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Determination of urinary steroid sulfate metabolites using ion paired extraction

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Abstract

The need for laboratories accredited by the World Anti-Doping Agency (WADA) to develop methods of analysis for steroids excreted primarily as their sulfate conjugates has faced significant analytical challenges. One of the issues relates to the extraction of these metabolites from urine in a relatively pure state. The use of (−)-*N*,*N*-dimethylephedrinium bromide as an ion pairing reagent was optimised to produce a method that is selective for the extraction of steroid sulfates prior to GC-MS or LC-MS analysis, with minimal contributions from the urine matrix. The recovery of androsterone from its sulfate conjugate was determined to be 67% with a relative quantitative uncertainty of ±14% ($k = 2$).

Keywords: Doping control; Steroid sulfates; Ion pairing

1. Introduction

The World Anti-Doping Agency (WADA) prohibits the use of anabolic (tissue building) androgenic (male sex hormone) steroids by athletes due to their performance enhancing and adverse health effects [1]. The process of steroid detection undertaken by WADA accredited laboratories makes use of the high sensitivity and selectivity of gas chromatography-mass spectrometry (GC-MS) in selected ion mode (SIM).

In order to be eliminated from the body, steroids must become soluble in urine by reducing their hydrophobicity. This is achieved by transformation of the steroid structure (phase I metabolism) and/or by formation of a conjugated species (phase II). The latter may form glucuronide or sulfate conjugates by reaction with UDP-glucuronic acid and 3′-phosphoadenosine 5′-phosphosulfate, respectively [2]. The phase II metabolism of androsterone (A) is illustrated by Fig. 1. Routinely, doping control laboratories screen urine samples collected from athletes for the presence of free (unconjugated) and glucuronide conjugated steroids by GC-MS [3]. In order to achieve this, glucuronide conjugates are first converted to their free form by enzymatic hydrolysis with β-glucuronidase from *Escherichia coli* (or *Helix pomatia*) which cleaves the glucuronide moiety from the steroid molecule. Once steroids are in their free form, they are isolated from the urine matrix by solid phase or solvent extraction. Many urinary steroids then require derivatisation to improve volatility, stability and peak shape for gas chromatography columns. Silylation is the preferred method used to form less polar steroid derivatives. Trimethylsilyl (TMS) enol–ether derivatives can be prepared using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) catalysed with trimethylsilyliodide (TMSI) [3,4]. This method of derivatisation can improve the selection of diagnostic ions.

There is a need for doping control laboratories to develop confirmation methods of analysis that are specific to individual steroid metabolism, which include a number of steroid metabolites primarily as their sulfate conjugates. Analysis of steroid sulfates would allow improvements to be made in the detection of some endogenous (i.e. naturally occur-

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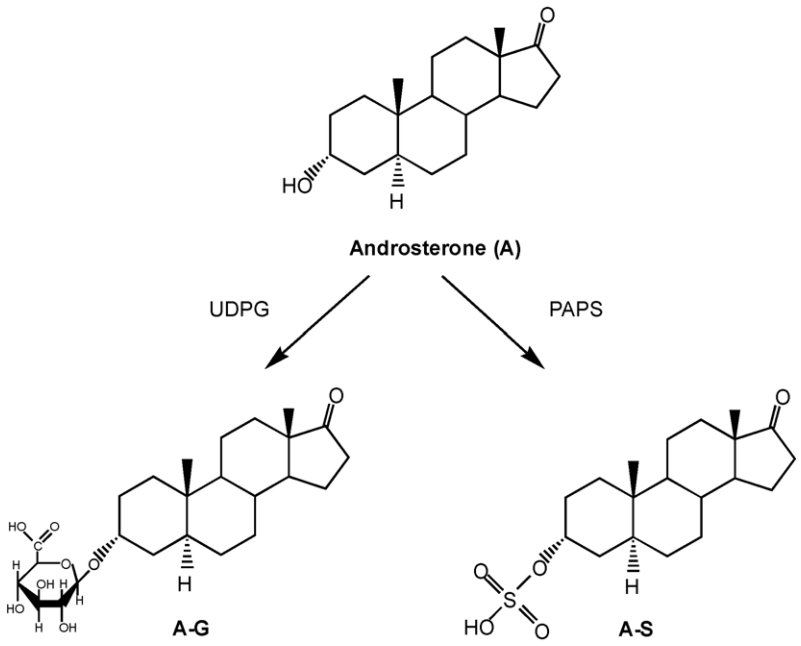

Fig. 1. Phase II metabolism of androsterone (A; 5α-androstane-3α-ol-17-one).

ring) steroids that are illegally administered as their synthetic analogues. Examples of these include the prohormones; dehydroepiandrosterone (DHEA) [5] and androstenedione [6]. Improvements are also sought for the detection of sulfate metabolites of exogenous steroids (i.e. those foreign to the body) that are excreted in greater quantities than their glucuronide analogues, resulting in greater retrospective detection capabilities. Numerous examples of these are discussed in the comprehensive review of steroid metabolism provided by Schänzer [2].

The development of a robust and reproducible analytical method for GC-MS analysis of steroids originating from their sulfate conjugates has not proved to be simple. Enzymatic hydrolysis using the digestive juices of the Roman snail (*H. pomatia*) do not contain all the sulfatases necessary for hydrolysis of all steroid sulfates. For instance, the hydrolysis of 3α-hydroxy-5β- and 3β-hydroxy-5-ene steroid sulfates using this enzyme has been found to be efficient, whereas 3α-hydroxy-5α-sulfates and C_{19}-steroids sulfated at C-17 have been found to be resistant to hydrolysis [7]. In addition, various authors have reported the possibility of *H. pomatia* induced enzyme hydrolysis resulting in artefact formation such as the conversion of 3β-hydroxy-5-ene steroids into 3β-hydroxy-5α-steroids and 3-keto-4-ene steroids [3,7–9].

The importance of hydrolyzing steroid sulfates using chemical hydrolysis (solvolysis) techniques was illustrated by Vestergaard [10] who reported that an average of 17% of A, 11% of etiocholanolone (Et) and 73% of DHEA is excreted as the sulfate conjugate. A and Et are the terminal metabolites of the androgen biosynthetic pathway while DHEA is their primary precursor. Chemical hydrolysis is preferable to enzymatic methods as it results in quantitative cleavage of the sulfate moiety. Many of the published chemical hydrolysis methods [11–13] relating to steroid sulfates are adaptations of the method described by Burstein and Lieberman [14]. For the purpose intended, however, these have been problematic to implement into the laboratory, presumably due to matrix effects.

Methodology was therefore required that would minimise urinary matrix effects during chemical hydrolysis procedures. Selectivity of the method toward the purification of steroid sulfates prior to hydrolysis was favoured in relation to all other method parameters such as time and labour. Siteri [15] has summarized the use of solvent extraction and ion exchange methods for the isolation of steroid conjugates from complex matrices. It was concluded that adsorption of sulfate conjugates to commonly employed ion exchange resins does not result simply from ionic interactions. The reported use of a liquid ion exchanger (Amberlite LA-1®) in ethyl acetate to extract estrogen conjugates from urine [16] was of particular interest. This concept of ion pairing was investigated in order to isolate sulfate conjugates from urine in a purified form. Novel ion pairing methods have been developed by Lisi et al. for doping control analysis using in situ extractive alkylation of diuretics [17,18], cannabis [19] and narcotic analgesic drugs such as buprenorphine [20]. These methods use phase transfer catalysis [21–23], the effectiveness of which is dependent on favourable ion pair extraction equilibrium between the aqueous and the organic phase.

In order to maximize this equilibrium there are a number of method parameters to consider:

- Initial cleanup: Solid phase cleanup techniques such as the use of polymerized resins can be employed to obtain

an organic fraction containing steroid conjugates from the aqueous urine matrix and subsequently remove inorganic anions that may compete with the ensuing ion pairing reaction.
- The nature of the organic phase used for ion pairing extraction is important, as it has a strong influence on the ion pairing extraction efficiency. Ion pairs have a polar character and have been observed to be poorly extracted by nonpolar solvents such as aliphatic hydrocarbons, but the extraction increases rapidly with increasing polarity of the organic phase [24].
- The choice of ion pairing reagent (also called phase transfer catalyst): The most common reagents are quaternary ammonium salts containing a lipophilic cation.
- Removal of ion pairing reagent: Once effective ion pairing extraction has been achieved the problem of co-extracted quaternary ammonium salts that are soluble in the organic phase exists. Inside the GC injection port these have been reported [25] to undergo Hofmann elimination (pyrolysis) to produce their corresponding tertiary amines, resulting in chromatographic interferences and a rapid deterioration in the efficiency of capillary columns. A solution to this problem was reported by Lisi et al. [18] who demonstrated removal of greater than 99% of co-extracted ion pairing reagent using a commercially available acrylic co-polymer (XAD-7) resin.

Following purification of urinary steroid sulfate metabolites using ion pairing the chemical hydrolysis technique needs to be defined. Androsterone was chosen as the analyte for which to develop this method due to its importance in endogenous steroid testing and the availability of the necessary sulfate reference materials. Following successful ion pairing extraction of androsterone hydrogen sulfate (AS) and removal of problematic reagents, the acid hydrolysis technique chosen was thought to be clean, gentle and less hazardous than other reported methods [11–14]. Trimethylchlorosilane (TMCS) is used to generate anhydrous hydrogen chloride (HCl) in methanol, in a temperature-controlled manner due to its exothermic nature [26].

The validation of the method described herein is presented in a manner that highlights the importance that ISO 17025 compliance has for doping control laboratories. A comprehensive and up to date review of bioanalytical method validation has been provided by Peters and Maurer [27] that discusses the implications for forensic and clinical toxicology laboratories.

2. Experimental

2.1. Steroids and reagents

Steroid reference materials of androsterone (D550) and (2,2,4,4-d_4)-androsterone (D549) were obtained from the National Analytical Reference Laboratory (NARL, Sydney, Australia). Androsterone hydrogen sulfate (AS) and (2,2,4,4-d_4)-androsterone hydrogen sulfate (d_4-AS) were obtained by cation exchange, using AG® MP-50 resin (Bio-Rad, Richmond, USA), of the respective triethylammonium salt reference materials (D610 and D587) provided by NARL. A steroid reference material of DHEA was obtained from Sigma Chemical Co., St. Louis, MO, USA. PADII resin was obtained from Serva (Heidelberg, Germany). The ion pairing reagents: (−)-*N*,*N*-dimethylephedrinium bromide, (−)-*N*-dodecyl-*N*-methylephedrinium bromide, tetraoctylammonium bromide, tetrakis(decyl)ammonium bromide, tetradodecylammonium bromide, tetraphenylphosphonium chloride and tetrahexylammonium hydrogen sulfate were obtained from Fluka (Buchs, Switzerland). XAD-7 resin (20–50 mesh) was obtained from Fluka (Steinheim, Switzerland); TMCS and silanized glass wool from Alltech (Deerfield, IL, USA); MSTFA from Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany); sodium hydroxide pellets, potassium carbonate, potassium bicarbonate, ammonium iodide and ethanethiol from BDH (Poole, UK). HPLC grade methanol together with analytical grade hexane, toluene and *tert*-butyl methyl ether were purchased from EMD Chemicals Inc. (Darmstadt, Germany). Analytical grade dichloromethane (DCM) was purchased from Mallinkrodt (Paris, Kentucky, USA). The water used was obtained using a Milli-Q® water purification system purchased from Millipore (Bedford, MA, USA).

2.2. Isolation of steroid sulfate conjugates from aqueous matrix

PADII resin was prepared for use by washing 100 g resin with methanol (2×200 mL) followed by water (3×200 mL). To a 3 mL aliquot of aqueous matrix (water or urine used in this study), d_4-AS (10 μg/mL, 30 μL) was added before being passed through columns containing PADII resin (2 cm in a pasteur pipette). The resin was washed with water (2 mL) before the steroid conjugates were eluted with methanol (2 mL) into a clean dry test tube. The eluate was evaporated to dryness under nitrogen in a Turbovap apparatus at 35 °C.

2.3. Ion pairing of steroid sulfates

Seven ion pairing reagents were evaluated to determine the most suitable for the extraction of steroid sulfates. Their relative efficiencies were determined by a simple recovery experiment, whereby AS (100 ng/mL equivalence of A) was spiked in water followed by ion pairing with the particular reagent (0.2 M, 100 μL), acid hydrolysis and hexane extraction before comparison to d_4-A (100 ng/mL) added as internal standard.

Following its selection, the concentration of (−)-*N*,*N*-dimethylephedrinium bromide required for efficient ion pairing was determined by the comparison of 5, 10, 20, 30, 40, 50 and 60 μM relative concentrations of (−)-

N,*N*-dimethylephedrinium bromide used to extract AS (1000 ng/mL equivalence of A) measured against d_4-A (100 ng/mL) added after acid hydrolysis. This was done by the addition of (−)-*N*,*N*-dimethylephedrinium bromide (0.2 M; 25, 50, 100, 150, 200, 250 and 300 μL) to deprotonated aqueous solutions containing AS followed by acid hydrolysis, hexane extraction and derivatisation.

In the optimised method, the dry steroid conjugate fraction obtained from PADII solid phase extraction was reconstituted with water (3 mL) before sodium hydroxide (6 M, 200 μL) was added to deprotonate the steroid sulfates. Dichloromethane (5 mL) was added followed by (−)-*N*,*N*-dimethylephedrinium bromide (0.5 M, 100 μL). The test tube was capped then shaken on a rotary mixer for 1 h before being centrifuged at 2000 rpm for 30 min.

2.4. Removal of co-extracted ion pairing reagent

The XAD-7 resin (20–50 mesh) was gently ground in a mortar and pestle and sieved to collect the 200–400 mesh fraction. The fines were removed from this material by suspending the sorbent in methanol, gently shaking, allowing to settle for a few minutes and decanting the supernatant. This procedure was repeated until the supernatant was clear. Columns (1 cm i.d.) containing XAD-7 resin were prepared by fitting small plugs of silanized glass wool to act as a bed support for 3 cm resin. Before use the columns were conditioned with methanol (3 mL) followed by DCM (2 × 2 mL). The separated organic layer from the ion pairing extraction was passed through the XAD-7 resin with collection of the eluate in a clean dry test tube. The column was washed with DCM (1 mL) and the combined eluate was then evaporated to dryness under nitrogen in a Turbovap apparatus at 35 °C.

2.5. Hydrolysis of steroid sulfates

TMCS (10%) was prepared for each batch of analysis by the dropwise addition of TMCS (2 mL) to methanol (18 mL) chilled in an ice bath. One milliliter of this reagent was added to the dry steroid sulfate residue and heated in a water bath at 50 °C for 30 min. The efficiency of this hydrolysis method was evaluated by a simple recovery experiment (based on the certified equivalence of 600 ng A from 1000 ng AS), whereby acid hydrolysis was conducted on seven replicates of AS (1000 ng/mL equivalence of A) before comparison to d_4-A (1000 ng/mL) added as internal standard post-hydrolysis.

2.6. Organic extraction of hydrolysed steroids

The hydrolysate was allowed to cool to room temperature before water (3 mL) was added then the pH adjusted to 9.0 using potassium carbonate/potassium bicarbonate (1:2) buffer (20% w/v, 1 mL). Hexane (5 mL) was added and the test tube sealed before shaking using a rotary mixer for 30 min, and centrifugation at 2000 rpm for 10 min. The hexane layer was transferred to a clean dry test tube and evaporated to dryness under nitrogen.

2.7. Derivatisation of steroids for GC-MS analysis

Steroid standards and extracts were derivatized by reconstitution of the dry extract in MSTFA-TMSI-ethanethiol (200:2:3, 55 μL) reagent with heating in a sealed test tube at 60 °C for 15 min. The final products were then transferred to sample vials and sealed.

2.8. GC-MS SIM analysis of steroids

GC-MS was carried out using an Agilent technologies HP 5973 electron-impact (EI) mass selective detector coupled via direct capillary interface to a HP 6890 gas chromatograph with a HP 7673 automatic injector and sample tray (Palo Alto, CA, USA). All analysis was carried out by obtaining peak areas from SIM for ions of mass to charge ratio (*m*/*z*) of 434 for A, and 438 for d_4A, corresponding to the molecular ions of their respective TMS enol–ether derivatives, with dwell times of 20 ms. The carrier gas was helium with a flow rate of 0.8 mL/min, split ratio 12:1 and pressure of 15.9 psi. The column was an Agilent HP Ultra-1 fused silica (0.22 mm i.d. × 17 m), cross-linked methylsilicone (0.11 μm film thickness) (Palo Alto, CA, USA), operated in constant pressure mode. The column temperature was programmed from 187 °C for 0.2 min to 238 °C at 3 °C/min, then to 310 °C at 50 °C/min and held for 0.5 min. The injector and transfer line temperatures were 250 and 300 °C, respectively. The injection volume was 3 μL and a solvent delay of 5 min was programmed.

2.9. Method selectivity

The selectivity of steroid sulfate extraction was evaluated by comparison to the routine steroid extractions, commonly used by doping control laboratories that utilise β-glucuronidase from *E. coli* (at pH 7) or *H. pomatia* (at pH 5) for enzymatic hydrolysis of glucuronides. A suitable volume (500 mL) of blank urine (QCU) was collected from an individual that was representative of a normal sample analysed by the laboratory. This determination was based on the concentrations of endogenous steroids (i.e. the steroid profile) determined by regular screening analysis of a 3 mL urine sample [28,29]. In addition, QCU was certified to contain no evidence of illegal steroid administration [1]. The comparison was made from full scan (50–550 amu) GC-MS analysis using the same chromatographic conditions described previously for SIM.

2.10. Limit of detection (LOD) and limit of quantification (LOQ)

To determine the LOD and LOQ of the method, water spikes containing AS were prepared at equivalent A concen-

trations of 1, 2, 5, 10, 15, 20, 30, 40, 50 and 100 ng/mL, without addition of d_4-AS, prior to ion pairing extraction, acid hydrolysis, hexane extraction and derivatisation.

2.11. Method linearity and recovery

The linearity and recovery of the method was evaluated by measuring the relative responses of A hydrolyzed from AS in water spikes to d_4-A (100 ng/mL) added after acid hydrolysis. AS was spiked into water (3 mL) at equivalent A concentrations of 2, 5, 10, 100, 500, 1000, 2000 and 5000 ng/mL prior to ion pairing, acid hydrolysis, solvent extraction and derivatisation.

2.12. Precision of the method

Seven replicates of QCU were prepared by addition of d_4-AS (10,000 ng/mL, 30 μL) to 3 mL of sample. These were subject to the ion pairing-acid hydrolysis method prior to GC-MS analysis. The AS concentration of QCU was determined relative to calibration curves obtained from A concentrations of 2, 5, 10, 100, 500, 1000, 2000 and 5000 ng/mL measured relative to d_4-A (100 ng/mL). Repeatability of the method was determined from three separate sets of analysis for seven aliquots of QCU.

2.13. LC-MS-MS analysis

The feasibility of direct analysis of steroid sulfate conjugates was evaluated by LC-MS-MS analysis of extracts obtained following ion pairing without subsequent acid hydrolysis required for GC-MS analysis. A (10,000 ng/mL; 30 μL) was added to water (3 mL) prior to PADII and ion pairing extraction. The dried steroid sulfate extract obtained following removal of the (−)-*N*,*N*-dimethylephedrinium cation was reconstituted in methanol (100 μL) for LC-MS-MS analysis using a Waters Alliance 2795 separation module equipped with a quaternary pump, coupled to a Micromass Quattro Micro triple stage quadrupole equipped with a Z spray ESI interface. A C18 column (Alltech Alltima, 150 mm × 2.1 mm × 5 μm) protected by a C18 guard column (Phenomenex Security Guard 4 mm × 2 mm) was used. The injection volume was 10 μL. The following mobile phase gradient was formed by solvent A (2% aqueous formic acid), solvent B (methanol) and solvent C (water) at a flow rate of 0.2 mL/min: 10% A/30% B/60% C (0–1 min), 10% A/70% B/20% C (1–9 min), 10% A/90% C (9–9.5 min), 1% A/99%B (9.5–13.5 min) and 10% A/30% B/60% C (13.5–20 min). The spray conditions of the ESI interface were: desolvation temperature 200 °C, desolvation gas (N_2) flow 560 L/h and cone gas (N_2) flow 60 L/h. The source temperature was 115 °C. The capillary was set at 3.5 kV and the AS specific cone voltage and collision energy were optimised at 25 V and 40 eV, respectively. Argon was used as the collision gas at a pressure of 3.9×10^{-3} mbar. Multiple reaction mode (MRM) analysis was conducted in negative ionisation mode for $[M-1]^{+\bullet} = 369$ and 373 corresponding to the molecular ions of AS and d_4-AS, respectively.

Table 1
Comparison of seven commercially available ion pairing reagents

Ion pairing reagent	Relative extraction efficiency	Approximate recovery (%)
(−)-*N*,*N*-Dimethylephedrinium bromide	1.0	62
(−)-*N*-Dodecyl-*N*-methylephedrinium bromide	0.8	50
Tetrahexylammonium hydrogen sulfate	0.7	43
Tetraoctylammoniuim bromide	0.0	0
Tetrakis(decyl)ammonium bromide	0.0	0
Tetradodecylammonium bromide	0.0	0
Tetraphenylphosphonium chloride	0.0	0

3. Results and discussion

3.1. Ion pairing of steroid sulfates

Table 1 summarises the relative extraction efficiencies and approximate recovery values from the survey of ion pairing reagents. The use of (−)-*N*,*N*-dimethylephedrinium bromide (Fig. 2) was observed to result in the highest relative recoveries of AS (62%) using 20 μM equivalence of reagent. Interesting, however, was the trend observed in relation to the structure of the ion pairing reagent, whereby smaller alkyl groups attached to the alkylammonium ion resulted in higher extraction efficiencies. This was unexpected, considering previous studies [21–24] that describe ion pairing efficiency to increase with increasing alkyl chain length of reagents.

The optimal equivalent (−)-*N*,*N*-dimethylephedrinium bromide concentration from the comparison study was found to be 50 μM (Fig. 3), at which an approximate recovery of 75% was achieved. No improvement in recovery was observed for a relative concentration of 60 μM, so 50 μM (i.e. addition of 100 μL of 0.5 M) was chosen as the optimised method parameter in order to minimise the possibility of incomplete removal of excess reagent by the XAD-7 resin.

The only solvent to result in effective ion pairing of AS with the (−)-*N*,*N*-dimethylephedrinium counter ion was

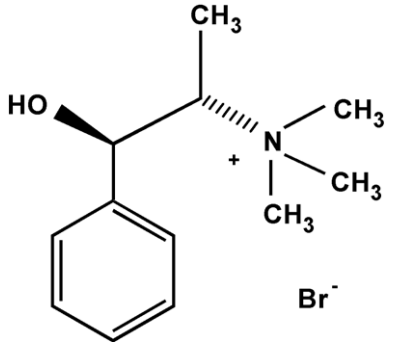

Fig. 2. Structure of (−)-*N*,*N*-dimethylephedrinium bromide.

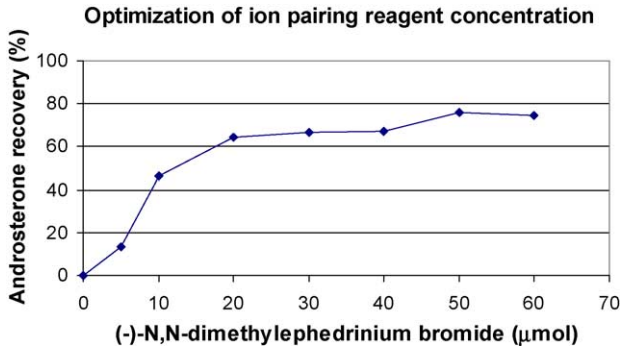

Fig. 3. Optimization of (−)-*N,N*-dimethylephedrinium bromide concentration.

DCM. Other solvents; hexane, toluene and *tert*-butyl methyl ether resulted in zero extraction efficiencies. While the use of DCM and hexane under these conditions was expected to provide the most and least favourable results, respectively, the extremely poor results obtained for toluene and *tert*-butyl methyl ether were unexpected.

3.2. Hydrolysis of steroid sulfates

Excellent yields of A compared to d_4-A were obtained from the seven replicates of AS subject to the acid hydrolysis procedure. The average yield was 102%, which within experimental error, indicates complete hydrolysis of A.

3.3. Method selectivity

Comparison was made between the urine sample that underwent enzyme hydrolysis prior to solid phase extraction, and for ion pairing prior to acid hydrolysis and solvent extraction. The QCU urine was declared to represent a normal sample that would usually be analysed by the laboratory following the regular steroid profile analysis showing the concentrations of A, Et and DHEA to be 1534, 1051 and 35 ng/mL, respectively. Fig. 4 shows the GC-MS full scan total ion chromatograms obtained from the ion pairing-acid hydrolysis method and two methods of enzymatic hydrolysis. Excellent selectivity was observed for the isolation of A and DHEA using the ion pairing-acid hydrolysis method

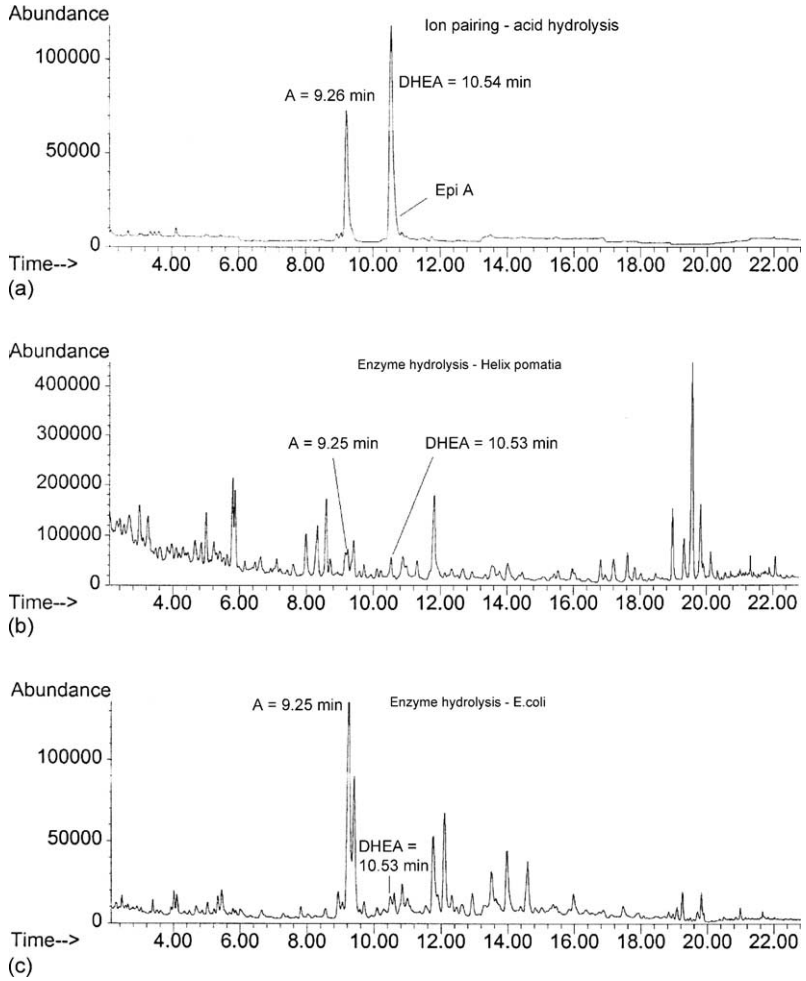

Fig. 4. Total ion chromatograms obtained from GC-MS full scan (50–650 amu) analysis of QCU showing (a) the selectivity of the ion pairing method for the extraction of urinary steroid sulfates by comparison to direct enzyme hydrolysis with β-glucuronidase from (b) *H. pomatia* and (c) *E. coli*.

in comparison to the two methods of enzymatic hydrolysis. The 3β-epimer of A (epiA) was also identified from standard mass spectra [30] to be present as a coeluting species located at the tailing edge of the DHEA peak. DHEA and epiA are 3β-hydroxy steroids expected to be primarily excreted as their sulfate conjugates in urine [10]. Further work will be required to determine whether the method is only selective to 17-keto steroids such as A, DHEA and epiA, therefore, limiting the analysis of 3-keto steroids that are excreted as their sulfate conjugates. While β-glucuronidase from *H. pomatia* (pH 5) was shown to hydrolyse significant proportions of steroid sulfates and glucuronides, it produced an extract containing many other peaks. As expected, β-glucuronidase from *E. coli* (pH 7) was ineffective in the hydrolysis of steroid sulfates, determined from the extremely low amount of DHEA recovered. Direct ether extraction of QCU at pH 7 demonstrated that no steroids were present in their unconjugated form.

3.4. Limit of detection (LOD) and limit of quantification (LOQ)

The International Conference on Harmonization (ICH) determines the LOD of an analytical method to be the lowest concentration of analyte in a sample, which can be detected but not necessarily quantified as an exact value [31]. This can be based on the signal to noise ratio *S*/*N*, defined as the ratio between the height of the analyte peak (signal) and the lowest point of the baseline (noise) in a certain area around the analyte peak. For LOD, *S*/*N* is usually required to be greater than or equal to 3 [32,33]. This condition represents an AS concentration of 2 ng/mL for the method reported. The LOQ of an analytical method has been defined as the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy [31]. For LOQ based on *S*/*N* a value of greater than or equal to 10 is usually required [32]. This represents an AS concentration of 5 ng/mL for this method. It should be emphasized, however, that these determinations were based on AS spiked in water and both the LOD and LOQ may be higher for the urine matrix containing interferences.

3.5. Method linearity and recovery

The endogenous nature of A prevented the determination of method linearity and recovery in the more representative urine matrix due to the effect that urinary A would have on standard additions. In water the optimised method was determined to be linear, in relation to the range of A concentrations derived from AS for the 8-point analysis in the range of 2–5000 ng/mL. Regression analysis of AS concentrations plotted against the response ratio of A to d_4—A showed the slope to be 0.0041 with an intercept of 0.1570. The standard deviations of the slope and intercept were 0.000045 and 0.0867, respectively, while the standard error was 0.2043. The correlation coefficient was determined to be 0.9993. The recovery of the method, accounting for the mass equivalence of A from AS, was determined to be 67% by comparison of the A response from spiked AS at the equivalent concentration of d_4-A. The loss of analyte is expected to be found in the PADII solid phase extraction, the ion pairing and hexane extractions, as well as the derivatisation step.

3.6. Precision of the method

Excellent precision was observed for the method. Table 2 provides the concentrations of A determined from QCU for the three sets of analysis. The mean concentration of A from AS determined for QCU was 464 ng/mL. The intra-assay precision was found to be within 1.0%, while the inter-assay precision between the three sets of analysis was 1.1%. Since the intra- and inter-assay variations were similar it is assumed

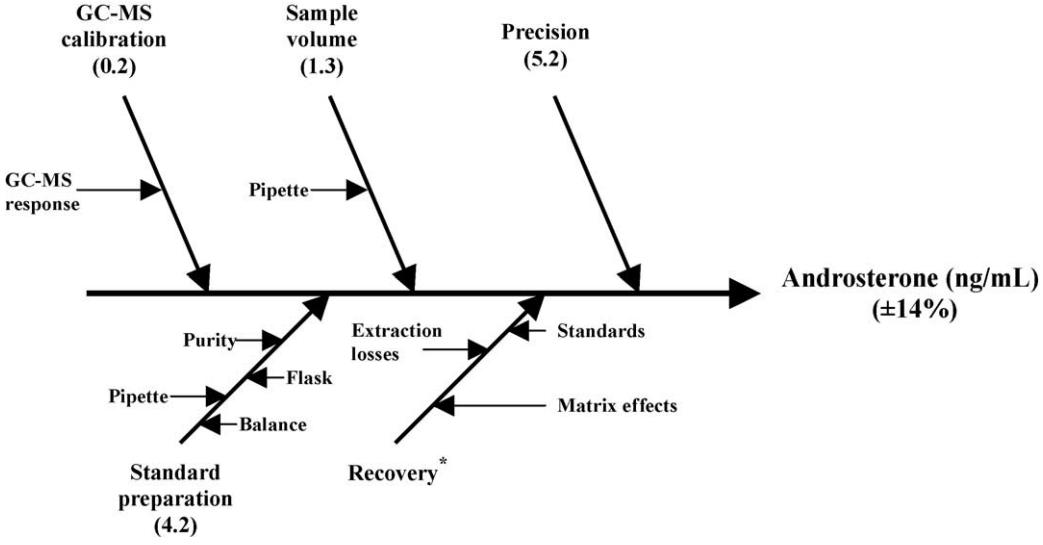

Fig. 5. Cause and effect diagram of MU components and their quantities resulting in an expanded relative uncertainty (*U*) of A determined by this method to be ±14%. (*) *Note*: a contribution from recovery would be required when isotopically labeled internal standards are not used.

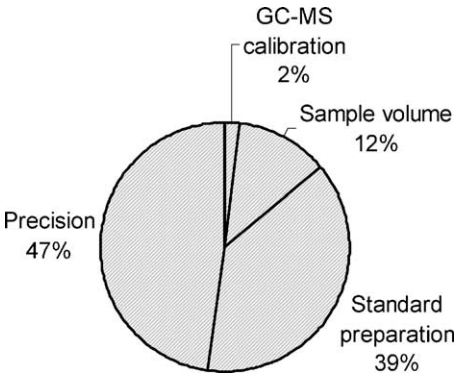

Fig. 6. Relative contribution of uncertainty components.

Table 2
Intra- and inter-assay repeatability of determining the concentration of A in QCU

Sample	Analysis 1 (ng/mL)	Analysis 2 (ng/mL)	Analysis 3 (ng/mL)	Inter-assay
1	473	470	465	
2	463	473	465	
3	462	467	463	
4	460	466	454	
5	462	470	464	
6	466	470	456	
7	460	460	458	
Mean	464	468	461	464
S.D.	4.6	4.2	4.6	5.2
% c.v.	1.0	0.9	1.0	1.1

that the overall precision of the method includes homogeneity of the sample.

3.7. Estimation of measurement uncertainty (MU) for the method

To satisfy the requirements of ISO 17025, a reasonable estimate of measurement uncertainty (MU) can be proposed for the method described here based on the reference material information, calibration data and validation data available [34,35]. In relation to the method reported here, the measure and is the endogenous steroid androsterone (A) concentration. A is a well defined chemical entity having the formula $C_{19}H_{30}O_2$, a molecular weight of 290.4, CAS registry number (53-41-8) and the structure shown in Fig. 1. The sources

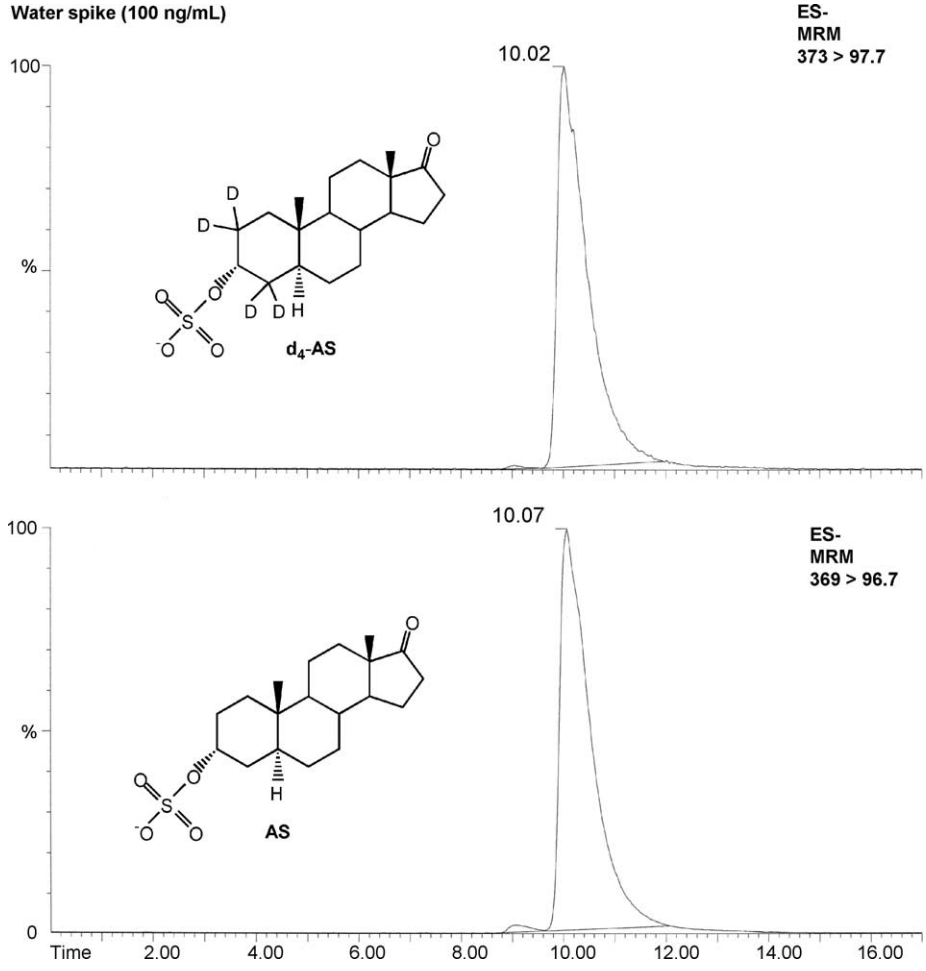

Fig. 7. LC-MS-MS analysis of d_4-AS ($[M-1]^{+\bullet} = 373$) and AS ($[M-1]^{+\bullet} = 369$) obtained from ion pairing of water spike.

of uncertainty for this method were summarized by cause and effect analysis (Fig. 5) that also includes the quantities of these components. These individual standard uncertainties (u values) were added as u^2 values using the square root of the sum of the squares method [34] to obtain a combined u value of ±32 ng/mL estimated at an A concentration of 464 ng/mL. The MU estimate is reported as an expanded uncertainty (U-value) of ±64 ng/mL using a coverage factor of $k = 2$, which represents a relative uncertainty of 14% that is thought to be fit for the intended purpose.

There was no requirement for a separate contribution for the uncertainty associated with recovery since an isotopically-labeled analogue of the analyte was used as surrogate. If an external standard method was used, however, a recovery factor would need to be included. The issue of bias is also alleviated by the use of certified reference materials with defined purities (98 ± 2%), such that any uncertainty associated with bias is included in the component representing the preparation of standard solutions. Fig. 6 shows the relative uncertainty that each component contributes to the overall MU estimate.

3.8. Further applications

3.8.1. LC-MS-MS analysis

Together with GC-MS analysis of hydrolyzed steroids obtained from their sulfate conjugates, the ion pairing method enables direct analysis of steroid sulfates by LC-MS-MS. Fig. 7 demonstrates an example of LC-MS-MS analysis of a steroid sulfate extract obtained following ion pairing of AS spiked in water. For quantitative and qualitative purposes, simultaneous analysis of d_4-AS added as internal standard can be conducted.

3.8.2. Carbon isotope ratio analysis

By taking advantage of the selectivity of the method, the analysis of steroid sulfate metabolites by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) [36–38] is also made possible. The high recovery and purity of steroid peaks will ensure that carbon isotopic fractionation is minimized. This will enable both pure and applied aspects of steroid analysis to be improved by the investigation of steroid metabolism and the ability to conduct confirmatory analyses.

4. Conclusion

A method has been presented that provides a significant improvement in the reliability of the extraction and analysis of steroid sulfates excreted in urine. While this has been extensively studied for AS, its application to other sulfates appears to be reasonable but requires further investigation. It is envisaged that this method will allow quantitative analysis of urinary steroid sulfates as needed for the confirmation of particular steroid abuse together with studies to improve the understanding of complex steroid metabolism. Of particular interest is the qualitative isolation of sulfates for LC-MS-MS and GC-C-IRMS analysis where the purity of the final product is of importance. Information obtained from the validation of this method was used to propose a reasonable estimate of measurement uncertainty for its intended purpose. This has been demonstrated to convey the importance of ISO 17025 compliance for forensic toxicology laboratories.

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