column in the second dimension focuses the selected analytes into narrower peaks for more sensitive and reliable IRMS and scan MS analysis.

Analysis of urine (5 mL) for testosterone (T) (around 38 ng/mL following T administration) by the developed method, resulted in the T delta value (-27.8 ‰) being clearly distinct from 11-ketoetiocholanolone (-22.5 ‰), which was used as an endogenous reference marker (ERC), indicating T as being of exogenous origin. The simultaneous analysis by the quadrupole mass spectrometer yielded a full scan mass spectrum of the same chromatographic peak thus confirming the peak to be T.

Introduction

The administration of endogenous steroids, particularly T remains a significant problem in sports drug testing, with the data interpretation complicated by their presence in all anti-doping specimens [1]. While thresholds based on steroid concentrations or concentration ratios (i.e. testosterone/epitestosterone) are useful [2], in some instances these thresholds can also be exceeded due to natural variation. To differentiate these cases from instances of exogenous administration, IRMS has been employed [3-5]. The isotopic composition of a molecule is not constant but determined by the origin of its elements, and due to isotopic fractionation, the processes involved in its production [6]. By precisely measuring the isotopic ratio of an element (commonly carbon for steroid analysis), IRMS can distinguish between the same molecule derived from different sources. Thus IRMS has come to be used to discriminate between endogenous and exogenous steroids excreted in urine. Rather than rely solely on absolute delta values, steroids considered markers of administration can be compared to steroids from a separate metabolic pathway (endogenous reference compounds, ERCs) which are not influenced by the exogenous steroid administration. A significant difference in the delta value of the steroid administration marker and the ERC indicates exogenous administration [2].

Initial work in this area focused on the analysis of T metabolites such as androsterone (A) and etiocholanolone (E) due their relative abundance in urine compared to T [7, 8]. These compounds however do not solely derive from T and so any perturbation in the isotope ratio caused by the administration of T is therefore diluted, which limits retrospection when using these analytes. While successful methods have subsequently been developed for the analysis of T, these typically rely on multiple extensive and thus time consuming sample preparation steps such as liquid chromatography (LC) fraction collection [9]. The derivatisation of steroids prior to analysis improves the chromatography and thus sensitivity but also involves the addition of extra carbons to the analyte, which will subsequently alter 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, as such the analysis of underivatised steroids can be considered preferable if adequate chromatography can be achieved. Furthermore, one of the major problems with IRMS analysis is its lack of confirmatory power. As all analytes are converted to CO_2 prior to analysis, identification by traditional mass spectral approaches is impossible post combustion. Therefore peak identification in GC-IRMS analysis relies on

retention time matching, which has previously been disputed in at least one major anti-doping case [10].

This paper describe how an Isoprime GC-IRMS has been modified to improve the sensitivity and confirmatory power of GC-IRMS data without the need for extensive sample preparation. A large volume injector (programmable temperature vaporisation, PTV) with solvent venting was used to increase the amount of analyte on column and thus sensitivity. Microfluidics were employed to improve the splitting of the column eluate between the IRMS and a conventional quadrupole mass spectrometer (> 5:1), allowing the simultaneous acquisition of IRMS data with full scan mass spectra of eluting analytes. Microfluidics were then further employed along with a LTM oven to facilitate GC+GC analysis and thus improve chromatographic separation. This approach differs from comprehensive GC (also termed GC x GC) [11, 12] as it relies on heart-cutting with a Deans Switch, rather than cryogenic slices/modulation periods. Our approach utilises a polar 60 m primary column VF-624ms (6 % cyanopropyl/phenyl, 60 m x 0.32 mm x 1.8 µm) to maximise the separation of steroids and a more conventional column, DB-5 ((5 %-phenyl)-methylpolysiloxane, 25 m x 0.32 mm x 0.5 µm) to optimise peak shape prior to analysis by mass spectrometry.

Materials and Methods

Reagents

T, epitestosterone (EpiT), A, E, 11-K, pregnanediol (PD) and *E. coli* were purchased from Sigma-Aldrich Company Ltd, Poole, UK. Methanol, ethyl acetate (EA), pyridine, acetic anhydride, 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, UK. BondElut® Certify (C8/SCX) cartridges were purchased from Phenomenex, Macclesfield, UK.

Standards

Individual standards were prepared in methanol (200 ng/µL). All subsequent standards were made from dilutions of these initial stocks with EA prior to injection.

Extraction method: Urine

Urine (5 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 FU/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. After cooling, samples were centrifuged for 5 min (1900 *g*). The hydrolysed urine was then loaded onto BondElut® Certify (C8/SCX) cartridges conditioned with 2 mL methanol and 2 mL water [13]. Cartridges were then washed with 2 mL water and 2 mL methanol/water (90:10 v/v) and vacuum dried for 10 minutes. Following further washing with 2 mL hexane, cartridges were again dried under vacuum for 10 minutes. Elution was performed using 2 mL methanol/ethyl acetate (5:95 v/v). The eluate was dried under nitrogen at 60 ±5 °C and derivatised using 50 µL of pyridine and 50 µL of acetic anhydride for 1 hour at 60 ±5 °C. Derivatised samples were then evaporated to dryness and reconstituted in 55 µL of EA for analysis by IRMS.

PTV-GC+GC-MS/C-IRMS - Instrument description

Figure 1 shows a schematic of the instrument, which consists of a Gerstal MPS-2 autosampler in conjunction with a Gerstal CIS 4 injection system, fitted with a baffled liner. Chromatographic separation was achieved in the first dimension by a VF-624ms (60 m x 0.32 mm x 1.8 µm) column installed in an Agilent 6890N GC, with the eluate from this column being diverted either to a flame ionisation detector (FID) or second GC column (DB-5, 25 m x 0.32 mm x 0.5 µm) by a microfluidic Deans Switch controlled by an Agilent EPC. The second column was situated in a LTM oven residing outside of the primary oven and under separate thermal control. Eluate from this second column was split by a microfluidic plate between a 5973N MSD and an Isoprime IRMS via a GC-IV furnace and Nafion® membrane.

Instrument conditions

Sample extract (40 µL) was injected at 3.2 µL/s into the cooled CIS (70 °C for 0.1 min). The CIS temperature was increased at 12 °C/sec to 280°C (3 min) with the pressure constant at 38.25 psi. The vent flow was 100 mL/min, the vent time 0.1 min and the purge time 2.1 min. The primary GC conditions were 80 °C (1 min), 35 °C/min to 280 °C (180 min).

Secondary GC conditions were 80 °C (68 min), 35 °C/min to 260 °C (0 min), 2 °C/min to 300 °C (180 min). Pressure was kept constant at the Deans Switch at 24.9 psi, and at the splitter at 13.0 psi.

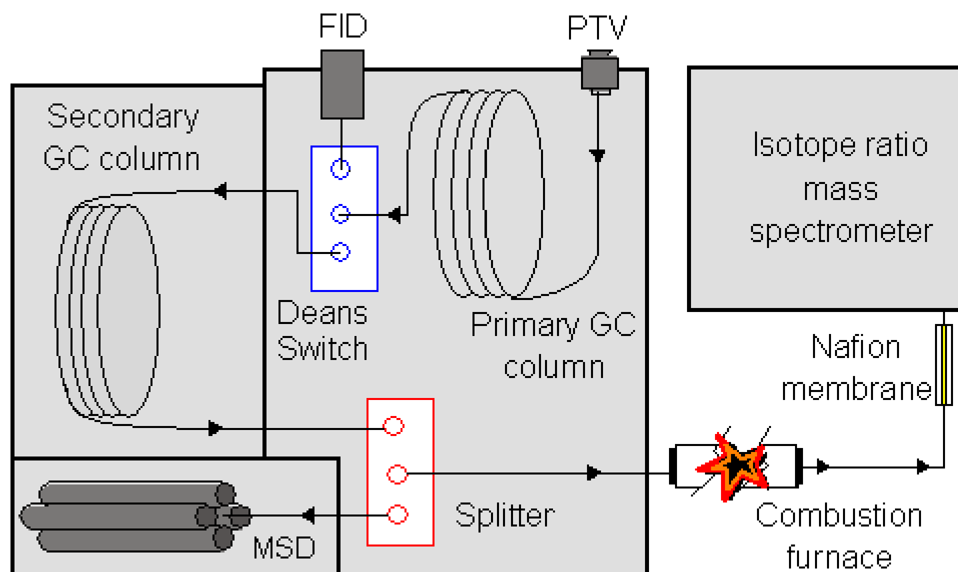

Figure 1. Simplified schematic of the developed PTV-GC+GC-MS/IRMS instrument.

Results and Discussion

A standard mix of E, A, T, EpiT, 11-K and PD was used for initial method development representing both potential markers of endogenous steroid abuse (T, A and E) and those commonly used as ERCs (PD and 11-K). Chromatographic separation of the underivatised steroids was optimised using a conventional GC-MS with particular emphasis on the separation of T and EpiT, which co-elute when analysed without derivatisation under conventional chromatographic conditions and therefore represents a significant problem to the analysis of T by IRMS without derivatisation or LC clean-up. Resolution between T and EpiT was found to be greatest using a 6 % cyanopropyl column (VF-624ms), with 60 m used for maximal separation. This column was then used for the PTV-GC+GC-MS/IRMS experiments outlined here, with the elution order of the steroids known from the earlier GC-MS work. Figure 2 shows a FID trace obtained from the steroid mix (200 ng injected) on the developed PTV-GC+GC-MS/IRMS after the primary GC column. Excellent separation was achieved for A, E and 11-K, and T was almost completely resolved from the co-eluting PD and EpiT.

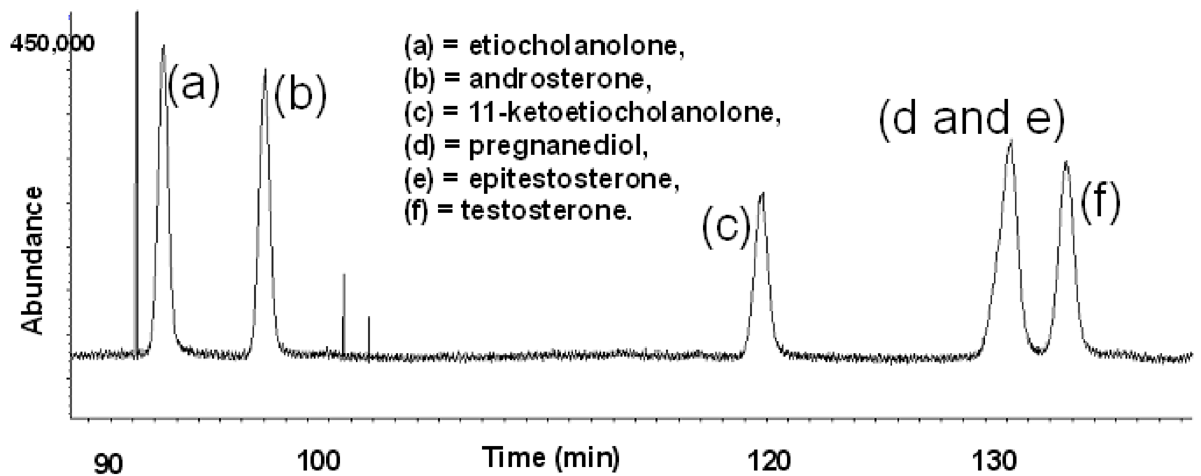

Figure 2. FID trace following the analysis of A, E, 11-K, PD, T and EpiT (200 ng injected) by PTV-GC+GC-MS/IRMS.

Based on the retention times observed on the FID trace, "cut-times" were calculated to be able to transfer specified regions of the primary chromatogram containing peaks of interest onto the secondary GC column. T, A were chosen to be "cut" and thus diverted towards the quadrupole and IRMS as markers of endogenous steroid abuse while 11-K was selected for "cutting" as an ERC. Figure 3 displays the FID trace 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), with the steroids not selectively "cut" still being detected on the FID (EpiT, PD and E).

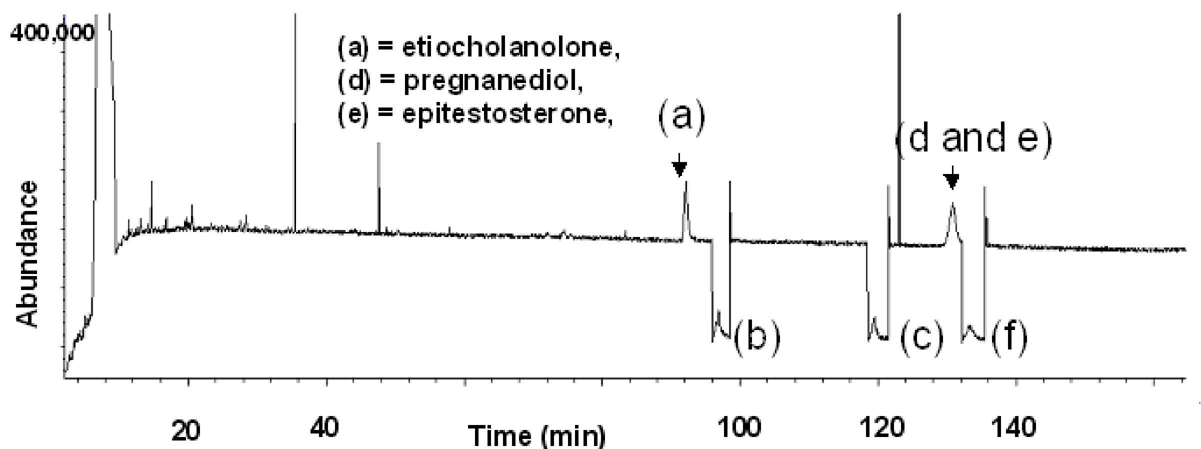

Figure 3 FID trace following the analysis of A, E, 11-K, PD, T and EpiT (20 ng injected) by PTV-GC+GC-MS/IRMS. A, T and 11-K have been heart-cut onto the second GC column, with the "cut" times clearly demonstrated by the decrease in signal response on the FID.

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 conventional quadrupole mass spectrometer and the IRMS. Figure 4 shows the MSD chromatogram for the peaks selectively cut from column 1. The 3 steroids (A, T and 11-K) are all clearly resolved with minimal peak tailing. The spectrum in Figure 4 is for T [14], demonstrating that full scan data, allowing analyte identification, is achievable.

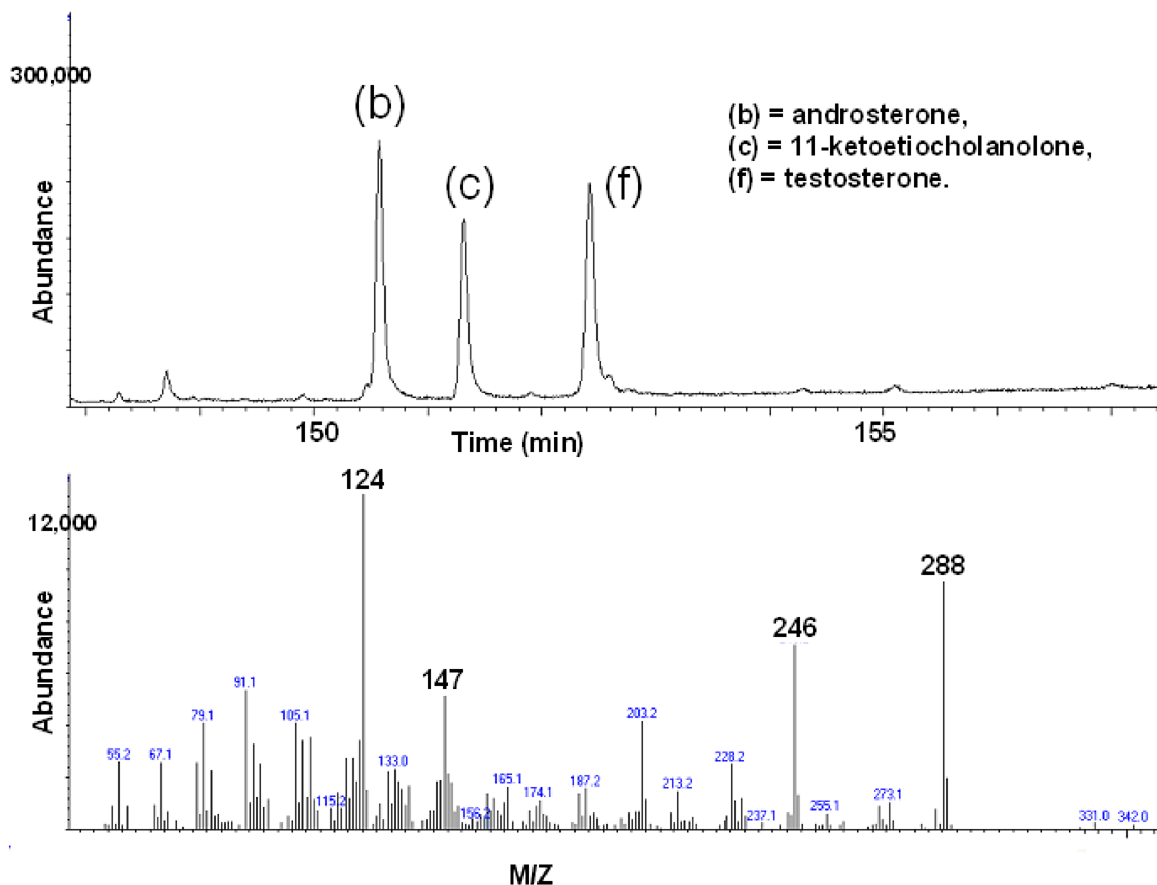

Figure 4. MSD data (total ion chromatogram – top and T spectrum – bottom) following the analysis of A, E, 11-K, PD, T and EpiT (20 ng injected) by PTV-GC+GC-MS/IRMS. A, T and 11-K have been heart-cut onto the second GC column, and then analysed by the quadrupole and IRMS mass spectrometers.

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

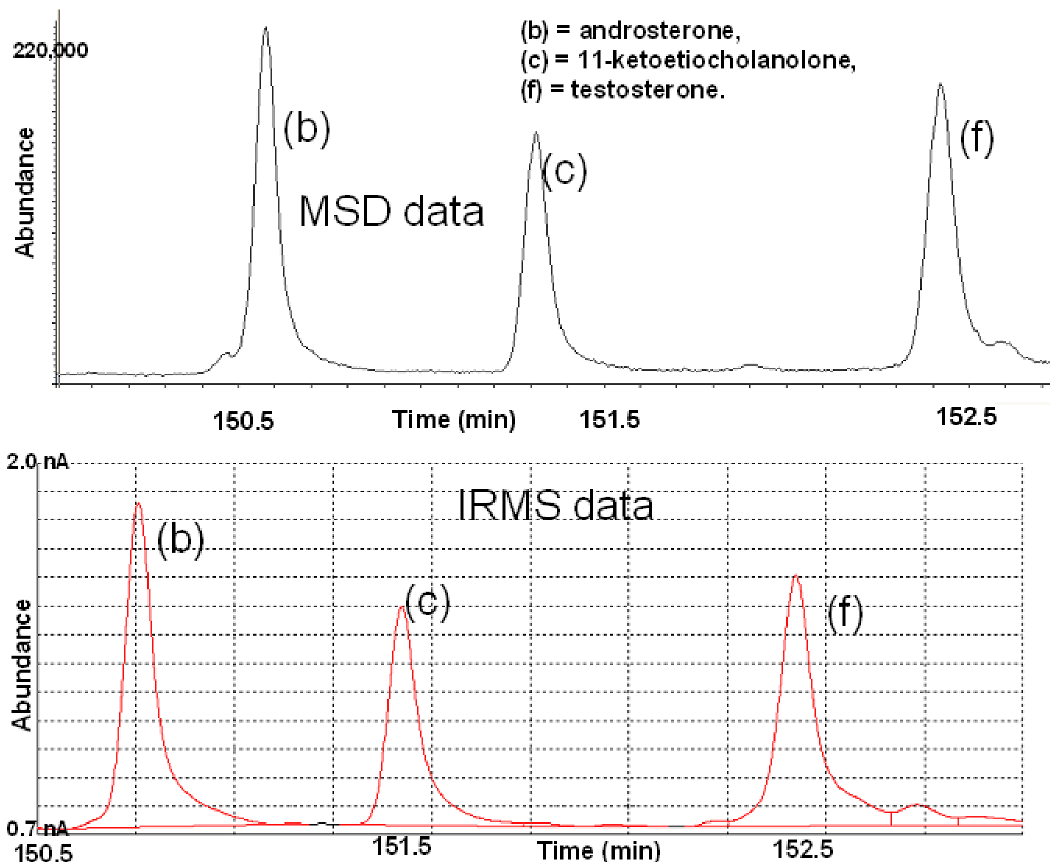

Figure 5. MSD data (top) and IRMS data (bottom) following the analysis of A, E, 11-K, PD, T and EpiT (20 ng injected) by PTV-GC+GC-MS/IRMS. A, T and 11-K have been heart- cut onto the second GC column, and are subsequently analysed by the quadropole and IRMS mass spectrometers.

Urine extracts

Figures 6 and 7 show data acquired from a urine extract analysed by the developed method. Urine containing exogenous T (from administration of T) at a concentration of 38 ng/mL was extracted and analysed by the developed method. Sample extraction prior to the analysis consisted simply of deconjugation and solid phase extraction and took about 2 h. The delta values for the 3 peaks were -27.8 ‰ for T, -25.6 ‰ for A and -22.5 ‰ for 11-K. While the delta difference between A and 11-K of 3.1 ‰ is very close to the limit for positive IRMS finding, the difference between T and 11-K of 5.3 ‰ is strongly indicative of the T administration. These results demonstrate the improved sensitivity is achieved by targeting T, and also that this approach may allow the targeting of T for IRMS analysis without the use of LC clean-up. Further optimisation of the method and associated sample preparation should increase peak purity removing the small co-eluting compounds noted with 11-K and T.

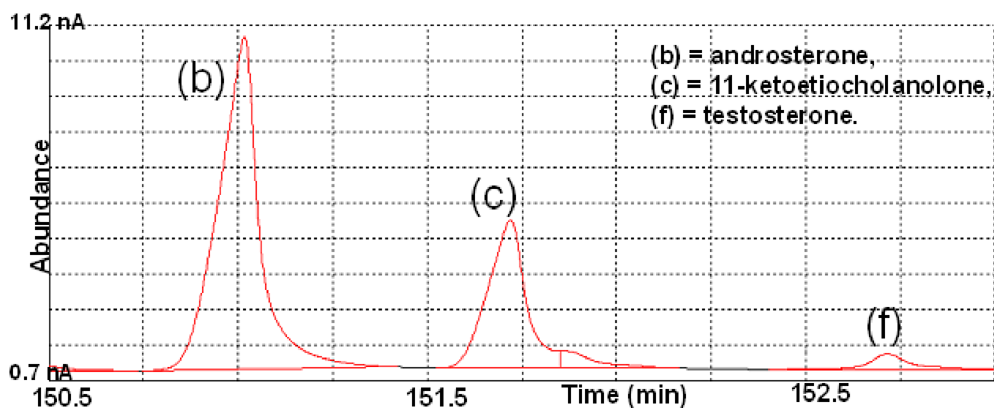

Figure 6. IRMS chromatogram (m/z = 44) following the analysis by PTV-GC+GC-MS/IRMS, of an extracted T administration urine (5 mL), with a T concentration of 38 ng/mL.

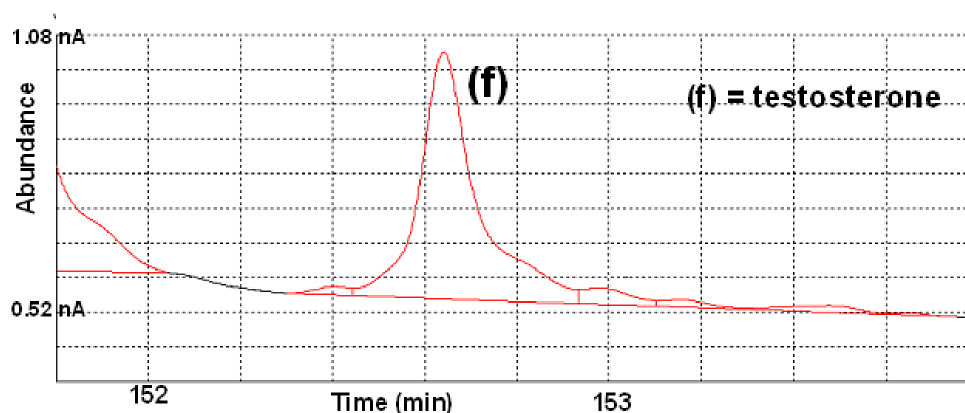

Figure 7. IRMS chromatogram (m/z = 44) following the analysis by PTV-GC+GC-MS/IRMS, of an extracted T administration urine (5 mL), with a T concentration of 38 ng/mL. (Focus on T peak.)

Conclusions

PTV-GC+GC-MS/IRMS may offer an alternative approach for the analysis of urinary steroids. The use of a PTV allowed increased sensitivity by increasing the amount of analyte on column. A simple flow splitting device allowed the simultaneous acquisition of full scan mass spectra thus enhancing the confirmatory power of the technique. GC+GC facilitated the direct analysis of T without the need for extensive sample preparation. While further optimisation of this technique and more comprehensive validation is required, these results confirm the merit of the approach. While the IRMS analysis time in excess of 2 hours is longer than currently available methods, the simplified sample preparation stage could result in a decrease in the total analysis time (sample preparation and instrumental analysis). This may be particularly useful if smaller sample numbers are analysed overnight.

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