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Direct measurement of steroid sulfate and glucuronide conjugates with high-performance liquid chromatography–mass spectrometry

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Abstract

Direct detection of several steroid glucuronide and sulfate conjugates was achieved with electrospray reversed-phase HPLC–mass spectrometry. Separation of steroid 17-OH or 5-H epimers conjugated with glucuronide or sulfate could be achieved using gradient elution. Testosterone glucuronide, testosterone sulfate, epitestosterone sulfate and epitestosterone glucuronide were chromatographically resolved, although significant variation in solvent strength was observed between methanol and acetonitrile. Positive ionization mode MS and MS–MS spectra were employed to obtain both quantitative and structural information. Some differences were noted with respect to steroid structure and adduct formation, including significant differences in the stability of epimers in the declustering region of the interface. Negative ionization mode, although having lower limits of detection, did not provide useful structural information in either the MS or MS–MS mode. Using a packed capillary column (300 μm I.D.), a detection limit of 25 pg was achieved for epitestosterone glucuronide.

Keywords: Steroid sulfates; Steroid glucuronides; Testosterone; Epitestosterone; Androsterone; Etiocholanolone

1. Introduction

The measurement of steroids in plasma and urine has been used for a number of years for the diagnosis of disease [1,2] and for identifying the abuse of anabolic agents in sports [3]. The use of urine has been advocated for obtaining a profile of steroid concentrations, in part due to the higher concentration of steroid conjugates found in urine relative to plasma. Most steroids are excreted in urine with their hydroxyl function being substituted by either sulfate or β-glucuronide. While the complexity of the urine matrix and the low concentration of steroid have required analysis with GC–MS, the polar conjugates have mandated hydrolysis of the conjugates with either an enzyme (e.g., β-glucuronidase) or solvolysis prior to analysis. This approach has a number of limitations. The enzymatic hydrolysis of some steroids may be incomplete in a particular urine matrix due to competitive or non-competitive inhibition of the enzyme. Although the enzymes used have broad specificity, there are some conjugates that are not readily cleaved or that are labile. Cleavage of both glucuronide and sulfate conjugates requires either two separate steps, or the use of an enzyme preparation containing both a β-glucuronidase and an aryl sulfatase. The latter approach again raises the issue of incomplete hydrolysis, since only simple experiments have been carried out on the enzyme kinetics of steroid conjugates. The inability of the aryl sulfatase to cleave sulfate from the 17-position has been documented [4,5]. The variation in hydrolysis with different β-glucuronidase preparations has been well documented for other drugs [6,7]. In

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addition, there has been some suggestion that contaminants in the enzyme preparations used for combined hydrolysis may convert one steroid into another under some conditions [8].

We report here on our development of an HPLC–MS method for the direct measurement of steroid conjugates in urine. Our goal was to evaluate the methodology for both quantitation and identification of a limited number of steroid conjugates, such as testosterone and epitestosterone, as well as the screening of a wider range of steroids (i.e., profiling). Our specific interest in testosterone and epitestosterone arises from the fact that these substances are used to detect testosterone abuse in athletes [3]. At present, GC–MS methods are used for quantitation and confirmation after deconjugation with β-glucuronidase and derivatization [9]. Testosterone is reported to be present in urine primarily as the glucuronide conjugate, whereas both the sulfate and glucuronide conjugates of epitestosterone are present [10]. In addition to these metabolic considerations, a recent report documents a matrix effect on the determination of testosterone and epitestosterone by GC–MS [11]. Thus, sensitive, direct measurement of the steroid conjugates should be useful in resolving basic issues about steroid excretion.

2. Experimental

2.1. Materials

Epitestosterone glucuronide and sulfate were synthesized by a previously reported method [12]. Androsterone glucuronide (AG), etiocholanolone glucuronide (EG), testosterone glucuronide and sulfate, epiandrosterone glucuronide and sulfate and dehydroepiandrosterone sulfate were purchased from Steraloids (Wilton, NH, USA) or Sigma (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile (Burdick and Jackson, MI, USA) were used throughout.

2.2. Instrumentation

A Beckman (Beckman Instruments, San Ramon, CA, USA) Model 126 programmable solvent module was used for solvent delivery and a Model 166 UV detector was used on some occasions after post-column splitting of the eluent. Samples were injected with a Rheodyne (Cotati, CA, USA) 8125 injection valve with a 20-μl loop housed in a DuPont (Wilmington, DE, USA) 850 forced air oven. A PE-Sciex (Norwalk, CT, USA) API-III^{Plus} triple quadrupole mass spectrometer equipped with an IonSpray interface was used for detection. The electrospray ionization (ESI) voltage was 4 kV. The potential of the orifice was 75 V, unless otherwise stated. The column was connected to the Ionspray probe by a piece of fused-silica capillary (50 μm I.D., 150 or 180 μm O.D.; Polymicro Technologies, Phoenix, AZ, USA). HPLC separations were performed either on a 150×1 mm I.D. Hypersil C-18 BDS column (Keystone Scientific, Bellefonte, PA, USA) or on a 250×300 μm I.D. packed fused-silica capillary packed with 3 μm Hypersil C-18 BDS stationary phase (LC Packings, San Francisco, CA, USA). In the case of the 1 mm I.D. column, a flow-rate of 50 μl/min was used and all of the eluent was directed to the mass spectrometer. For work with the packed capillary column, the eluent (50 μl/min) was split prior to the injection valve and a piece of fused-silica capillary of sufficient length to assure a flow-rate through the column of about 5 μl/min was used. All of the eluent was directed to the mass spectrometer.

Gradient elution was used throughout. An aqueous solution of 0.11% glacial acetic acid and 7.5 mmol/l ammonium acetate was used as solvent A, unless otherwise stated. Solvent B consisted of either methanol or acetonitrile which was 0.11% (v/v) in glacial acetic acid and 7.5 mmol/l in ammonium acetate.

MS–MS spectra were obtained using argon as the collision gas in a high-pressure collision cell. The collision energy was 30 eV (laboratory frame of reference) and the collision gas thickness was $1.2 \cdot 10^{11}$ molecules/cm^2 unless otherwise stated.

3. Results

3.1. Chromatographic optimization

Interfacing an HPLC with a mass spectrometer requires the use of volatile mobile phase additives. Acetic acid and ammonium acetate (NH_4OAc) were

selected for this purpose. In the case of glucuronide and sulfate conjugates, we anticipated an influence of pH and ionic strength on the retention behavior of the various steroid structures. As expected, variation of pH in the region of 3.5 to 5.5 had virtually no effect on the retention of the sulfates. Increasing the concentration of NH_4OAc (0–25 m*M*), however, increased the retention times (t_R) for the sulfate conjugates. The retention of the glucuronide conjugates increases with decreasing pH as well as with an increase in the concentration of NH_4OAc, as would be expected for an acid with a pK_a of 2.8. At pH values above 5.0, for example, the glucuronide and sulfate conjugates are fully ionized and may co-elute for any steroid. The retention behavior of the glucuronide and sulfate conjugates of testosterone and epitestosterone as a function of the concentration of NH_4OAc at pH 4.0 is shown in Fig. 1. Based on these studies, mobile phase conditions of pH 4.3 and 7.5 mmol/l NH_4OAc were selected.

Separation of $3\alpha/3\beta$- and $17\alpha/17\beta$-hydroxysteroid conjugates was relatively easy, since the conjugate was in close proximity to the epimeric site. The presence of the polar substituent on one face of the non-polar steroid nucleus would be expected to change its hydrophobic interaction with the stationary phase. We were also able to achieve the separation of $5\alpha/5\beta$-isomers when the conjugate was on the 3α-hydroxyl group (Fig. 2). We were unable to test the situation for the $5\alpha/5\beta$-isomer when the conjugate was on the 17β-hydroxyl group, due to the unavailability of standard materials. It was also found that conjugated steroids with no double bonds in the ring structure were eluted later than their unsaturated analogs.

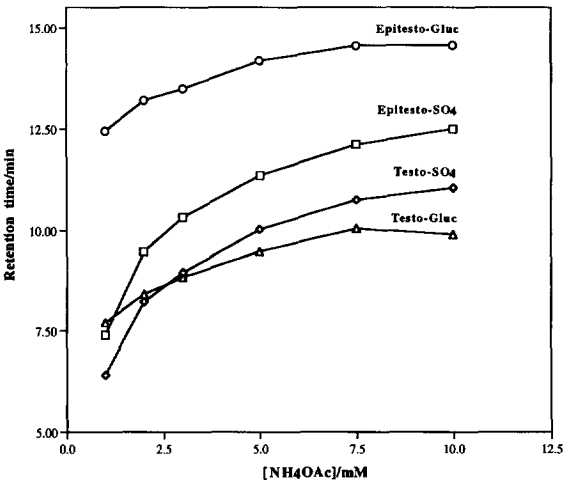

Fig. 1. Retention time of testosterone sulfate (◊), testosterone glucuronide (△), epitestosterone sulfate (□) and epitestosterone glucuronide (○) as a function of ammonium acetate concentration at pH 4.0.

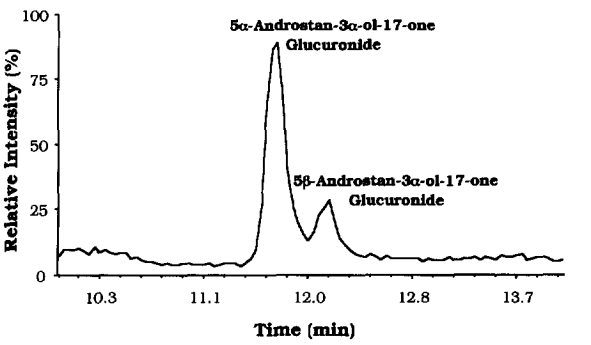

Fig. 2. Separation of androsterone- and etiocholanolone glucuronide. Calculated elution composition: 77% methanol for AG and 79% methanol for EG. (Gradient conditions: 50 to 95% B in 15 min).

3.2. Mass spectra of steroid conjugates

Several isomeric steroids (Table 1) were selected to study whether their structures could be distinguished on the basis of their ESI mass spectra. Androsterone, etiocholanolone, epiandrosterone and estrone glucuronides, each conjugated at the 3 position, all showed predominantly $[M+NH_4]^+$ adducts (Fig. 3). Testosterone, epitestosterone and dehydroepiandrosterone glucuronides, each conjugated at the 17-position, yielded strong $[M+H]^+$ ions. All the steroid conjugates studied, glucuronides and sulfates, showed a strong tendency to form ammonium adducts at low orifice voltages (~40–50 V). While the glucuronides tend to form a singly ammoniated ion $[M+NH_4]^+$, the sulfates form di-ammonium adducts. The 17-sulfates form the singly and doubly ammoniated ions in about equal abundance. In the case of 3-sulfate conjugates, an adduct with two ammonia molecules ($[M+NH_3{\cdot}NH_4]^+$) was the predominant ion at low orifice voltage, exceeding the amount of NH_4^+ adduct and loss of sulfate ($[M-SO_3+H]^+$). With increasing orifice voltage the 17-

Table 1
Model steroid conjugate structures

Conjugates studied	Steroid moiety	Conjugate type	Abundant adduct[a]
Testosterone glucuronide	Androst-4-en-3-one-17β-ol	17β-Glucuronide	H^+
Epitestosterone glucuronide	Androst-4-en-3-one-17α-ol	17α-Glucuronide	H^+
Epiandrosterone glucuronide	5α-Androstan-17-one-3β-ol	3β-Glucuronide	NH_4^+
Androsterone glucuronide	5α-Androstan-17-one-3α-ol	3α-Glucuronide	NH_4^+
Dehydroepiandrosterone glucuronide	Androst-5-en-17-one-3β-ol	3β-Glucuronide	NH_4^+
Etiocholanolone glucuronide	5β-Androstan-17-one-3a-ol	3α-Glucuronide	NH_4^+
Estrone glucuronide	Estra-1,3,5(10)-trien-17-one-3-ol	3-Glucuronide	NH_4^+
Dehydroepiandrosterone sulfate	Androst-5-en-17-one-3β-ol	3β-Sulfate	$NH_3 \cdot NH_4^+$
Etiocholanolone sulfate	5β-Androstan-17-one-3α-ol	3α-Sulfate	$NH_3 \cdot NH_4^+$
Testosterone sulfate	Androst-4-en-3-one-17β-ol	17β-Sulfate	H^+
Epitestosterone sulfate	Androst-4-en-3-one-17α-ol	17α-Sulfate	H^+

[a] Adducts formed at 75 V orifice voltage.

glucuronide and -sulfate conjugates can be transformed to the protonated adduct $[M+H]^+$ ion, whereas for the 3-sulfate and -glucuronide conjugates the ammonium adduct $[M+NH_4]^+$ is still the predominant species at an orifice voltage as high as 90 V. In general, the 3-hydroxysteroid conjugates gave rise to more abundant ions due to loss of H_2O from both the conjugate and the aglycone or asulfate.

Since both glucuronide and sulfate are anions at the pH used for separation, we explored the possibility that negative ionization might have lower limits of detection. Not surprisingly, both types of conjugates gave good signals from proton abstraction in the negative ion mode. As shown in Fig. 4, however, it was impossible to obtain additional structural information from the sulfate conjugates, since the only product ion was from HSO_4^- (m/z 97). This would not provide criteria for identification of the steroid, as required by IOC regulations for documentation of a positive result. For comparative purposes, the MS–MS spectrum of testosterone glucuronide ($[M+H]^+$ precursor) is shown in Fig. 5. In addition to prominent ions from the protonated

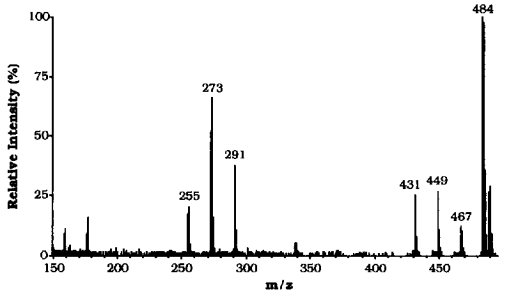

Fig. 3. Electrospray mass spectrum of androsterone glucuronide. The m/z 467, 484 and 489 ions are the H^+, NH_4^+ and Na^+ adducts, respectively. Other significant ions are the result of the loss of H_2O from the conjugate (m/z 449, 431) or the aglycone (m/z 273, 255).

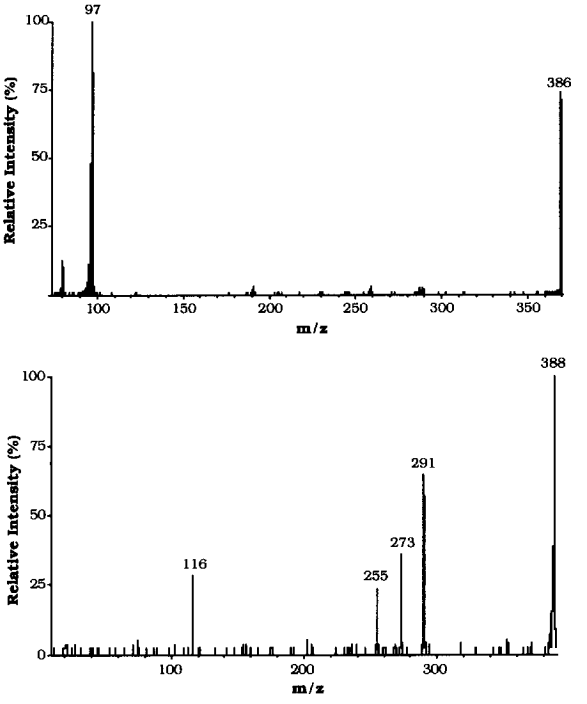

Fig. 4. Tandem mass spectrum of the $[M-H]^-$ ion (top) and the $[M+H]^+$ ion (bottom) of androsterone sulfate. Note that the only significant product ion present in the negative ion spectrum is due to HSO_4^-.

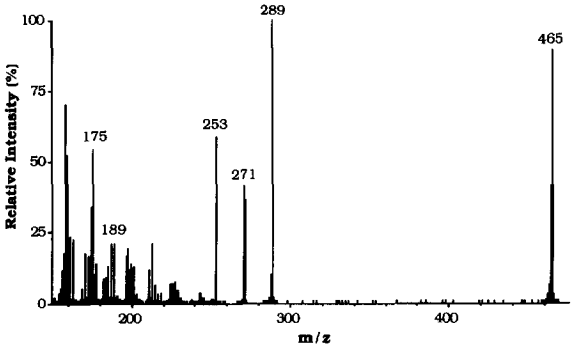

Fig. 5. Tandem mass spectrum of the $[M+H]^+$ ion (m/z 465) of testosterone glucuronide. Significant ions are present from the protonated aglycone (m/z 289), loss of H_2O (m/z 271, 253) and from fragmentation of the steroid nucleus.

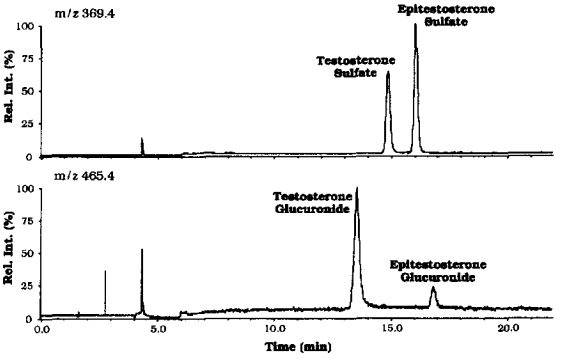

Fig. 6. Packed capillary column separation of 65 pg each of testosterone- and epitestosterone glucuronide (m/z 465) and sulfate (m/z 369). Calculated elution composition: 31% methanol for TG; 34% methanol for TS; 38% methanol for ES; 40% methanol for EG. (Gradient conditions: 8% B, 1 min hold to 60% B in 15 min).

aglycone (m/z 289) and sequential losses of H_2O, a series of low mass ions from the ring fragmentation can be seen. The m/z 175 and 187 ions arise from fragments containing the D-ring, since these ions were shifted to higher mass when [16,16,17]-$^2H_3$-testosterone glucuronide was analyzed. The same type of MS–MS fragmentation pattern was observed for all of the glucuronide and sulfate conjugates tested. Differences in the relative abundance of ions resulting from the loss of H_2O appear to be correlated with the ring substitution. Thus, it should be feasible to select a number of ions in the MS–MS mode that can be correlated to the steroid structure.

Although the observed limit of detection for the testosterone and epitestosterone conjugates in the positive ion mode was about 1 ng on the 150×1 mm I.D. column, a packed capillary column was evaluated to determine if lower limits could be achieved. Using selected-ion monitoring, standards could be detected at a concentration of 65 pg on-column (Fig. 6). Based on this performance, we estimate the detection limits for epitestosterone glucuronide at 25 pg and those for testosterone glucuronide and the two sulfate conjugates at about 3 pg, using five times the noise level as the criteria for limit of detection. The relatively poor response of the epitestosterone glucuronide is due to fragmentation at the orifice to form the aglycone [12], which was not monitored. Attempts to analyze a urine sample extract on the packed capillary column resulted in rapid deterioration of the column performance and increase in column back-pressure. This suggests that the solid-phase extraction procedure used for urine clean-up is not adequate for packed capillary columns.

A problematic situation was noted when high concentrations of acetonitrile were used in the mobile phase, whether alone or in the presence of methanol. High background signals and peaks of various masses were observed during the gradient, even in the absence of an injection (Fig. 7). Inspection of the terminal end of the fused-silica capillary under a microscope revealed the removal of the polyimide coating, notable for the sharpness of the concentric line dividing the uncoated area from the remaining coating. Repeated experiments indicate that this is not due to mechanical abrasion. It also appeared that the acetonitrile had penetrated between

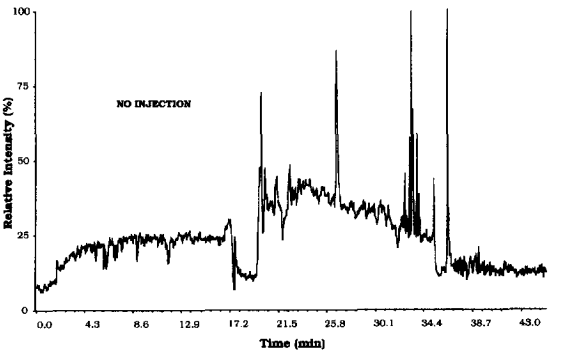

Fig. 7. Background signal from fused-silica polyimide coating observed after a blank injection into an acetonitrile gradient.

the polyimide coating and the fused-silica tube, loosening an additional section of the protective coating. It was not observed with methanol alone. It is not clear whether the electrospray voltage is necessary to produce this effect. We therefore recommend caution when using high fractions of acetonitrile with fused-silica tubing coated with polyimide.

4. Discussion

The direct detection and confirmation of steroid conjugates in urine is a difficult problem. A wide variety of steroids are present in urine in a range of concentrations that vary by more than 100-fold, a situation made more complex by the potential presence of glucuronide and sulfate conjugates of each steroid nucleus. The need for chromatographic resolution may be moderated somewhat by the use of a detector, the mass spectrometer, which potentially provides some structural information about co-eluting compounds. The use of near-atmospheric pressure interface techniques such as electrospray, however, results predominantly in adduct formation between the molecule and H^+, NH_4^+ or Na^+. As shown above, the mass spectra of steroid conjugates are relatively simple and do not provide conclusive identification information. Thus, it is important both to separate the various steroid conjugates and to obtain MS or MS–MS spectra with sufficient ability to discriminate between steroid structures.

The effect of ionic strength on the separation of ionic compounds is well known [13]. It was interesting to note the greater effect of NH_4OAc concentration on sulfate relative to glucuronide anion (see Fig. 1). As is apparent from the figure, careful control of the salt concentration is necessary to avoid co-elution of various steroid species. Although in purely chromatographic situations, selection of the highest concentration of salt might provide the most robust assay, the concentration of salt can decrease the ionization efficiency in the electrospray interface. Thus, optimization of the analytical system is important.

There is a significant difference between methanol and acetonitrile in the resolution of steroid conjugates. Acetonitrile was clearly a stronger solvent than methanol. With acetonitrile, it was difficult to separate a number of compounds, such as testosterone sulfate and epitestosterone glucuronide. Although this did not present a problem with mass spectrometric detection, it does suggest that co-elution could be a serious problem in a complex matrix such as urine. The resolution of the $5\alpha/5\beta$-androstane epimers was also better with methanol. It is interesting to note that similar solvent effects were observed for unconjugated steroids [14], although the corticosteroids reported previously had signficantly different structures than those studied here.

It was interesting to note that steroids conjugated at the 3-hydroxyl group, regardless of the other structural features of the A-ring (e.g., androsterone vs. etiocholanolone vs. estrone), formed stable ammonium adducts. It was not easy to break the adducts with the 3-hydroxysteroid conjugates using higher orifice voltages. The stereochemistry of the 3-hydroxyl group did not appear to have an effect, as exemplified by androsterone and epiandrosterone glucuronide. In general, 3-hydroxysteroids gave rise to more intense ions by the loss of two molecules of H_2O. In contrast, 17-hydroxysteroids formed predominantly protonated molecular ions. It was relatively easy to break any ammonium adducts of 17-hydroxysteroids by adjusting the orifice voltage. Within each grouping, there were some variations in the extent of adduct formation. For example, epitestosterone glucuronide formed ammonium adducts to a much greater extent than testosterone glucuronide. The potential of the orifice was an important factor not only in determining the ion adducts observed, but also in fragmentation of the steroid-conjugate bond. As we reported earlier [12], the glucuronide bond to epitestosterone is signficantly more labile than that of testosterone. This limits the voltage that could be applied to dissociate adducts, since the protonated aglycone was the predominant ion at high orifice voltages.

In agreement with our observations, Kobayashi et al. [15] observed a pattern of H_2O loss from the protonated molecular ion using atmospheric pressure chemical ionization (APCI) for unconjugated 3-hydroxysteroids. The 17-hydroxysteroids produced abundant protonated molecular ions. Edlund et al. [16] also found that androst-1,4-dien-17α-methyl-17β-ol-3-one (dianabol) and epidianabol produced abundant $[M+H]^+$ ions and a weak fragment ion

due to loss of water under APCI conditions. Mück and Henion [17] made a similar observation for stanozolol and several of its metabolites, although the presence of a pyrazol ring makes stanozolol a unique case among steroids with respect to positive ion formation. Weidolf et al. [18] developed a negative ion pneumatically assisted electrospray ionization HPLC–MS method for androst-1,4-dien-17β-ol-3-one-17-sulfate. The limit of detection was 10 pg on-column by selected-ion monitoring with a linear response observed over a 500-fold concentration range.

Although some information about the site of conjugation appears to be obtained from ESI–MS spectra, the identity of the steroid nucleus is difficult to ascertain from molecular adducts. We observed significant differences in product ion ratios from H_2O loss from the aglycone among the various steroid isomers (e.g., androsterone and etiocholanolone; testosterone and epitestosterone). For epiandrosterone, m/z 273, resulting from the loss of one H_2O from the deconjugated steroid (m/z 291), dominated the MS–MS spectra. Both androsterone and etiocholanolone glucuronide had m/z 255 [which resulted from the loss of two H_2O molecules from the aglycone (m/z 291)] as the most abundant ion in the MS–MS spectrum, although the ratios of m/z 255/273 were significantly different. Cole et al. [19], using fast atom bombardment ionization and a sector instrument, also found differences for MS–MS spectra of androsterone and etiocholanolone glucuronides. They reported that the isomers could be distinguished on the basis of their relative intensities of the product ions, with m/z 291 being greater than m/z 273 and the m/z 273 to 255 ratio being greater than two for androsterone glucuronide, while the opposite was observed for etiocholanolone. This difference could be the result of differences in collision energies or gas thicknesses. Cole et al. [19] did demonstrate a diminished difference in the spectra at high concentration, although it is unlikely that this is the explanation for the results observed here.

The structural data for the metabolites of dianabol [16], stanozolol [17] and boldenone [18] were obtained from positive ion MS–MS spectra. In the latter case, although the analysis was carried out in the negative ion mode, no negative ion MS–MS spectra were reported. Using positive ion MS–MS spectra, boldenone could be detected for seventeen days after a single dose. Poon and co-workers [20] have also identified a number of conjugated metabolites of 4-hydroxyandrost-4-en-3,17-dione using positive ionization MS–MS from individuals receiving this chemotherapeutic agent [20].

In summary, we were able to detect steroid glucuronide and sulfate conjugates directly by HPLC–ESI–MS. Reversed-phase HPLC is capable of resolving all of the isomers studied. The detection limits of 3–25 pg on-column with a packed capillary column are among the best reported, relative to other steroid studies. The detection limits on both the 1 mm and the 300 μm column are readily compatible with testing in urine. In order to obtain spectra that contain useful data for structural confirmation, it was necessary to work in the positive ion mode. Significant differences were observed for steroids with hydroxyl substituents at the 3- or 17-position for the most abundant adduct ions produced from ESI–MS mode. Spectral differences in the MS–MS mode appear to be sufficient to base identification criteria on ion ratios.

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