Published in final edited form as:

Analyst. 2012 July 7; 137(13): 3102–3110. doi:10.1039/c2an35087d.

Highly sensitive and selective analysis of urinary steroids by comprehensive two-dimensional gas chromatography combined with positive chemical ionization quadrupole mass spectrometry

Ying Zhang*, Herbert J. Tobias, and J. Thomas Brenna*

Cornell University, Division of Nutritional Sciences, Savage Hall, Ithaca NY 14853

Abstract

Comprehensive two dimensional gas chromatography (GC×GC) provides greater separation space than conventional GC. Because of fast peak elution, a time of flight mass spectrometer (TOFMS) is the usual structure-specific detector of choice. The quantitative capabilities of a novel GC×GC fast quadrupole MS were investigated with electron ionization (EI), and CH₄ or NH₃ positive chemical ionization (PCI) for analysis of endogenous urinary steroids targeted in anti-doping tests. Average precisions for steroid quantitative analysis from replicate urine extractions were 6% (RSD) for EI and 8% for PCI-NH₃. The average limits of detection (LOD) calculated by quantification ions for 12 target steroids spiked into steroid-free urine matrix (SFUM) were 2.6 ng mL⁻¹ for EI, 1.3 ng mL⁻¹ for PCI-CH₄, and 0.3 ng mL⁻¹ for PCI-NH₃, all in mass scanning mode. The measured limits of quantification (LOQ) with full mass scan GC×GC-qMS were comparable with the LOQ values measured by one-dimensional GC-MS in single ion monitoring (SIM) mode. PCI-NH₃ yields fewer fragments and greater (pseudo)molecular ion abundances than EI or PCI-CH₄. These data show a benchtop GC×GC-qMS system has the sensitivity, specificity, and resolution to analyze urinary steroids at normal urine concentrations, and that PCI-NH₃, not currently available on most GC×GC-TOFMS instruments, is of particular value for generation of structure-specific ions.

Introduction

Anti-doping tests for the use of synthetic steroids target the analysis of endogenous and exogenous urinary steroids used as performance enhancers in competitive sport. Common targets include measurement of the concentration of testosterone (T) to epitestosterone (EpiT) ratio (T/EpiT) using gas chromatography mass spectrometry (GC/MS)^{1–2}. The concentrations of steroids excreted from humans vary over 3 orders of magnitude, with some approaching limits of quantification of the typical analyses. The World Anti-Doping Agency (WADA) specifies that the urinary steroid detection limit should not be higher than 2 ng mL⁻¹ in urine for T or EpiT, and 5 ng mL⁻¹ in urine for 5α-androstane-diol (5αA) or 5β-androstane-diol (5βA)³. Conventional steroid analysis employs 1D GC coupled to quadrupole MS (qMS), and is often conducted with selected ion monitoring (SIM), rather than with full mass scans, in order to achieve the mass scanning rate and sensitivity required

*Corresponding authors. <jtb4@cornell.edu>; v: (607)255-9182; f: (607)255-1033. <yzy92@cornell.edu>; v: (607)255-9009; f: (607)255-1033.

for effective detection and quantification of steroids. SIM requires setting of retention windows with pre-selected quantification and confirmation masses for each steroid or metabolite, which can only be developed for known or targeted compounds, making it transparent to unknowns or non-targeted compounds.

Although comprehensive two-dimensional gas chromatography (GC $\times$ GC) is usually coupled to a FID^{4–6} or TOFMS^{7–10}, GC $\times$ GC coupled to a fast qMS has been shown to be a powerful technique with advantages over conventional GC-MS¹¹. GC $\times$ GC employs two columns with orthogonal stationary phases resulting in improved peak capacity and compound separation in complex mixtures, when compared to 1D GC. The effluent from a 1D GC column (GC1) is trapped cryogenically at intervals (i.e. 6 s), and will normally elute from the 2D GC column (GC2) within one modulation period. Flow modulation has also been used for GC $\times$ GC. An added benefit of modulation is the considerable increase in signal to noise (S/N) attained through focusing by cryogenic modulation or time compression by flow modulation, enabling more sensitive detection than with 1D GC¹¹. In order to detect low concentration or coeluting compounds with GC-MS, targeting of specific single ions using SIM is required. GC $\times$ GC-qMS has greater sensitivity and separation capabilities and may detect unknown compounds at low concentration using full scans.

The enhanced component resolving power of GC $\times$ GC-MS over GC-MS provide a greater opportunity for the detection of non-targeted and unknown illicit anabolic androgenic steroids, where mass spectra can be obtained in the complex urine matrix without targeting specific single m/z ions. In addition, the easily visualized peak patterns provided by GC $\times$ GC could be used to relate steroid patterns to specific endocrine disorders, and changes in steroid profiles due to medical treatments or doping practices¹². The non-targeted serial recording of urinary metabolite profiles employing GC $\times$ GC-qMS may be useful as “biological passports” to detect abrupt shifts in steroid abundances or the use of designer steroids, where the unknown steroids and/or their metabolites would appear as GC $\times$ GC peaks or m/z ion signal in regions where endogenous steroids are normally absent. The concept of the athlete biological passport in anti-doping has been previously demonstrated using 1D GC-MS with the Bayesian inferential technique which has been adopted by WADA^{13–15}.

Applications of GC $\times$ GC-qMS have appeared since 1999¹⁶, demonstrating it as an excellent tool for both qualitative and quantitative classification of compounds and the detailed identification of individual components in complex mixtures^{17–21}. The application of GC $\times$ GC-time of flight MS (TOFMS) for analysis of a suite of endogenous steroids spiked into human urine was first reported in 2008⁸; subsequently, Mitrevski et al.²² and Silva et al.²³ demonstrated the analysis of exogenous anabolic agents (AA) in urine using GC $\times$ GC-TOFMS.

Although TOFMS is currently the most popular MS detector for GC $\times$ GC, similar performance can be achieved with the recently available fast qMS. Rapid scanning, up to 20000 u s⁻¹, enables the acquisition of mass spectra with duty cycles up to 50 Hz. In this work, a qMS with a scan rate of 10000 u s⁻¹ at 25 Hz was used. This is suitable for derivatized steroids of mass up to 650 g/mol. Apart from a report of electron-capture

negative ionization²⁴, most commercial GC×GC-TOFMS systems are limited to electron impact ionization (EI), which is the most commonly used ionization method in GC-MS of steroids^{25–27}. While EI often generates mass spectra rich with structural information, EI of steroid acetates causes extensive fragmentation often yielding negligible molecular ion abundance, M^+ . Compared to GC×GC-TOFMS systems, the benefits of GC×GC-fast qMS include that they have a smaller footprint (or size) than most of the commercially available TOFMS systems, they are obtainable at considerably lower cost, and they are available with positive chemical ionization (PCI) and negative CI (NCI)²⁸. CI produces ions that depend on the reagent gas used, usually with little excess energy that yields abundant (pseudo)molecular ions that can be of great value in assignment of unknown structures, and provide strong signal for quantitative analysis.

One goal of this work was to simplify and standardize urine sample preparation procedures in anti-doping tests between GC×GC-qMS and gas chromatography combustion isotope ratio mass spectrometry (GCC-IRMS), an important and powerful technique used for the confirmation of synthetic steroid use by the stable carbon isotope analysis of testosterone and its metabolites^{1, 29–31}. Coupling the GC×GC separations with fast-qMS as well as IRMS is of interest as future screening technology for complete detection and confirmation of synthetic steroid use in sports doping. Initial results and considerations for GC×GCC-IRMS applied to urine extracts is presented elsewhere³².

In this work, we report on the evaluation of GC×GC-qMS with a fast quadrupole mass spectrometer for the analysis of steroids, of anti-doping interest, derived from human urine. Conditions to achieve baseline separation for most steroids with GC×GC-qMS are evaluated for precision, LOD, and LOQ using EI and PCI with NH_3 and CH_4 .

Experimental

Standards and chemicals

Standard mixtures were prepared with 12 targeted endogenous steroids [5β -estrane- 3α -ol-17-one (19-Noretiocholanolone, 19-NE), 5β -androstan- 3α -ol-17-one (etiocholanolone, E), 5α -androstan- 3α -ol-17-one (androsterone, A), 5-androsten- 3β -ol-17-one (dehydroepiandrosterone, DHEA), 5β -androstan- 3α -ol-11, 17-dione (11-ketoetiocholanolone, 11-KE), 5α -androstan-17 β -ol-3-one (dihydrotestosterone, DHT), 5β -androstan- 3α , 17 β -diol (5β A), 5α -androstan- 3α , 17 β -diol (5α A), 4-androsten-17 α -ol-3-one (epitestosterone, EpiT), 4-androsten-17 β -ol-3-one (testosterone, T), 5α -androstan- 3α , 11 β -diol-17-one (11 β -hydroxyandrosterone, 11-OHA), 5β -pregnane- 3α , 20 α -diol (5β -pregnanediol, 5β P)], and three steroids as internal standard candidates [5α -androstan- 3β -ol (5α -androstanol), 5α -androstan- 3β -ol-17-one (epiandrosterone, EpiA), and 5α -cholestane (Cne)]. All steroids were of 99% purity (Steraloids, Newport RI), and 2-propanol and methanol were HPLC grade (Mallinckrodt Baker, Phillipsburg, NJ). Each steroid in the SM15 mixture (all 15 steroids above) and SM14 mixture (SM15 without Cne) was prepared at equal mass concentration (i.e. $1\text{ ng }\mu\text{L}^{-1}$) in 2-propanol, as required. Pyridine, acetic anhydride, tert-butylmethylether (TBME), β -glucuronidase from *Escherichia coli* (50% glycerol solution $2.3\text{ mg protein mL}^{-1}$ Biuret, 33539556 units g^{-1} protein), sodium phosphate buffer (0.2M, PH=7), and potassium carbonate buffer ($K_2CO_3/KHCO_3$ 1:1, w/w,

200 g L⁻¹) were purchased from Sigma-Aldrich (St. Louis, MO). All solvents and reagents were of analytical grade.

Sample Preparation

Fifteen steroid acetate standard mixture (SM15-AC)

A standard, SM15, was prepared by dissolving an equal amount of each steroid (~1 mg) in 1 mL 2-propanol and diluting to a concentration of 100 ng μL^{-1} , and it was stored at 4°C when not in use. A 200 μL aliquot of SM14 (100 ng μL^{-1}), with all steroids excluding Cne, was dried under nitrogen, acetylated by adding 100 μL pyridine and 100 μL acetic anhydride and heating at 60°C for 1 hour, and then evaporated to dryness under nitrogen. The derivatized steroids were reconstituted and diluted to create a series of solutions with concentrations of 0.001 ng μL^{-1} , 0.002 ng μL^{-1} , 0.01 ng μL^{-1} , 0.02 ng μL^{-1} , 0.1 ng μL^{-1} , 0.2 ng μL^{-1} , 1 ng μL^{-1} , 2 ng μL^{-1} , 10 ng μL^{-1} , 20 ng μL^{-1} , 200 ng μL^{-1} , for each steroid. Then each solution was spiked with the same amount of EpiA-AC, resulting in 20 ng μL^{-1} EpiA-AC. These were used to generate calibration curves and measure LOD and LOQ. Two replicates of the low concentrations of SM15-AC (EI and PCI-CH₄: 0.02 ng μL^{-1} or 0.2 ng μL^{-1} ; PCI-NH₃: 0.001 ng μL^{-1} , 0.002 ng μL^{-1} , or 0.02 ng μL^{-1}) were run on GC×GC-qMS, and analyzed where the S/N ratio was calculated using the Shimadzu GC-MS software. Calibration curves for LOQ measurement were built at numerous concentration levels, performing two replicates at each level. The repeatability and accuracy were evaluated by two replicates of the SM15-AC in both as a standard and spiked into urine matrix at low end of the known endogenous concentration range of each steroid in urine.

Urine samples

Steroids, as glucuronide conjugates, were extracted from a 20 mL urine sample pooled from of a large population of athletes. Chromabond® C₁₈ cartridges (500 mg, 6 mL, Macherey-Nagel, Bethlehem, PA) were conditioned with 2 mL of MeOH and 2 mL of water. Twenty mL of urine spiked with 100 μL of 20 ng μL^{-1} EpiA glucuronide as an internal standard, was applied to the column. After washing with 2 mL of water, the residue was eluted with 2 × 1 mL of MeOH and evaporated to dryness under nitrogen. The dried eluate was dissolved in 1 mL of sodium phosphate buffer (0.2 M, PH=7) and 5 mL of TBME was added. After shaking for 5 min and centrifugation at 1200 g for 5 min, the organic layer was discarded. Then 100 μL of β -glucuronidase was added to the sample and incubated at 50°C for 1 hour in order to hydrolyze components into free steroids. After cooling to room temperature, 500 μL of potassium carbonate buffer (K₂CO₃/KHCO₃ 1:1, w/w, 200 g L⁻¹) was added. The aqueous layer was extracted with 2 × 5 mL of TBME, shaken for 5 min and centrifuged (1200 g, 5 min), and the organic layers were combined in a conical test-tube and evaporated to dryness under nitrogen. The residue was then acetylated by adding 100 μL pyridine and 100 μL acetic anhydride and heating at 60°C for 1 hour, and then evaporated to dryness under nitrogen. The derivatized steroids were reconstituted in 2 × 50 μL of 20 ng μL^{-1} internal standard Cne and transferred to an auto-sampler vial for GC analysis. Before the urinary steroid acetate sample was analyzed by GC×GC-qMS, it was further diluted 10-fold times. No HPLC cleanup was performed on the steroid extract samples.

Steroid free urine matrix (SFUM)

SFUM was prepared by percolating 20 mL urine sample through an SPE column which was conditioned with 2 mL of MeOH and 2 mL of water. In this way, urinary steroid glucuronides or sulfates and other potential interfering compounds were retained in the SPE cartridges. After this, the water eluates were collected for liquid-liquid extraction and acetylation, using the same procedure as in the above urinary steroid extraction. The final SFUM samples were reconstituted into 100 μL of 2-propanol, further diluted 10 times, and then used as a matrix for LOD and LOQ measurements of spiked steroids.

Evaluation of repeatability and recovery of urine steroid preparation

The repeatability of the urine steroid extraction procedure was determined by 4 replicate aliquots of acetylated normal pooled urine extracts, spiked with the internal standard Cne after preparation of urine extract just before GC $\times$ GC-qMS analysis. The four acetylated urine steroid extracts were diluted 10 times, followed by GC $\times$ GC-qMS analysis of two replicate 1 μL injections for each extract.

Steroid recovery was measured for four replicate aliquots of acetylated urine extract spiked with the internal standard EpiA-glucuronide (100 μL of 20 ng μL^{-1}) before the SPE step, and two replicate aliquots of acetylated urine extract spiked with the internal standard EpiA acetate (100 μL of 20 ng μL^{-1}) after the extraction, but prior to the injection into the GC $\times$ GC-qMS. All the acetylated urine steroid extracts were diluted 10-fold. Two replicate 1 μL injections of each extract were analyzed by GC $\times$ GC-qMS.

Evaluation of urinary steroid LOD and LOQ

Fifty μL of SFUM was spiked with SM15-AC at each of 20 pg μL^{-1} , 100 pg μL^{-1} , 200 pg μL^{-1} , 1 ng μL^{-1} and 2 ng μL^{-1} . One μL of SM15-AC spiked SFUM sample was injected into GC $\times$ GC-qMS for determination of LOD and LOQ. Detection limits at a S/N = 3 were calculated from these results based on the S/N of a quantifier ion in the slice of highest intensity (apex peak slice).

GC $\times$ GC-qMS Setup

High purity He (99.999%) and NH_3 (99.9995%) from Airgas East (Salem, NH) and high purity CH_4 (99.999%) from Matheson Tri-Gas were used. All steroid analyses were carried out on a novel Shimadzu GC $\times$ GC-qMS system, consisting of a single GC (GC2010, Shimadzu, Columbia, MD), and a QP2010 Ultra quadrupole MS (Shimadzu, Columbia, MD) equipped with an AOC-20i autoinjector and a split/splitless inlet (300°C). A Zoex double focusing Loop Modulator (ZX1-LN2 Cooled Loop Modulation GC $\times$ GC system, Zoex Corp., Houston, USA) was mounted on the GC oven between GC1 and GC2 and consisted of a 1.5 m $\times$ 0.1 mm i.d. deactivated fused-silica capillary. The system delivers nitrogen gas cooled by liquid nitrogen to create a cold jet to trap GC eluate, as well as heated nitrogen gas for rapid desorption with a hot jet pulse (350°C for 375 ms) at a modulation period of 6 s. Preliminary experiments showed that the cold jet trapped significant amounts of column bleed because of the high temperatures required for steroid chromatography and the 1 μm film used for GC1. Experiments with the modulator temperature led to an optimal trapping temperature of approximately +130°C achieved by switching off the nitrogen

chiller (which cools to -90°C) and by adjusting ambient temperature nitrogen gas flow rate. At this modulation temperature, most of the column bleed passed through the cool modulator though steroids were trapped quantitatively, thus stabilizing the baseline. The procedure offers the advantage of obviating the need for the gas chiller normally supplied with the system, reducing complexity and cost for systems dedicated to steroid analyses.

GC1 was a 30 m $\times$ 0.25 mm i.d. $\times$ 1.0 μm film ZB-1ms (100% dimethylpolysiloxane, Phenomenex, Torrance, California) and GC2 was a 1.5 m $\times$ 0.1 mm i.d. $\times$ 0.1 μm film BPX50 (50% diphenyl and 50% dimethyl polysilphenylene-siloxane, SGE, Austin, TX). The GC1 oven temperature was held at 70°C for 1 min, ramped at $40^{\circ}\text{C min}^{-1}$ to 300°C and held for 35 min, ramped at $40^{\circ}\text{C min}^{-1}$ to 340°C and held for 5 min. Helium carrier gas was used at a constant flow rate of 1.3 mL min^{-1} , with an initial head pressure of 376.6 kPa at 70°C . The sample was injected into a split/splitless inlet held at 300°C in splitless mode (splitless time=1 min).

A 1 m $\times$ 0.1 mm i.d. deactivated fused-silica capillary transfer line was used to connect the GC2 column with the MS ion source. The MS transfer block was held at 330°C during EI, and 320°C during PCI- CH_4 and PCI- NH_3 analyses. The ion source temperature was held at 290°C during EI and held at 250°C during PCI- CH_4 and PCI- NH_3 . The MS was run in the full scan mode at 25 Hz with a scan speed of 10000 u s^{-1} and with mass ranges of 50–390 u for EI, 70–420 u for PCI- CH_4 , and 200–440 u for PCI- NH_3 at a detector voltage of 0.8 V. EI was operated at an electron energy of 70 eV. The NH_3 and CH_4 gas pressures were 30 psi and 35 psi, respectively during PCI. Data analysis and presentation of the GC $\times$ GC chromatograms were performed using the Shimadzu GCMS solution software version 2.53 and GC image software (Zoex Corp.), version 2.1b.

Results and discussion

GC $\times$ GC chromatography of androgenic anabolic steroids

Using SM-15, GC $\times$ GC column parameters were investigated to optimize separations of target steroids, including stationary phase (e.g., non-polar, polar), stationary phase thickness (e.g., 0.10 to 1.0 micron), and column length. The target steroids were chosen based on relevance to molecular and isotopic (IRMS) doping analysis. A non-polar, polar column set with the best optimized separation of target steroids and other components in urinary steroid extracts was developed comprising a 30 m long, 0.25 mm inner diameter (i.d.), 1 μm film thickness ZB-1ms stationary phase column for GC1 and a 1.5 m long, 0.10 mm i.d., 0.10 μm BPX50 polar stationary phase column for GC2. A 1 μm film thickness column was selected due to superior capacity and dynamic range and, under our conditions, similar separation properties, as a 0.5 μm film column.

In preliminary studies, GC $\times$ GC peak shape and chromatography was characterized and optimized for native steroids (underivatized), acetate (AC) and trimethylsilyl (TMS) steroid derivatives. As expected, the optimized GC $\times$ GC chromatographic separations of derivatized steroids were superior to that of underivatized steroids with respect to peak shapes and time of analysis. TMS did not offer obviously superior results to acetates and are incompatible with IRMS analysis³³ which was a goal for this preparation and chromatography system.

Therefore, focus was on analysis of steroid acetates (steroids-AC) and resulted in GC2 peak slice widths of 96–276 ms full width half maximum (FWHM).

Figure 1A presents a TIC of SM-15-AC analyzed by GC×GC-qMS using PCI-NH₃, showing that all steroids are baseline resolved including EpiT-AC and T-AC, highly significant steroids for T/EpiT ratio measurement in anti-doping tests. Using the same conditions, Figure 1B shows the TIC of endogenous steroids in a normal urine extract, demonstrating similar baseline separation except for partial urine matrix co-elutions with DHT-AC and 5αA-AC.

Validation of urine sample preparation and analytical procedure

Sample preparation for GC×GC-qMS of endogenous urinary steroids was designed to be compatible with GC×GCC-IRMS analyses³² where the methodology would allow application to carbon isotope ratio measurement without a change in sample preparation or chromatography. Conventional GCC-IRMS analysis of steroids requires additional HPLC purification steps, and is restricted to the use of underivatized steroids or their acetate derivatives. Taking into account the desire for a common sample preparation between molecular (qMS) and isotope (IRMS) analyses, the selection of a molecular quantification internal standard required the following considerations. Deuterated versions of normally endogenous steroid glucuronides, such as testosterone itself, while appropriate for GC-MS where SIM analysis is used, were avoided since any overlap with the nondeuterated steroid in urine would interfere with the IRMS measurement. An exogenous steroid glucuronide might work well if defined by unique elution times in the 2D chromatogram; however, they are not readily available. We settled on epiandrosterone (EpiA) glucuronide, an endogenous steroid glucuronide, as the internal standard in the urine extraction procedure because EpiA-AC is well separated from other substances in the urine matrix (Figure 1); its concentration in urine is very low, about 10% (1 ng mL⁻¹ urine) of the amount of testosterone (10 ng mL⁻¹ urine) excreted in urine³⁴ and about 1% of an intended IS spike amount of 100 ng mL⁻¹ urine. Finally, the repeatability and recovery of the urine steroid extraction procedure was evaluated by spiking the internal standard EpiA glucuronide into urine samples prior to the urine extraction procedure, followed by GC×GC-qMS analysis.

The precision of the sample preparation procedure was assessed by GC×GC-qMS analyses of 4 replicate preparations for 11 extracted endogenous urinary steroids, in addition to a spike of Cne IS, and 2 injections for each replicate preparation. The average precision for quantitative analysis of steroids from replicate urine extractions was 6% (RSD) for EI, and 8% for PCI-NH₃. This includes all sources of variation from extraction, hydrolysis and derivatization, and other sources of losses and variability. The recovery of the EpiA glucuronide spike was also measured and calculated separately from the repeatability test and was equal to 98±4%.

Linearity, limits of detection, and limits of quantification of endogenous urinary steroids

The GC×GC-qMS linearity for the endogenous urinary steroids targeted in this study was determined experimentally over five separate concentration ranges, from 0.001 to 20 ng μL⁻¹ for the LOD measurement, and 0.1 to 2 ng μL⁻¹, 0.1 to 20 ng μL⁻¹, 0.2 to 20 ng μL⁻¹,

and 2 to 200 ng μL^{-1} for LOQ measurement, chosen based on expected endogenous range in normal urine. The correlation coefficients of the linear fit for the calibration curves varied from 0.9850 for EpiT-AC to >0.999 for E-AC.

The detection limit for each steroid was defined as the lowest mass (in pg) which would result in a S/N equal to 3 for a single quantification ion, determined from extracted-ion chromatograms, and are listed in Table 1 for EI, Table 2 for PCI-NH₃, and Table 3 for PCI-CH₄. The S/N was calculated based on the quantifier m/z ion signal in the apex (most intense) peak slice for each steroid. Tables 1 and 2 show that, compared to EI, the endogenous steroid detection sensitivities for PCI-NH₃ under our conditions were 11-fold improved for the neat standard mixture SM15-AC, and 9-fold improved for the SM15-AC spiked in the steroid free urine matrix (SFUM). PCI-CH₄ detection sensitivity was similar to those of EI (Table 3). PCI-NH₃ had the lowest average absolute detection limit, in mass scanning mode, which was 0.8 pg for steroids in the SM15-AC standard and 3.2 pg for steroids in the urine matrix. The steroid AC detection limits in urine matrix using GC×GC-qMS in EI mode are comparable to the steroid TMS derivatives detection sensitivities in urine matrix, using GC×GC-TOF-MS with EI from our previous work⁸, indicating that GC×GC-qMS using EI achieves similar steroid detection limits as GC×GC-TOF-MS.

In Tables 1, 2 and 3, LOD are reported in units of ng mL^{-1} urine to simplify comparison with the WADA requirement³. The LODs average 2.6 ng mL^{-1} in urine using EI, 1.3 ng mL^{-1} in urine using PCI-CH₄, and 0.3 ng mL^{-1} in urine using PCI-NH₃, which are near or well below the WADA requirement³ for EpiT (2 ng mL^{-1} urine), the lowest among the relevant steroids.

To assist in automatic identification of the endogenous steroids in the complex urine matrix, we constructed a mass spectral library containing full scan mass spectra for the target compounds using 1 ng standards of each steroid in SM15-AC injected on column and analyzed by GC×GC-qMS under the same conditions as the samples. In GC Image software, we used the reverse match factor (RMF) to express the similarity (0–999 range) between the experimental and library spectra, which considered only those m/z ions present in the library spectra. Due to the scanning nature of qMS, peak skewing can be an issue where mass spectra are acquired across a sharp peak³⁵. We did not study skewing in detail in this work; however, our results show that using PCI-NH₃ (Table 2), the RMF was above 740, and using EI (Table 1), the RMF was above 700 for the target steroids in urine matrix at 2 to 1000 pg injected (depending on detection limit of steroid). These results indicate that high quality mass spectra without dramatic biases are generated by qMS.

In Tables 1, 2, and 3, LOQ and its repeatability and accuracy for the twelve steroids in both SM15-AC standard and urine matrix, are reported. Four different concentration ranges were used to create calibration curves for the steroids, based on their normal endogenous concentrations in urine^{36, 37}. The LOQ values achieved by full scan GC×GC-qMS for the twelve endogenous steroids were comparable to GC-MS used with SIM reported in other work^{38, 39}. The LOQ repeatability for both EI and PCI-NH₃ were less than 5% RSD, and the accuracy, reported as the deviation from the mean value, was better than $\pm 18\%$ for all the

steroids tested. These values are within the acceptable range for the measurements, where LOQ repeatability and error in accuracy are expected to be < 20%⁴⁰.

3.4. Identification of the endogenous urinary steroids—In addition to EI, we investigated various PCI reagent gases (isobutane, methane, and ammonia). The goal, in part, was to simplify mass spectra to concentrate signal into fewer, structure-specific ions, particularly more abundant steroid (pseudo)molecular ion signal. In this work, isobutane PCI resulted in low signals and data are neither discussed nor presented.

Of the various types of ion/molecule adducts observed with PCI reactant ions, only two reactions, proton transfer and NH_4^+ attachment for NH_3 , are common. The major positive ion present in ammonia plasmas under common PCI source operating conditions is the NH_4^+ adduct ion ($[\text{M}+\text{NH}_4]^+$); however, the molecular ion, $[\text{M}+\text{H}]^+$, is often observed in PCI- NH_3 as well. The ion source NH_3 pressure was set to achieve the most intense signals for the m/z ions $[\text{M}+\text{NH}_4]^+$ or $[\text{M}+\text{H}]^+$.

In general, PCI- NH_3 yielded more abundant high mass and (pseudo)molecular ions $[\text{M}]^+$ or $[\text{MH}]^+$, or $[\text{M} + \text{adduct}]^+$. PCI- CH_4 resulted in significant (pseudo)molecular ion signal for some, but not all steroid acetates, though it always produced high mass fragments. In contrast, PCI- NH_3 resulted in the most intense (pseudo) molecular ion mass signals for all compounds. In addition to dominant $[\text{M}+\text{H}]^+$ or $[\text{M}+\text{NH}_4]^+$ ions, PCI- NH_3 yielded simple mass fragmentation patterns that reveal additional chemical information, such as number of acetate groups. For example, Figure 2 shows a mass spectrum for $5\alpha\text{A-AC}$ generated using (2A) EI and (2B) PCI- NH_3 . EI results in extensive fragmentation with little molecular ion signal. PCI- NH_3 gives a significant peak for the molecular adduct ion $[\text{M}+\text{NH}_4]^+$ (m/z 394), in addition to the m/z 317, $[\text{MH}-60]^+$, which is due to a loss of a full acetate group, and the m/z 257, $[\text{MH}-120]^+$, which is due to a loss of two full acetate groups. Alternatively, EpiT-AC, a mono-acetate, PCI- NH_3 yields only one intense protonated molecular ion $[\text{M}+\text{H}]^+$ (m/z 331).

We note one limitation of PCI using NH_3 is an enhanced tendency of the ion source to foul with samples extracted from urine, requiring disassembly and cleaning after 30 or so runs under our conditions. Much longer ion source life is obtained with standards, indicating that contaminants that are extracted from urine itself are at issue, and that enhanced cleanup may mitigate the issue.

Conclusions

This work shows that GC×GC coupled to the qMS detector is promising for separation, quantification, and identification of steroids in complex matrices such as urine, even at a concentration below 1 ng mL^{-1} urine. In addition, unknown compounds, such as designer steroids, that elute in unique retention spots in the 2D chromatogram may be more easily detected and identified compared to using normal 1D GC/MS techniques. Our data indicate that steroid (EpiA) recovery from urine using our adapted preparation method was greater than 90% and average repeatability for quantitative analysis of steroids from replicate urine extractions was 8% RSD using PCI- NH_3 .

PCI-NH₃ steroid mass spectra were simpler than EI mass spectra, and consisted of intense (pseudo)molecular ions. Compared to EI and PCI-CH₄, sensitivity and detection limits were superior using PCI-NH₃, where detection of endogenous steroids of interest in anti-doping was possible. GC×GC-qMS using PCI-NH₃ in combination with EI may provide useful information for identification of unknown compounds, such as designer steroids, in the urine matrices. GC×GC-MS has also been demonstrated for its suitability in metabolomic studies, such as clinical pathology for steroid dysfunction¹², organic acid metabolite detection in human urine^{41, 42}, metabolomic analysis of mouse tissue extracts^{43, 44}, and metabolite analysis in plant studies^{45, 46}. Future work will entail evaluation of GC×GC-qMS analyses of a range of human urines and informatics development to handle complex data sets.

Acknowledgments

This work was supported by the Partnership for Clean Competition (PCC) <<http://www.cleancompetition.org>>. We thank Dr. Larry Bowers for many helpful discussions and Dr Jonathan Danaceau for the gift of pooled urine.

References

1. Donike M, Barwald KR, Klostermann K, Schanzer W, Zimmermann J. *Int. J. Sports Med.* 1983; 4:68–68.
2. Shackleton CHL. *J. Chromatogr. B.* 1986; 379:91–156.
3. World Anti-Doping Agency (WADA). Endogenous Anabolic Androgenic Steroids Testing, Reporting and Interpretive Guidance, 2009, WADA Technical Document - TD2009EAAS. Montreal, Canada: 2009.
4. Shellie R, Mondello L, Marriott P, Dugo G. *J. Chromatogr. A.* 2002; 970:225–234. [PubMed: 12350096]
5. Shellie R, Marriott P, Morrison P. *J. Chromatogr. Sci.* 2004; 42:417–422. [PubMed: 15516281]
6. Groger T, Schaffer M, Putz M, Ahrens B, Drew K, Eschner M, Zimmermann R. *J. Chromatogr. A.* 2008; 1200:8–16. [PubMed: 18539290]
7. Shellie R, Marriott P, Morrison P. *Anal. Chem.* 2001; 73:1336–1344.
8. Mitrevski BS, Brenna JT, Zhang Y, Marriott PJ. *J. Chromatogr. A.* 2008; 1214:134–142. [PubMed: 18980770]
9. Adahchour M, Beens J, Vreuls RJJ, Batenburg AM, Rosing EAE, Brinkman UAT. *Chromatographia.* 2002; 55:361–367.
10. Dalluge J, Vreuls RJJ, Beens J, Brinkman UAT. *J. Sep. Sci.* 2002; 25:201–214.
11. Mondello L, Tranchida PQ, Dugo P, Dugo G. *Mass Spectrom. Rev.* 2008; 27:101–124. [PubMed: 18240151]
12. Zhang Y, Tobias HJ, Auchus RJ, Brenna JT. *Drug Test. Anal.* 2011; 3:857–867. [PubMed: 22147458]
13. Van Renterghem P, Van Eenoo P, Sottas PE, Saugy M, Delbeke F. *Drug Test. Anal.* 2010; 2:582–588. [PubMed: 21204290]
14. Van Renterghem P, Van Eenoo P, Sottas PE, Saugy M, Delbeke F. *Clinical Endocrinology.* 2011; 75:134–140. [PubMed: 21521264]
15. Sottas PE, Robinson N, Rabin O, Saugy M. *Clin. Chem.* 2011; 57:969–976. [PubMed: 21596947]
16. Frysinger GS, Gaines RB. *Hrc-Journal of High Resolution Chromatography.* 1999; 22:251–255.
17. Kallio M, Hyotylainen T, Lehtonen M, Jussila M, Hartonen K, Shimmo M, Riekkola ML. *J. Chromatogr. A.* 2003; 1019:251–260. [PubMed: 14650619]
18. Shellie RA, Marriott PJ, Huie CW. *J. Sep. Sci.* 2003; 26:1185–1192.
19. Shellie RA, Marriott PJ. *Analyst.* 2003; 128:879–883.
20. Cordero C, Bicchi C, Joulain D, Rubiolo P. *J. Chromatogr. A.* 2007; 1150:37–49. [PubMed: 16973171]

21. Purcaro G, Tranchida PQ, Ragonese C, Conte L, Dugo P, Dugo G, Mondello L. *Anal. Chem.* 2010; 82:8583–8590. [PubMed: 20873720]
22. Mitrevski BS, Wilairat P, Marriott PJ. *Anal. Bioanal. Chem.* 2010; 396:2503–2511. [PubMed: 20091298]
23. Silva AI, Pereira HMG, Casilli A, Conceicao FC, Neto FRA. *J. Chromatogr. A.* 2009; 1216:2913–2922. [PubMed: 19013575]
24. Korytar P, Parera J, Leonards PEG, Santos FJ, de Boer J, Brinkman UAT. *J. Chromatogr. A.* 2005; 1086:71–82. [PubMed: 16130657]
25. Han E, Yim O, Beak SY, Chung J, Lee JH, Kim J, Kim Y. *Bull. Korean Chem. Soc.* 2009; 30:1489–1496.
26. Fragkaki AG, Tsantili-Kakoulidou A, Angelis YS, Koupparis M, Georgakopoulos C. *J. Chromatogr. A.* 2009; 1216:8404–8420. [PubMed: 19836752]
27. Revelsky AI, Samokhin AS, Virus ED, Rodchenkov GM, Revelsky IA. *Drug Testing and Analysis.* 2011; 3:263–267. [PubMed: 21500370]
28. Korytar P, Parera J, Leonards PEG, de Boer J, Brinkman UAT. *J. Chromatogr. A.* 2005; 1067:255–264. [PubMed: 15844531]
29. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG. *J. Mass Spectrom.* 1996; 31:169–176. [PubMed: 8799270]
30. Aguilera R, Becchi M, Grenot C, Casabianca H, Hatton CK. *J. Chromatogr. B.* 1996; 687:43–53.
31. Piper T, Mareck U, Geyer H, Flenker U, Thevis M, Platen P, Schanzer W. *Rapid Commun. Mass Spectrom.* 2008; 22:2161–2175. [PubMed: 18536069]
32. Tobias HJ, Zhang Y, Auchus RJ, Brenna JT. *Anal. Chem.* 2011; 83:7158–7165. [PubMed: 21846122]
33. Shinebarger SR, Haisch M, Matthews DE. *Anal. Chem.* 2002; 74:6244–6251. [PubMed: 12510745]
34. Winters, SJ. *Evaluation of Testicular Function.* 3rd edn.. Lippincott Williams & Wilkins; Philadelphia, PA, USA: 2001.
35. Mondello L, Casilli A, Tranchida PQ, Lo Presti M, Dugo P, Dugo G. *Anal. Bioanal. Chem.* 2007; 389:1755–1763. [PubMed: 17589835]
36. Van Renterghem P, Van Eenoo P, Van Thuyne W, Geyer H, Schanzer W, Delbeke FT. *J. Chromatogr. B.* 2008; 876:225–235.
37. Ayotte C, Goudreault D, Charlebois A. *J. Chromatogr. B.* 1996; 687:3–25.
38. Van Renterghem P, Van Eenoo P, Geyer H, Schanzer W, Delbeke FT. *Steroids.* 2010; 75:373–375.
39. Stopforth A, Grobbelaar CJ, Crouch AM, Sandra P. *J. Sep. Sci.* 2007; 30:257–265. [PubMed: 17390621]
40. Shah VP. *Aaps Journal.* 2007; 9:E43–E47.
41. Sinha AE, Hope JL, Prazen BJ, Nilsson EJ, Jack RM, Synovec RE. *J. Chromatogr. A.* 2004; 1058:209–215. [PubMed: 15595670]
42. Pierce KM, Hoggard JC, Hope JL, Rainey PM, Hoofnagle AN, Jack RM, Wright BW, Synovec RE. *Anal. Chem.* 2006; 78:5068–5075. [PubMed: 16841931]
43. Welthagen W, Shellie RA, Spranger J, Ristow M, Zimmermann R, Fiehn O. *Metabolomics.* 2005; 1:65–73.
44. Shellie RA, Welthagen W, Zrostlikova J, Spranger J, Ristow M, Fiehn O, Zimmermann R. *J. Chromatogr. A.* 2005; 1086:83–90. [PubMed: 16130658]
45. Pierce KM, Hope JL, Hoggard JC, Synovec RE. *Talanta.* 2006; 70:797–804. [PubMed: 18970842]
46. Ozel MZ, Gogus F, Lewis AC. *J. Chromatogr. A.* 2006; 1114:164–169. [PubMed: 16516906]

Figure 1. GCxGC-qMS PCI-NH₃ total ion chromatogram of (A) 20 ng SM15 acetate standard and (B) endogenous steroid acetates in a urine extract sample.

Figure 2. Mass spectra of 5αA-diAC, in a urine extract acquired using (A) EI and (B) PCI with ammonia reagent gas (PCI-NH₃). It can be seen that PCI-NH₃ results in a very strong relative intensity for the [M + NH₄]⁺ ion and a MH⁺ mass ion, making molecular weight identification more unambiguous than for EI.

Table 1

EI GC×GC-qMS peak table for the 12 targeted endogenous steroids in SM15-AC neat and SM15-AC spiked into steroid free urine matrix(SFUM). The table excludes the exogenous steroid 5α-androstanol-AC and the 2 internal standards EpiA-AC and Cne.

EI of SM15-AC											
Steroid#	Steroid	Nominal Mass	t _R (min)	t _R (s)	GC2 Peak Width FWHM (ms)	Injection Mass (pg)	m/z (S/N)	LOD ¹ (pg)	Mass Range ⁴ (ng)	LOQ ⁵ (ng)	A ⁷ (%) (n=2)
1	19-NE-AC	318	20.3	2.20	132	20	201(22)	2.7	0.1–2	0.1	1.2
2	E-AC	332	21.4	2.52	144	20	272(29)	2.1	2–200	2.0	0.2
3	A-AC	332	21.8	2.60	156	20	272(17)	3.5	2–200	2.0	1.8
4	DHEA-AC	330	22.6	2.84	120	20	255(17)	3.5	0.1–2	0.1	5.1
5	11-KE-AC	346	23.3	3.60	156	20	271(13)	4.6	0.1–2	0.1	3.4
6	DHT-AC	332	24.0	3.16	180	20	257(11)	5.5	0.1–2	0.1	0.9
7	5βA-DiAC	376	24.8	2.76	180	20	241(36)	1.7	0.1–20	0.1	9.6
8	5αA-DiAC	376	25.2	2.88	144	20	241(36)	1.7	0.1–20	0.1	6.8
9	EpiT-AC	330	24.9	3.96	180	200	228(32)	18.8	0.1–2	0.1	2.3
10	T-AC	330	26.2	4.12	180	200	228(38)	15.8	0.1–2	0.1	8.7
11	11-OHA-3-AC	348	27.4	5.48	252	200	255(15)	40.0	0.2–20	0.2	10.8
12	5βP-DiAC	404	32.1	4.32	276	200	215(60)	10.0	0.1–20	0.1	8.2
Mean								9.1			4.9

EI of SM15-AC Spiked into Urine Matrix										
Steroid#	Steroid	Injection Mass (pg)	m/z (S/N)	LOD ¹ (pg)	Urine LOD ² (ng/mL urine)	Reverse Match Factor ³	Mass Range ⁴	LOQ ⁵ (ng)	RSD ⁶ (%) (n=2)	A ⁷ (%) (n=2)
1	19-NE-AC	20	201(11)	5.5	0.5	780	0.1–2	0.1	2.2	10.1
2	E-AC	20	272(6)	10.0	1.0	801	2–200	2.0	1.3	5.2
3	A-AC	20	272(8)	7.5	0.8	810	2–200	2.0	1.5	3.2
4	DHEA-AC	200	255(34)	17.6	1.8	803	0.1–2	0.1	1.8	–8.4

EI of SMI15-AC Spiked into Urine Matrix										
Steroid#	Steroid	Injection Mass (pg)	m/z (S/N)	LOD ¹ (pg)	Urine LOD ² (ng/mL urine)	Reverse Match Factor ³	Mass Range ⁴ (ng)	LOQ ⁵ (ng)	RSD ⁶ (%) (n=2)	A ⁷ (%) (n=2)
5	11-KE-AC	20	271(10)	6.0	0.6	760	0.1–2	0.1	3.4	14.2
6	DHT-AC	20	257(8)	7.5	0.8	766	0.1–2	0.1	4.2	–6.2
7	5βA-DiAC	20	241(14)	4.3	0.4	750	0.1–20	0.1	8.0	15.2
8	5αA-DiAC	20	241(10)	6.0	0.6	790	0.1–20	0.1	3.1	–11.4
9	EpiT-AC	200	228(32)	18.8	1.9	788	0.1–2	0.1	2.9	7.5
10	T-AC	200	228(36)	16.7	1.7	745	0.1–2	0.1	5.1	–16.9
11	11-OHA-3-AC	1000	255(16)	187.5	18.8	700	0.2–20	0.2	5.2	3.5
12	5βP-DiAC	200	215(30)	20.0	2.0	731	0.1–20	0.1	4.8	14.2
Mean				25.6	2.6				3.6	

¹ Limit of detection (LOD) is defined as the lowest concentration calculated to yield S/N=3 for the quantification ion signal in the (most intense) apex peak slice.

² Based on preparation of 2 mL urine into 100 uL extract, where 1 μL of extract is injected onto GC.

³ The reverse match factor was measured at peak apexes with the injection mass of the SM15-AC. It ignores chemical noise peaks in the submitted spectrum.

⁴ Mass range of the calibration curve is defined by the normal endogenous steroid concentration range in the human urine.

⁵ Limit of quantification (LOQ) is defined as the lowest concentration on the experimental calibration curve which can be measured within acceptable precision (RSD<20%) and accuracy (A<20%).

⁶ RSD, relative standard deviation or repeatability/reproducibility at our experimental LOQ, was measured using 2 replicates (n=2).

⁷ A is accuracy at LOQ calculated using 2 replicates (n=2).

Table 2

PCI- NH₃ GC×GC-qMS peak table for the 12 targeted endogenous steroids in SM15-AC neat and SM15-AC spiked into SFUM.

PCI-NH ₃ of SM15-AC										
Steroid#	Steroid	GC2 Peak Width FWHM (ms)	Injection Mass (pg)	(M+18) ⁺ (S/N)	(M+H) ⁺ (S/N)	LOD ¹ (pg)	Mass Range ⁴ (ng)	LOQ ⁵ (ng)	RSD ⁶ (%) (n=2)	A ⁷ (%) (n=2)
1	19-NE-AC	120	2	336(39)		0.2	0.1–2	0.1	3.7	10.5
2	E-AC	102	1	350(28)		0.1	2–200	2.0	0.2	1.3
3	A-AC	96	1	350(44)		0.1	2–200	2.0	2.4	2.1
4	DHEA-AC	180	20	348(39)		1.5	0.1–2	0.1	4.3	–8.8
5	11-KE-AC	156	20	364(41)		0.3	0.1–2	0.1	1.1	10.5
6	DHT-AC	210	20	350(17)		3.5	0.1–2	0.1	0.9	3.5
7	5βA-DiAC	102	2	394(26)		0.2	0.1–20	0.1	5.4	–8.4
8	5αA-DiAC	132	2	394(14)		0.4	0.1–20	0.1	3.3	10.9
9	EpiT-AC	108	1		331 (11)	0.3	0.1–2	0.1	4.5	7.9
10	T-AC	96	1		331 (26)	0.1	0.1–2	0.1	9.9	–14.6
11	11-OHA-3-AC	114	20	366(38)		1.6	0.2–20	0.2	1.8	–0.9
12	5βP-DiAC	174	20	422(30)		0.8	0.1–20	0.1	7.4	10.5
Mean						0.8			3.7	

PCI-NH ₃ of SM15-AC Spiked into Urine Matrix											
Steroid#	Steroid	Injection Mass (pg)	(M+18) ⁺ (S/N)	(M+H) ⁺ (S/N)	LOD ¹ (pg)	Urine LOD ² (ng mL ⁻¹ urine)	Reverse Match Factor ³	Mass Range ⁴ (ng)	LOQ ⁵ (ng)	RSD ⁶ (%) (n=2)	A ⁷ (%) (n=2)
1	19-NE-AC	2	336(8)		0.8	0.1	790	0.1-2	0.1	2.4	-12.5
2	E-AC	2	350(10)		0.6	0.1	760	2-200	2.0	1.2	1.3
3	A-AC	2	350(21)		0.3	0.0	770	2-200	2.0	3.5	-2.4
4	DHEA-AC	20	348(15)		4.0	0.4	833	0.1-2	0.1	1.5	-12.8
5	11-KE-AC	20	364(15)		4.0	0.4	772	0.1-2	0.1	1.8	9.5
6	DHT-AC	20	350(21)		2.9	0.3	762	0.1-2	0.1	0.4	5.5
7	5βA-DiAC	20	394(46)		1.3	0.1	846	0.1-20	0.1	4.3	-6.2
8	5αA-DiAC	20	394(18)		3.3	0.3	814	0.1-20	0.1	2.9	10.5
9	EpT-AC	20		331 (25)	2.4	0.2	827	0.1-2	0.1	5.4	-6.4
10	T-AC	20		331 (38)	1.6	0.2	868	0.1-2	0.1	8.5	-18.2

PCl-NH ₃ of SM15-AC Spiked into Urine Matrix											
Steroid#	Steroid	Injection Mass (pg)	(M+18) ⁺ (S/N)	(M+H) ⁺ (S/N)	LOD ¹ (pg)	Urine LOD ² (ng mL ⁻¹ urine)	Reverse Match Factor ³	Mass Range ⁴	LOQ ⁵ (ng)	RSD ⁶ (%) (n=2)	A ⁷ (%) (n=2)
11	11-OHA-3-AC	20	366(7)		8.6	0.9	740	0.2–20	0.2	2.3	–3.5
12	5βP-DiAC	20	422(7)		8.6	0.9	780	0.1–20	0.1	7.9	10.3
Mean					3.2	0.3				3.5	

¹Limit of detection (LOD) is defined as the lowest concentration calculated to yield S/N=3 for the quantification ion signal in the (most intense) apex peak slice.

²Based on preparation of 2 mL urine into 100 uL extract, where 1 μL of extract is injected onto GC.

³The reverse match factor was measured at peak apexes with the injection mass of the SM15-AC. It ignores chemical noise peaks in the submitted spectrum.

⁴Mass range of the calibration curve is defined by the normal endogenous steroid concentration range in the human urine.

⁵Limit of quantification (LOQ) is defined as the lowest concentration on the experimental calibration curve which can be measured within acceptable precision (RSD<20%) and accuracy (A<20%).

⁶RSD, relative standard deviation or repeatability/reproducibility at our experimental LOQ, was measured using 2 replicates (n=2).

⁷A is accuracy at LOQ calculated using 2 replicates (n=2).

Table 3
PCI-CH₄ GC×GC-qMS peak table for the 12 targeted endogenous steroids in SM15-AC neat and SM15-AC spiked into SFUM.

PCI-CH ₄ of SM15-AC									
Steroid#	Steroid	GC2 Peak Width FWHM (ms)	Injection Mass (pg)	m/z (S/N)	LOD ¹ (pg)	Mass Range ⁴ (ng)	LOQ ⁵ (ng)	RSD ⁶ (%) (n=2)	A ⁷ (%) (n=2)
1	19-NE-AC	144	20	241(17)	3.5	0.1–2	0.1	2.6	–13.3
2	E-AC	132	20	255(16)	3.8	2–200	2.0	1.3	4.6
3	A-AC	120	20	255(16)	3.8	2–200	2.0	0.9	5.2
4	DHEA-AC	168	20	271(16)	3.8	0.1–2	0.1	3.8	–7.9
5	11-KE-AC	108	20	287(16)	3.8	0.1–2	0.1	5.2	–15.3
6	DHT-AC	180	20	273(8)	7.5	0.1–2	0.1	0.8	4.8
7	5βA-DiAC	156	20	257(40)	1.5	0.1–20	0.1	9.5	16.3
8	5αA-DiAC	168	20	257(16)	3.8	0.1–20	0.1	5.0	11.8
9	EpiT-AC	156	200	331(13)	46.2	0.1–2	0.1	4.2	–17.2
10	T-AC	156	200	331(16)	37.5	0.1–2	0.1	7.4	16.3
11	11-OHA-3-AC	240	200	271(51)	11.8	0.2–20	0.2	12.8	3.4
12	5βP-DiAC	180	200	285(58)	10.3	0.1–20	0.1	6.9	–18.3
Mean					11.4			5.0	

PCI-CH ₄ of SM15-AC Spiked into Urine Matrix										
Steroid#	Steroid	Injection Mass (pg)	m/z (S/N)	LOD ¹ (pg)	Urine LOD ² (ng mL ⁻¹ urine)	Reverse Match Factor ³	Mass Range ⁴ (ng)	LOQ ⁵ (ng)	RSD ⁶ (%) (n=2)	A ⁷ (%) (n=2)
1	19-NE-AC	20	241(14)	4.3	0.4	780	0.1–2	0.1	5.2	16.5
2	E-AC	20	255(18)	3.3	0.3	779	2–200	2.0	1.1	7.6
3	A-AC	20	255(13)	4.6	0.5	834	2–200	2.0	2.5	–5.5
4	DHEA-AC	20	271(10)	6.0	0.6	750	0.1–2	0.1	3.8	–14.9
5	11-KE-AC	20	287(12)	5.0	0.5	733	0.1–2	0.1	6.5	8.4
6	DHT-AC	20	273(9)	6.7	0.7	540	0.1–2	0.1	3.8	3.5
7	5βA-DiAC	20	257(12)	5.0	0.5	750	0.1–20	0.1	9.8	–8.2
8	5αA-DiAC	20	257(11)	5.5	0.5	750	0.1–20	0.1	4.7	17.3

PCI-CH ₄ of SM15-AC Spiked into Urine Matrix										
Steroid#	Steroid	Injection Mass (pg)	m/z (S/N)	LOD ^I (pg)	Urine LOD ² (ng mL ⁻¹ urine)	Reverse Match Factor ³	Mass Range ⁴ (ng)	LOQ ⁵ (ng)	RSD ⁶ (%) (n=2)	A ⁷ (%) (n=2)
9	EpiT-AC	200	331(24)	25.0	2.5	695	0.1-2	0.1	8.6	-16.8
10	T-AC	200	331(20)	30.0	3.0	683	0.1-2	0.1	9.4	-17.9
11	11-OHA-3-AC	200	271(16)	37.5	3.8	546	0.2-20	0.2	10.5	3.7
12	5βP-DiAC	200	285(22)	27.3	2.7	608	0.1-20	0.1	6.9	12.3
Mean				13.3	1.3				6.1	

¹ Limit of detection (LOD) is defined as the lowest concentration calculated to yield S/N=3 for the quantification ion signal in the (most intense) apex peak slice.

² Based on preparation of 2 mL urine into 100 uL extract, where 1 μL of extract is injected onto GC.

³ The reverse match factor was measured at peak apexes with the injection mass of the SM15-AC. It ignores chemical noise peaks in the submitted spectrum.

⁴ Mass range of the calibration curve is defined by the normal endogenous steroid concentration range in the human urine.

⁵ Limit of quantification (LOQ) is defined as the lowest concentration on the experimental calibration curve which can be measured within acceptable precision (RSD<20%) and accuracy (A<20%).

⁶ RSD, relative standard deviation or repeatability/reproducibility at our experimental LOQ, was measured using 2 replicates (n=2).

⁷ A is accuracy at LOQ calculated using 2 replicates (n=2).