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Direct determination of anabolic steroid conjugates in human urine by combined high-performance liquid chromatography and tandem mass spectrometry

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Abstract

A novel screening procedure for the sulfate and glucuronide conjugates of testosterone (T) and epitestosterone (E) in human urine was developed based on liquid–solid extraction and microbore high-performance liquid chromatography combined on-line with ion-spray tandem mass spectrometry. Confirmation of the sulfate and glucuronide conjugates of testosterone and epitestosterone isolated from normal human urine was achieved by selected reaction monitoring of characteristic product ions of the parent compounds. Endogenous levels of the steroid conjugates are detected in normal male urine and an increase is observed when the sample is fortified with authentic analytical standards of the conjugates. Calibration curves of all steroid conjugates in urine are linear over a range of twenty. Deuterated internal standards of testosterone glucuronide and epitestosterone sulfate were used for quantitation of the endogenous conjugates. T/E ratios were determined based on the glucuronide fractions of six replicates from a normal male and were shown to be statistically reproducible and below the accepted T/E threshold of 6:1. Sulfate conjugates were shown to be present at significantly lower levels in the urine. The method has potential as an alternative for monitoring anabolic steroid conjugates in human urine.

Keywords: Steroids; Anabolic steroids; Testosterone; Epitestosterone

1. Introduction

Anabolic steroids are currently an important class of abused drugs. The determination of these compounds in biological samples is an analytical challenge due to their chemically neutral character and extensive metabolism. Metabolism of the parent drugs yields polar, involatile end-products, including sulfate and glucuronide conjugates. Conjugation of the parent drugs increases the rate of their excretion. Due to their low volatility and thermal instability, drug conjugates are not directly amenable to gas chromatography–mass spectrometry (GC–MS), so these compounds are commonly hydrolyzed enzymatically for further characterization. Following isolation and derivatization, the released steroids are then confirmed by GC–MS. From the amount of testosterone (T) and epitestosterone (E) released from the glucuronide conjugates arises the T/E ratio currently used in doping control to determine whether or not an individual has abused anabolic steroids to enhance athletic performance.

Many sulfate conjugates are resistant to enzymatic hydrolysis and are either overlooked or hydrolyzed

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by other means, such as hydrolysis with aqueous hydrochloric acid or mild acidic hydrolysis in organic solvent [1]. Hydrolysis is usually performed using the enzyme β-glucuronidase/arylsulfatase from *Helix pomatia*. A major disadvantage to the use of this preparation, however, is that it may contain an isomerase that can convert androstenediol totestosterone [2,3]. Massé et al. [3] demonstrated that this conversion takes place in incubations with extracts from normal subjects, which can lead to false positive results when hydrolysis conditions are not carefully maintained. False positives may occur when T, resulting from the conversion of androstenediol, increases the numerator of the T/E ratio. Since none of the steroidal substrates are converted to epitestosterone [3], the epitestosterone level remains constant, causing the T/E ratio to increase. The use of β-glucuronidase from *E. coli* eliminates the conversion problem, but because the *E. coli* preparation does not have arylsulfatase activity, some anabolic and many endogenous steroid conjugates are not hydrolyzed [2].

Numerous studies have revealed that very few steroids are excreted without being metabolized [4–9]. In equine studies oftestosteronemetabolism, greater than 85% of the urinary metabolites are excreted in conjugated form [10–15]. Human metabolic studies [16,17] have also shown that the predominant urinary conjugate oftestosteroneand epitestosterone is the glucuronide. Houghton and Dumasia [10] investigated the hydrolysis step in sample preparation and found that further neutral metabolites were liberated from the *Helix pomatia* reaction mixture by subsequent hot acid hydrolysis, thus demonstrating that the enzymatic hydrolysis is not effective in liberating all of the steroid conjugates present in the urine [10]. It was also discovered by the same laboratory that hot acid hydrolysis was unreliable because it caused some decomposition of the steroids, which resulted in low recoveries [12]. Because the steroid conjugates dominate the urinary profile, it would be to great advantage to directly determine the intact steroid conjugates, thereby eliminating the time-consuming and potentially inaccurate hydrolysis procedures.

An alternative to capillary GC–MS techniques [18] is combined high-performance liquid chromatography–mass spectrometry (HPLC–MS). We have employed a liquid–solid extraction (LSE) technique [19] to isolate target analytes from the conjugated fraction of human urine in which most of the anabolic steroid metabolites are found [2,4–15]. Since ion-spray LC–MS techniques [20] provide only soft ionization and no fragmentation of these compounds, a means for providing more structural information for identification purposes is needed. Tandem mass spectrometry (MS–MS) provides the structural information required. Confirmation of the parent conjugate in human urine is achieved by selected reaction monitoring (SRM) of the characteristic precursor ion to product ion transitions.

In this work, sulfate and glucuronide conjugates of testosterone and epitestosterone are isolated from human urine by LSE and characterized directly by ion-spray LC–MS–MS techniques. Normal male urines are analyzed as negative controls. It is shown that the method is effective for the extraction and quantitation of anabolic steroid conjugates in human urine using the title method.

2. Experimental

2.1. Chemicals

The structural formulae and fragmentation diagram of the anabolic steroid conjugates determined are shown in Fig. 1. Testosterone β-D-glucuronide sodium salt (TG) was purchased from Steraloids, (Wilton, NH, USA). Epitestosterone glucuronide (EG) was purchased from Research Plus (Bayonne, NJ, USA). Testosterone sulfate (TS) and epitestosterone sulfate (ES) were synthesized from T and E (Steraloids) in this laboratory by Dr. Wu Nan Wu of the R.W. Johnson Pharmaceutical Research Institute. Deuterated internal standards of 16,16,17-d_3-testosterone glucuronide (D_3TG) and 16,16,17-d_3-epitestosterone sulfate (D_3ES) were generously provided by Dr. Larry Bowers of the Indiana University School of Medicine. Methanol, water and ammonium acetate (HPLC-grade) and reagent-grade glacial acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Liquid–solid extraction cartridges (J.T. Baker, Phillipsburg, NJ, USA), packed with 200 mg of C_{18}-bonded silica (Model 7020-2, 40 μm, 60 Å), were used with a Gilson automated solid-phase extraction system with extraction Cartridges (Aspec)

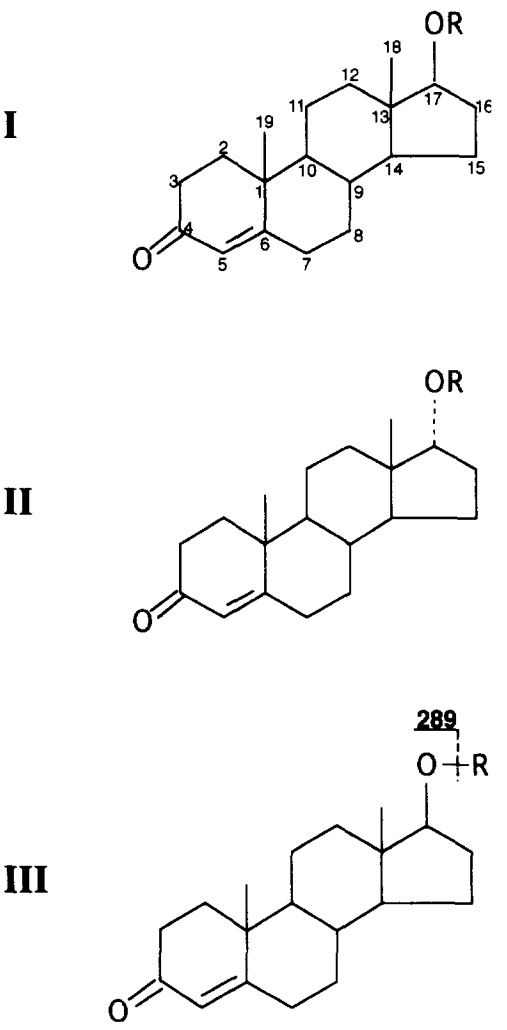

Fig. 1. Structures of the investigated compounds. I: R=OSO_3, 17β-testosterone sulfate (TS), M_r=368; R=$C_6H_8O_6$, 17β-testosterone glucuronide (TG), M_r=464. II: R=OSO_3, 17α-epitestosterone sulfate (ES), M_r=368; R=$C_6H_8O_6$, epitestosterone glucuronide (EG), M_r=465. III: Fragmentation resulting in the most abundant product ions under collision-induced dissociation conditions (see Section 2).

233XL instrument (Middleton, WI, USA). Control human urine samples were donated by co-workers.

2.2. Liquid–solid extraction

The development of LSE conditions for the isolation of anabolic steroid conjugates has been described in detail elsewhere [19]. Samples of human urine (2 ml) were diluted with 1 ml of 24 m*M* ammonium acetate, pH 5.5, mixed on a vortex-mixer, ultra-sonicated and centrifuged. The LSE cartridges were conditioned with 2 ml of methanol and 1 ml of 24 m*M* ammonium acetate, pH 5.5, prior to sample application. Following the addition of the diluted urine sample to the LSE cartridges, weakly retained compounds were eluted by the sequential addition of 1 ml of 24 m*M* ammonium acetate, 2 ml of methanol–24 m*M* ammonium acetate, pH 5.5 (45:55, v/v) and 3 ml of water. The steroid conjugates were then eluted with 2 ml methanol–water (40:60, v/v). The eluate was then concentrated to dryness under vacuum in a Savant Instruments (Farmingdale, NY, USA) SpeedVac SVC100. The dried extracts were reconstituted in 20 μl of water and mixed on a vortex-mixer. Aliquots (5 μl) were used for LC–SRM-MS analysis.

2.3. Microbore chromatography

The liquid chromatographic system consisted of a PerSeptive Biosystems INTEGRAL micro-analytical workstation (Framingham, MA, USA). The autosampler, injection valve and liquid chromatography pumps of the INTEGRAL were used for this work. The injection valve was fitted with a 5-μl sample loop. Methanol–7 m*M* ammonium acetate (70:30, v/v), pH 4.5, at 70 μl/min was used for the isocratic separation of the steroid conjugates on a Betasil-C_{18} (5 μm, 100×1 mm I.D.) column (Keystone Scientific, Bellefonte, PA, USA). The mobile phase was pre-filtered through a 0.45-μm Nylon-66 filter (Rainin, Woburn, MA, USA) and degassed by sparging with helium prior to use. Authentic analytical standard mixtures of the compounds were prepared from 1 mg/ml stock solutions and had solvent compositions that were close to, or equal to, the mobile phase composition. The eluent from the column was directly transferred to the ion-spray interface with no post-column split.

2.4. Mass spectrometry

A Sciex (Thornhill, Canada) API 300 triple quadrupole mass spectrometer was used in this work. The interface electrode was set at 4.5 kV for the production of positive ions. The ion-spray interface

received the total 70 μl/min from the chromatographic column eluent. Nitrogen was used as the nebulizing gas. Ions were sampled from the API source into the mass analyzer through a 0.254 mm I.D. orifice in a flat plate in front of a 0.75-mm I.D. orifice in the tip of a skimmer projected towards the sprayer region. The area in front of the orifice was flushed with dry nitrogen, which acts as a curtain gas to prevent solvent vapor and contaminants from entering the orifice [20]. Nitrogen was also used for collision-induced dissociation in the second quadrupole region. The collision energy in the laboratory frame was 17 eV. SRM was based on the observation of product ions from m/z 468 $[M+H]^+$ for D_3TG, m/z 372 $[M+H]^+$ for D_3ES, m/z 369 $[M+H]^+$ for TS and ES, and m/z 465 $[M+H]^+$ for TG and EG. The dwell time for each transition in the SRM mode was 200 ms. The compounds were separated on the LC column, but standard 2 μ*M* mixtures of each steroid conjugate in methanol–10 m*M* ammonium acetate (50:50, v/v) were also introduced to the API source by infusion at 4 μl/min using an infusion pump (Harvard Apparatus, South Natick, MA, USA) to obtain representative product ion spectra (Figs. 2–4).

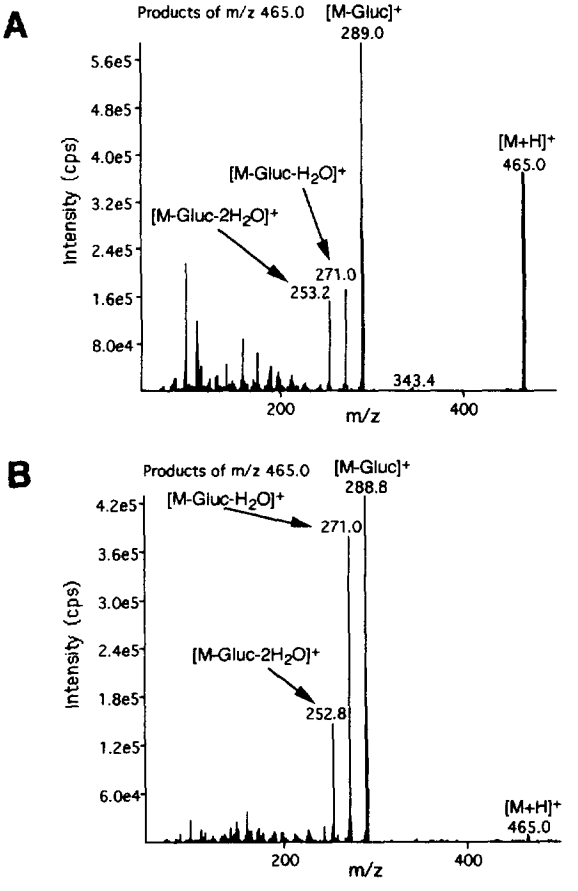

Fig. 2. Full-scan product ion spectra from 50–500 a.m.u. of (A) testosterone glucuronide (TG) resulting from infusion of a 2 μ*M* solution of TG at 4 μl/min and (B) epitestosterone glucuronide (EG) resulting from infusion of a 2 μ*M* solution of EG at 4 μl/min. For mass spectrometric conditions, refer to Section 2.

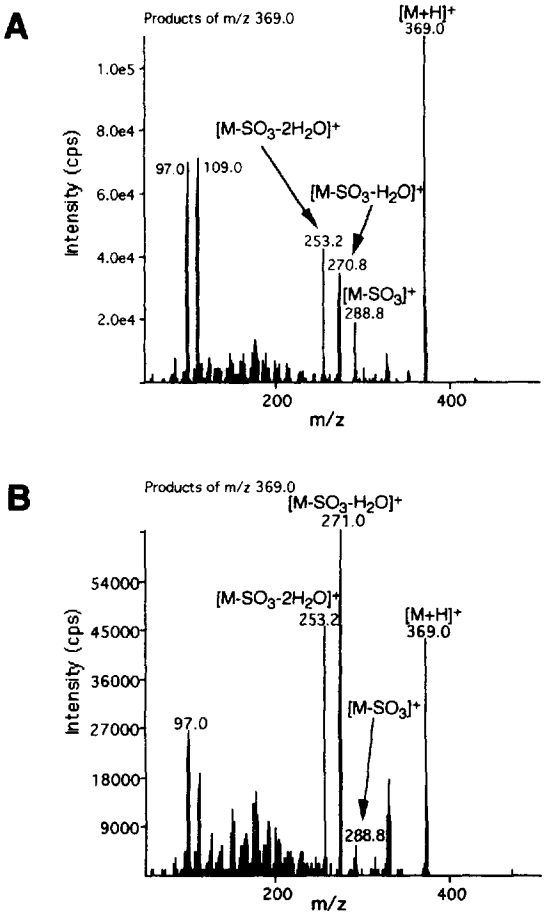

Fig. 3. Full-scan product ion spectra from 50–500 a.m.u. of (A) testosterone sulfate (TS) resulting from infusion of a 2 μ*M* solution of TS at 4 μl/min and (B) epitestosterone sulfate (ES) resulting from infusion of a 2 μ*M* solution of ES at 4 μl/min. For mass spectrometric conditions, refer to Section 2.

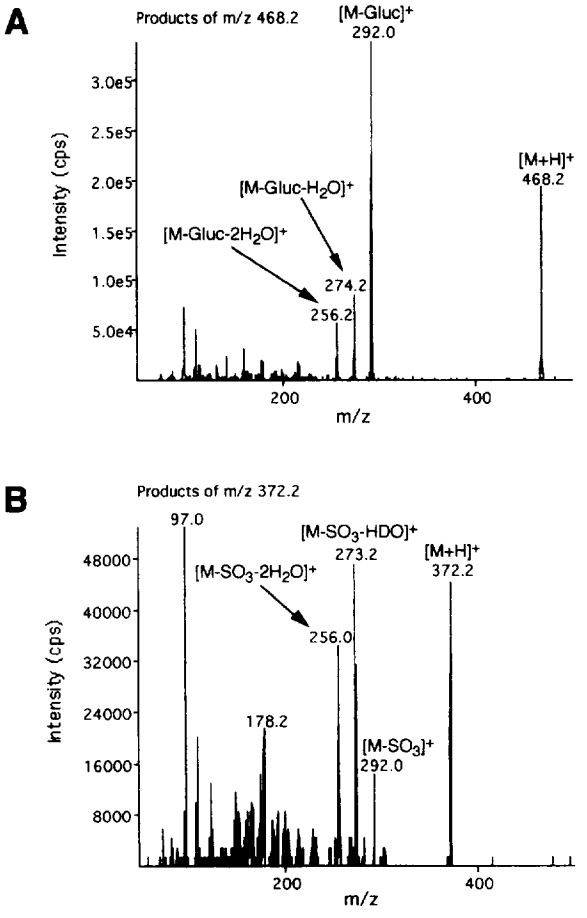

Fig. 4. Full-scan product ion spectra from 50–500 a.m.u. of (A) 16,16,17-d_3-testosterone glucuronide (D_3TG) resulting from infusion of a 2-μM solution of D_3TG at 4 μl/min and (B) 16,16,17-d_3-epitestosterone sulfate (D_3ES) resulting from infusion of a 2-μM solution of D_3ES at 4 μl/min. For mass spectrometric conditions, refer to Section 2.

3. Results and discussion

3.1. Sample preparation

A LSE procedure was used to isolate the sulfate and glucuronide conjugates of testosterone and epitestosterone from human urine [19]. During extraction, eluent composition is altered to produce different elution behavior. This allows the selective elution of the polar steroid conjugates while minimizing the recovery of many interfering compounds that are common to urine. Dilution of the urine with buffer, sonication and centrifugation help remove particulate matter and prevent cartridge clogging.

Extraction recovery of the sulfate and glucuronide conjugates was evaluated at the 100 nM and 1 μM levels for all four steroid conjugates and the two deuterated internal standards in human male urine. Urine samples (2 ml) were divided into three groups: One group was fortified by addition of authentic standards to produce aliquots that contain 100 nM of each steroid conjugate and deuterated internal standard, while the second group was fortified at the 1 μM level with each of the six components. None of the conjugates or the internal standards were added to the third group. Each of the urine samples was extracted as described above. The group of extracts to which no conjugates or internal standards had been added prior to extraction were then fortified with the six components so that the concentrations would be the same as expected if there had been 100% recovery by LSE. LC–SRM-MS ion current profile peak areas of the conjugates extracted from the urine fortified before liquid–solid extraction were divided by the peak areas of the conjugates that were added to the extracts following LSE. This ratio provided the percentage recovery of each conjugate and each internal standard. The internal standards D_3TG and D_3ES were recovered at 82 and 70%, respectively. TG, TS, ES and EG were recovered at 70, 75, 90 and 90%, respectively. Whereas the LSE step is the only extraction step, no further losses of analyte occur.

3.2. Microbore HPLC separation

The four steroid conjugates of interest (TG, EG, TS, ES) were separated by isocratic microbore HPLC with selected reaction monitoring mass spectrometric detection (Fig. 5). The internal standards (D_3TG, D_3ES) co-eluted with their respective D_0 analogs. An important factor in the HPLC separation was to resolve TS from ES (as, being epimers, they have similar retention times). Whereas they have the same molecular mass, their different retention times would be crucial for confirming their presence in urine. Isocratic separation was chosen to speed up the analysis time. Even with an autosampler, the large number of urine extracts prepared would take

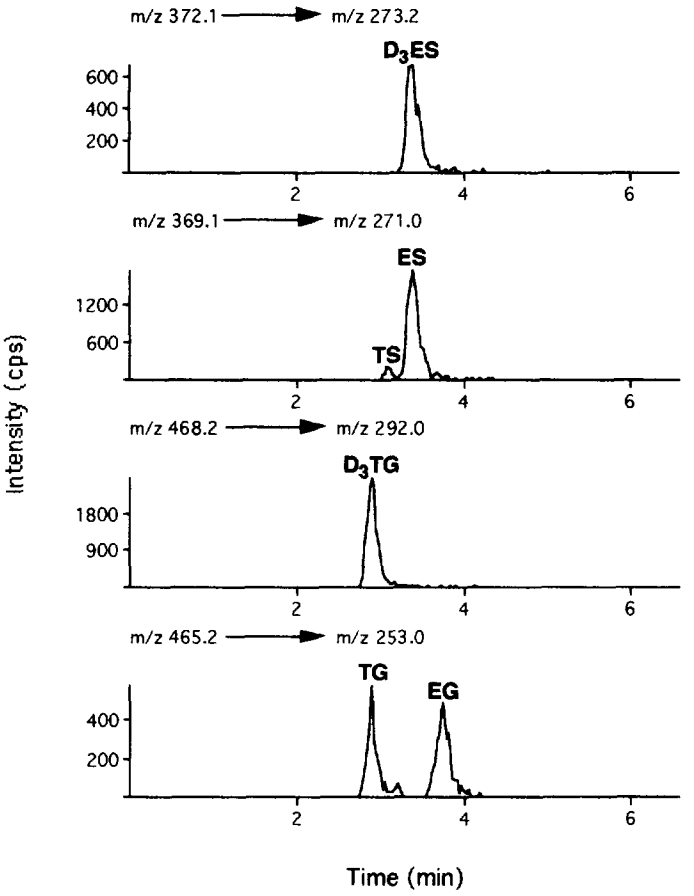

Fig. 5. Ion current profiles from the LC–SRM-MS analysis of a synthetic mixture of authentic reference standards containing TG, EG, TS, ES and the internal standards D_3TG and D_3ES. TG, EG and D_3TG (22 pmol) and 27 pmol of TS, ES and D_3ES were injected on-column. For liquid chromatographic and mass spectrometric conditions, refer to Section 2.

several days to analyze under gradient conditions. Therefore, a chromatographic separation that reproducibly retained the analytes, resolved the epimers and was compatible with the ion-spray interface was essential. An octadecyl–silica packing material was chosen for its strong retention characteristics so that a mobile phase with higher organic content could be used. The use of higher methanol concentrations in the mobile phase also enhances the sensitivity of the ion-spray LC–MS interface [21]. Under isocratic conditions, the separation was best achieved with a mobile phase composition of methanol–water (70:30, v/v) with 7 m*M* ammonium acetate added and with the pH adjusted to 4.5 with glacial acetic acid. Without ammonium acetate in the mobile phase, chromatographic peaks had variable retention times and sometimes were not well retained. The combination of methanol content and ammonium acetate in the mobile phase is a compromise between chromatographic performance and ion-spray mass spectrometric sensitivity and stability [21]. Adjustment of pH was also necessary to prevent ES and EG from co-eluting.

3.3. MS–MS of authentic reference material

CID product-ion mass spectra of the five reference compounds were obtained under the conditions described in Section 2 (Figs. 2–4). From earlier experiments in this laboratory, it was found that

testosterone and epitestosterone have identical product ions in their mass spectra. Comparisons of other steroids with common A-ring functionalities provided similar results. Similar mass spectra are obtained for conjugates as well as for the parent steroids studied, with the exception of the protonated molecule ion. The exception to this trend is epitestosterone glucuronide. It exhibits differences in abundances of fragment ions from testosterone glucuronide, presumably due to the orientation of the bulky glucuronide group, alpha, to the steran nucleus.

The most abundant ions present in the CID product ion spectra of TG, EG, TS and ES (Figs. 2 and 3) represent losses of their respective conjugate moiety followed by the subsequent loss of molecules of water. These fragment ions are observed at *m/z* 289, 271 and 253. For the sulfate conjugates, the ions representing losses of water were the most abundant, while the ions resulting from the loss of the conjugated moiety were most abundant for the glucuronides. The product-ion mass spectra of these conjugates are shown in Figs. 2 and 3. The deuterated internal standards (D_3TG, D_3ES) exhibited product ion spectra with ion abundances that were similar to the ions in the corresponding D_0 spectra. Their product-ion mass spectra are shown in Fig. 4.

For the analysis of urine extracts, the precursor ion-product ion transitions of *m/z* 465 to 289, *m/z* 465 to 271, and *m/z* 465 to 253 were monitored by SRM for the quantitative and qualitative determination of TG and EG. The precursor ion-product ion transitions of *m/z* 369 to 289, *m/z* 369 to 271, and *m/z* 369 to 253 were monitored for TS and ES. The precursor ion-product ion transitions of *m/z* 468 to 292 and of *m/z* 372 to 273 were monitored for the internal standards D_3TG and D_3ES, respectively. Fig. 5 shows the results of the isocratic micro-bore HPLC separation with SRM mass spectrometric detection.

3.4. Determination of steroid conjugates isolated from human urine

Detection limits for the target analytes and the internal standards found by monitoring only the precursor ion-product ion transitions described above using ion-spray LC–MS–MS in the positive ion mode are in the low nanomolar range in urine. While better limits of detection have been reported in the negative ion mode by this laboratory [22], the positive ion mode was chosen due to significantly improved sensitivity for the glucuronide conjugate. The sulfates provided a good response even in the positive ion mode, although they were not as good as in the negative ion mode reported previously [22]. It was not practical to implement a polarity change from positive ion detection to negative ion detection, due to the small differences in the retention times of the respective conjugates. The sensitivity obtained for the glucuronide and sulfate conjugates studied, combined with the selectivity of MS–MS make the described method suitable for the determination of these conjugates in human urine with the added advantage of simplified sample preparation.

Standard curves of all four anabolic steroid conjugates were prepared from fortified standards in human male urine. Duplicate values of each standard were obtained to prepare a single standard curve. The levels of TG and EG added to the urine were 50, 100, 200, 500 and 1000 n*M*. TS and ES were added at the 10, 25, 50, 100 and 200 n*M* levels.

The differences between the levels of sulfates and glucuronides added to the urine reflect the differences in endogenous levels of these conjugates. The D_3TG and D_3ES internal standards were added at 125 and 50 n*M*, respectively, for all of the aliquots of urine extracted. One set of standard curve extracts was analyzed at the beginning of the autosampler tray, before the analysis of the six normal male extracts for quantitation, which were then followed by the second set of standard curve extracts. The peak area from the extracted ion current profiles of each steroid conjugate was divided by the peak area of the corresponding deuterated internal standard resulting in a TG/D_3TG, EG/D_3TG, TS/D_3ES, or ES/D_3ES ratio. The average ratio for the two points was then plotted versus concentration to produce a standard curve to be used for the quantitation of the conjugates. Linear curve fitting yielded an equation which described the line and from which concentrations of the conjugates could be calculated when an unknown level of conjugate/internal standard ratio is determined.

Three quality control (QC) samples were used in duplicate to ensure that the method produced satis-

factory results. These extracts were randomly placed throughout the autosampler tray. The analytical standards used to prepare the QC samples were prepared by a co-worker to reflect any differences in measurement that may exist between two individuals. Two sets of aliquots from a normal male urine specimen were fortified with authentic analytical standards at a low, medium and high level within the standard curve range, to produce two sets of QC samples. Each of the four steroid conjugates were fortified at concentrations of 200, 500 and 750 n*M* TG and EG, and 50, 100 and 150 n*M* TS and ES. The intra-assay precision, calculated from the QC samples, was less than 15% for EG and ES and less than 20% for TG and TS.

Linear regression of standard curves for the four steroid conjugates resulted in the following equations: $y=0.001x+1.205$, $R^2=0.956$ for TG; $y=0.007x+0.490$, $R^2=0.988$ for EG; $y=0.84x-0.467$, $R^2=0.979$ for TS; $y=0.074x+4.681$, $R^2=0.984$ fro ES. The correlation coefficients for the standard curves varied between the sulfate and glucuronide conjugates with the latter typically producing lower R^2 values. It is possible that the variations observed are due to different conjugate levels in the control urines that were used to prepare the standards in the standard curves.

The ion current profiles resulting from the SRM LC–MS analysis of a normal human male urine sample are shown in Fig. 6. The ion current profiles shown represent the endogenous levels of the steroid conjugates in the sample. The same urine sample fortified at 500 n*M* TG and EG and 100 n*M* TS and ES is shown in Fig. 7. The chromatographic peaks

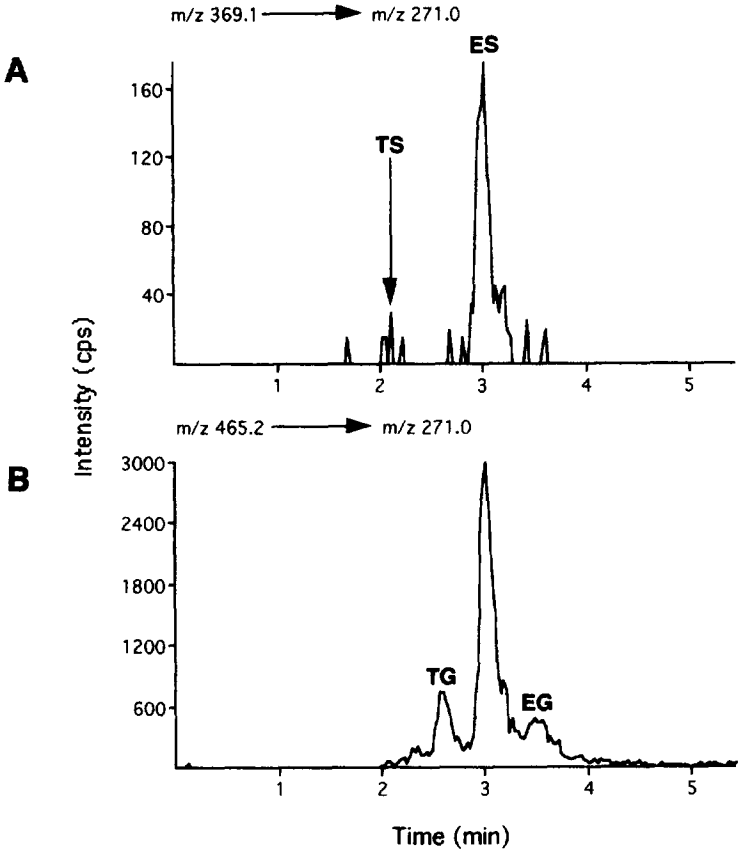

Fig. 6. LC–SRM-MS determination of endogenous anabolic steroid conjugates in normal human male urine. (A) Endogenous ES. TS is below the level of detection. (B) Endogenous TG and EG. The peak at 3 min is an unknown endogenous component. For liquid chromatographic and mass spectrometric conditions, refer to Section 2.

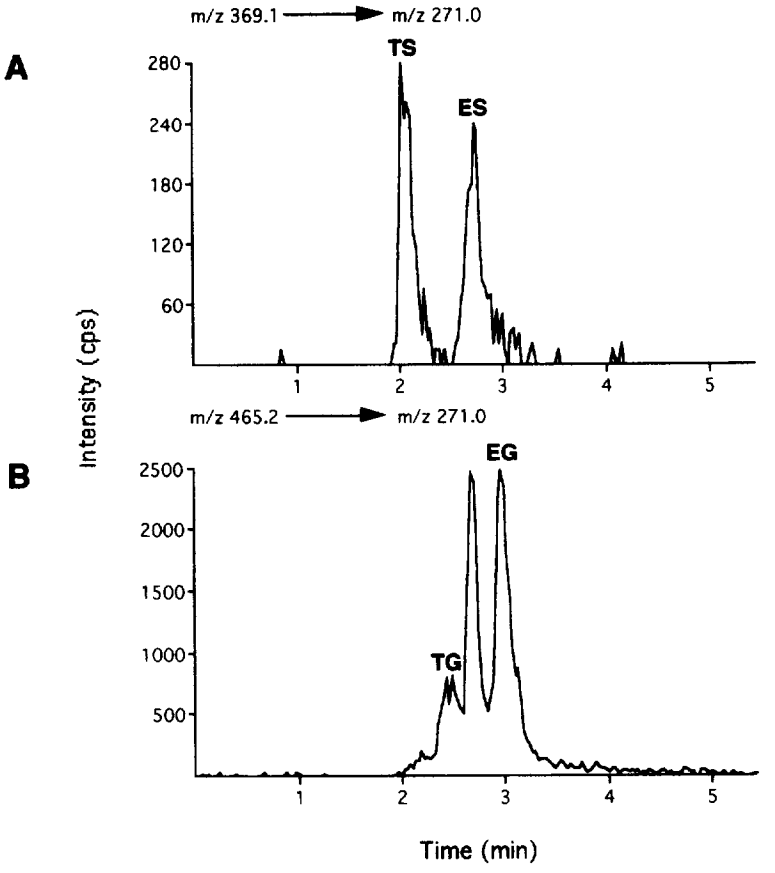

Fig. 7. LC–SRM-MS determination of human male urine fortified with (A) 100 n*M* TS and ES and (B) 500 n*M* TG and EG. The peak at 3 min is an unknown endogenous component. For liquid chromatographic and mass spectrometric conditions, refer to Section 2.

associated with the conjugates in the blank are increased in the sample that is fortified with authentic reference standards. The peak-area ratios of the sulfate and glucuronide conjugates of testosterone and epitestosterone for this male were calculated and are shown in Table 1. It can be seen that the T/E ratio, based on the peak areas of the glucuronide conjugates, corresponds to the current belief that a normal male exhibits a T/E ratio of less than 6:1 [23]. In human metabolism studies of epitestosterone by Wilson and Lipsett [16], it was found that there is very little metabolism of epitestosterone and that it is primarily excreted unchanged as its glucuronide conjugate. This is in contrast to testosterone, which is metabolized to etiocholanolone and androsterone [2,16]. By observing the ordinates in Fig. 6, it is shown that the intensity of the ion current profile of

Table 1
Anabolic steroid conjugate ratios from a normal human male sample

Steroid	Ratio
$\frac{TG}{EG}$	1.01
$\frac{TG + TS}{EG + ES}$	0.24
$\frac{TS}{TG}$	[a]
$\frac{ES}{EG}$	3.2
$\frac{TS/TG}{ES/EG}$	[a]

[a] TS is below the level of detection.

TS and ES, as monitored by the selected transition of m/z 369 to 271 demonstrates that the sulfate conjugates are present at lower levels than the glucuronide conjugates. For the male sample in Fig. 6, TS is below the level of detection for this method. By observing the ratios of the total T (TG+TS) compared to total E (EG+ES) (Table 1), the inclusion of the sulfate conjugates in the T/E ratio only serves to drive the ratio lower and even further from the legal limit of 6:1 in this case.

In a recent study, Dehennin and Matsumoto [17] reported a poor correlation between the glucuronide conjugates of testosterone and epitestosterone in normal men undergoing long-term administration of testosterone enanthate. The authors suggest that the excretion of epitestosterone sulfate should also be considered in the T/E ratio. However, the ratio of total T to total E in Table 1 demonstrates that such a comparison still does not lend enough information to determine whether or not the individual has abused anabolic steroids. As demonstrated with the normal male in Fig. 7, ES alone can skew the T/E ratio in favor of the athlete. In their study, Wilson and Lipsett [16] stated that a method which attempts to determine either the production or excretion rate of testosterone and that also measures epitestosterone will be subject to considerable error. It is important to note that both of these studies relied on enzymatic hydrolysis of the steroid conjugates in order to quantitate the ratios and rates reported. While Wilson and Lipsett [16] stated that they believed their inability to recover a large fraction of radioactivity was not due to inefficient hydrolysis, but to preparation of the fractions, the LC–MS–MS method described here has fewer sample preparation steps where analyte can be lost and involves the direct determination of the target conjugates.

Because the glucuronide conjugates of testosterone and epitestosterone are more abundant in the urine than are the sulfate conjugates, it would seem reasonable to compare the ratios of both of the conjugates of testosterone to the ratios of both of the conjugates of epitestosterone. It would then be possible to monitor changes in each steroid, individually, and to determine the ratio of T/E based on the ratios of the conjugates of each steroid. Table 1 lists the ratios of the glucuronide and sulfate conjugates of testosterone (TS/TG) and epitestosterone (ES–EG) as well as the ratio of these ratios [(TS/TG)/(ES/EG)]. In the case of this normal male, the level of TS is below the method's level of detection, so the overall ratio is zero. It is clear that the behavior of all conjugated metabolites of testosterone and epitestosterone can give further information regarding an athlete's urinary steroid profile. However, further studies are required to determine the relevance and importance of the measured quantities of each conjugate regarding an individual's use or abuse of anabolic steroids.

4. Conclusions

The described LC–MS–MS method is suitable for the direct determination of the sulfate and glucuronide conjugates of testosterone and epitestosterone in human urine. The LSE procedure employed is simple and achieved recoveries of the steroid conjugates from urine that were satisfactory at all levels of fortification. Isocratic LC separation provides resolved steroid epimers, with confirmation of the analytes by MS–MS.

The advantages of LC–MS over current GC–MS procedures include the elimination of hydrolysis and derivatization steps prior to the chromatographic separation. Whereas the conjugates are determined directly, the ratios of the conjugates can be more accurately calculated, thus reducing the risk of false positives or misleading results.

Because some individual variations in endogenous conjugate levels exist, it is essential that extensive statistical analysis of normal human males, based on the title method, be conducted to determine a normal response. By considering the effects of exogenous testosterone on urinary conjugate levels and by understanding the relationship between those conjugates, it could then be possible to accurately determine whether or not an athlete has abused anabolic steroids.

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