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Microbore high-performance liquid chromatography–electrospray ionisation mass spectrometry of steroid sulphates

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Abstract

With the increasing abuse of steroids in both athletic and equine sports, there is a growing need for more sensitive and specific methods of detection of these compounds. Steroid sulphates are products of steroid metabolism and may be used as markers of steroid administration as well as endogenous steroid production. Electrospray negative ion mass spectra of 23 steroid monosulphates, 5 steroid disulphates and 4 bile acid sulphates have been recorded. The most intense ions in the mass spectra are due to molecular anions or dianions and in most cases these are the only ions present. The daughter ion mass spectra of these compounds vary depending on the location of the sulphate group in the molecule. Alicyclic steroid sulphates have daughter ion mass spectra containing a single ion at m/z 97 (HSO_4^-) while, for aromatic sulphates, m/z 97 is absent. Combined microbore HPLC–electrospray ionisation mass spectrometry was used for the separation and identification of the individual components of a mixture of seven steroid monosulphates and detection of these compounds by scanning and multiple reaction monitoring is reported.

Keywords: Electrospray ionisation; Mass spectrometry; Steroid sulphates; Bile acid sulphates

1. Introduction

The profiling of steroids and their metabolites in plasma and urine is now increasingly used in the detection and diagnosis of various medical disorders [1–6] as well as in the identification of cases of steroid abuse. Steroid sulphates are products of steroid metabolism and these conjugates may be used as markers of endogenous steroid production and exogenous steroid administration. Gas chromatography–mass spectrometry (GC–MS) has been one of the most widely used methods for measuring steroid conjugates but the requirements of a thermally stable and volatile analyte for GC–MS necessitate prior hydrolysis of a sulphate conjugate to the parent steroid before analysis. This process has a number of drawbacks, not least of which are problems of incomplete hydrolysis and degeneration of the liberated steroid during the hydrolytic procedure.

Direct analysis of steroid conjugates by HPLC–mass spectrometry (HPLC–MS) does not have the potential problems discussed above but there have been considerable difficulties in interfacing these two analytical techniques. Until recently, the most successful approach to coupling HPLC and MS instrumentation has been via the thermospray interface [7] and HPLC–thermospray MS has been shown to be applicable to the analysis of intact steroid sulphates

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[8–10]. However, the thermospray interface requires heating of the vaporiser probe, which can cause decomposition of thermally labile compounds, and problems with ion current stability are often encountered.

The novel technique of ion spray or pneumatically-assisted electrospray ionisation MS [11] appears to offer some advantages over thermospray MS. The electrospray interface for HPLC–MS uses an atmospheric pressure ion source and has a higher efficiency of sample ionisation than thermospray. Weidolf et al. [12] have used microbore HPLC–electrospray MS for the analysis of sulphate conjugates of six steroids and the use of a triple quadrupole mass spectrometer allowed them to record daughter ion mass spectra of these compounds. Wong et al. [13] have used electrospray MS to identify several steroid monosulphates that were interfering with radioimmunoassays for 17α-hydroxyprogesterone in neonatal plasma. We have extended this work by recording the electrospray mass spectra of 23 steroid monosulphates, 5 steroid disulphates and 4 bile acid sulphates. Conditions for microbore HPLC separation of some of these compounds were developed and limits of detection were assessed. The use of a triple quadrupole mass spectrometer which permitted the recording of daughter ion mass spectra and detection of steroid sulphates by multiple reaction monitoring (MRM) is reported.

2. Experimental

2.1. Chemicals

Dehydroisoandrosterone sulphate, estriol 3-sulphate, estrone 3-sulphate, lithocholic acid 3-sulphate, taurolithocholic acid 3-sulphate and glycolithocholic acid 3-sulphate were purchased as their sodium salts from Sigma (Poole, Dorset, UK). The remaining compounds were originally obtained from the Medical Research Council Steroid Reference Collection (this collection is now available from Sigma). Steroid sulphates were dissolved in deionized water at a concentration of 200 μg/ml and these stock solutions were stored at 4°C. HPLC-grade water was obtained from Rathburn Chemicals (Walkerburn, Scotland, UK) and "far UV" HPLC-grade acetonitrile from BDH (Lutterworth, Leics., UK). HPLC mobile phases were filtered and degassed by sparging with helium before use.

2.2. Flow injection analysis–electrospray mass spectrometry

A VG Quattro II triple quadrupole mass spectrometer (VG Biotech, Altrincham, Cheshire, UK) equipped with an atmospheric pressure ion source and a pneumatically-assisted electrospray ionisation interface was used. Nitrogen was supplied as both drying gas and nebulising gas and flow injection analysis was used for the recording of negative ion mass spectra. Samples were introduced into the mass spectrometer through a Model 7125 injector (Rheodyne, Cotati, CA, USA) fitted with a 20-μl loop, which was connected to the electrospray probe by PEEK tubing (Michrom BioResources, Pleasanton, CA, USA) of 1 m length and 76.2 μm internal diameter. The solvent used was acetonitrile–water (50:50, v/v) and a flow-rate of 10 μl/min through the injector and tubing was maintained with the use of a fluid delivery module (Michrom BioResources) operating at a solvent reservoir head pressure of 103 kPa. The source temperature was maintained at 70°C and a capillary voltage of between 2 and 3 kV was used. Other source and quadrupole parameters were not kept at any preset values but were adjusted during the daily tuning procedure to give maximum sensitivity for the compounds being analysed. The cone voltage was kept below 50 V for these studies as, above this value, increased ion fragmentation was observed. For collision induced dissociation (CID) studies, argon was admitted into the hexapole collision cell at a pressure of $\sim 5 \times 10^{-5}$ kPa and a collision energy of 30–45 eV was used. Negative ion mass spectra were acquired over a range of m/z 50 to m/z 700 at a rate of one scan every two seconds and with unit mass resolution (10% valley) in both scanning quadrupoles.

2.3. Microbore HPLC–electrospray mass spectrometry

The HPLC system consisted of a Waters 616 liquid chromatography pump and 600S flow controller (Waters, Watford, Herts., UK), a Rheodyne

Model 8125 injector fitted with a 5-μl sample loop and a Waters 486 tunable UV absorbance detector equipped with a 2.5-μl flow cell. Isocratic separation of steroid sulphates was carried out on a 5 μm Exsil 80 ODS column, 15 cm×1 mm I.D., supplied by Hichrom (Theale, UK) using a solvent mixture of acetonitrile–water (15:85, v/v) at a flow-rate of 50 μl/min. The UV detector was placed "in line" between the HPLC column and the electrospray probe and PEEK tubing was used for the connections. Stock solutions of the sulphates in water were diluted with appropriate amounts of acetonitrile prior to injection to give a solvent composition the same as that of the mobile phase. Negative ion mass spectra were acquired over a range of m/z 300 to m/z 500 at a rate of one scan per second and with unit mass resolution (10% valley) in both scanning quadrupoles. Detection by MRM was carried out post-HPLC for five of the compounds. A dwell time of 0.10 s for each of the parent/daughter ion transitions, an inter-channel delay of 0.02 s, a cone voltage of 33 V and a collision energy of 45 eV were used. The recordings obtained for m/z 351/97 (17β-estradiol 17-sulphate), m/z 367/97 (testosterone 17-sulphate; dehydroisoandrosterone sulphate), m/z 369/97 (17α-hydroxy-5α-androstan-3-one 17-sulphate) and m/z 441/97 (cortisol 21-sulphate) are shown in Fig. 4 below.

3. Results and discussion

The electrospray mass spectra of nineteen alicyclic and four aromatic steroid monosulphates are summarised in Table 1. The base peaks in the mass spectra are due to the intact anions ($[M-H]^-$) and in most cases these are the only ions present. These spectra are similar to the thermospray mass spectra of these compounds which have been reported previously [8–10]. The electrospray mass spectra of five steroid disulphates and four bile acid sulphates are listed in Table 2. For the steroid disulphates, and

Table 1
Electrospray negative ion mass spectra of steroid monosulphates

Compound	Molecular mass of anion	Base peak (m/z)	Other ions (>5% base peak)
Alicyclic steroids			
5α-Androstan-17β-ol sulphate TEA salt	355	355	
Androst-5-en-3β,17β-diol 17-sulphate K salt	369	369	
17α-Hydroxy-5α-androstan-3-one 17-sulphate Na salt	369	369	
17β-Hydroxy-5α-androstan-3-one 17-sulphate pyr. salt	369	369	
Testosterone 17-sulphate K salt	367	367	
Androsterone sulphate Na salt	369	369	
3β-Hydroxy-5β-androstan-17-one sulphate Na salt	369	369	
Isoandrosterone sulphate K salt	369	369	
Dehydroisoandrosterone sulphate Na salt	367	367	
3β,19-Dihydroxyandrost-5-en-17-one 19-sulphate K salt	383	383	
3β-Hydroxy-17-oxoandrost-5-en-19-al 3-sulphate pyr. salt	381	381	
11β-Hydroxyandrosterone 3-sulphate Na salt	385	385	369(43)
3α,11β-Dihydroxy-5β-androstan-17-one 3-sulphate Na salt	385	385	369(11), 367(22)
11,17-Dioxo-5β-androstan-3α-ol sulphate TEA salt	383	383	
5β-Pregnan-3α,20α-diol 20-sulphate Na salt	399	399	201(5), 113(5)
Pregnenolone sulphate Na salt	395	395	
17α-Hydroxypregnenolone 17-sulphate K salt	411	411	395(28)
Cortisol 21-sulphate Na salt	441	441	411(6)
Tetrahydrocortisone 21-sulphate Na salt	443	443	429(7), 383(32)
Aromatic steroids			
17β-Estradiol 3-sulphate TEA salt	351	351	219(6), 212(6)
17β-Estradiol 17-sulphate Na salt	351	351	
Estriol 3-sulphate Na salt	367	367	
Estrone 3-sulphate Na salt	349	349	

Cations: Na - sodium; K - potassium; pyr. - pyridinium; TEA - tetraethylammonium.

Table 2
Electrospray negative ion mass spectra of steroid disulphates and bile acid sulphates

Compound	Molecular mass of dianion	Base peak (m/z)	Other ions (>5% base peak)
Steroid disulphates			
Androst-5-en-3β,17β-diol disulphate di K salt	448	224	
Androst-5-en-3β,17α-diol disulphate di K salt	448	224	
3β,19-Dihydroxyandrost-5-en-17-one disulphate di K salt	462	231	113(18)
20-Oxopregn-5-en-3β,17α-diol disulphate di K salt	490	245	113(14)
17β-Estradiol-3,17-disulphate di K salt	430	215	
Bile acid sulphates			
3β-Hydroxycholest-5-en-24-oic acid 3-sulphate Na salt	452	453	
Lithocholic acid 3-sulphate Na salt	454	455	
Taurolithocholic acid 3-sulphate di Na salt	561	280.5	
Glycolithocholic acid 3-sulphate di Na salt	511	255.5	279(6)

Cations: Na - sodium; K - potassium.

taurolithocholic acid and glycolithocholic acid 3-sulphate, the principal ions in the mass spectra correspond to the doubly charged intact anions ($[M-2H]^{2-}$) and little fragmentation of these ions occurs. However for 3β-hydroxycholest-5-en-24-oic acid and lithocholic acid 3-sulphate, which both contain C_{24} carboxyl groups, only ions due to singly charged species at m/z 453 and m/z 455 respectively are visible, indicating that ionisation of the carboxyl groups is suppressed during the electrospray ionisation process. The spectra of the disulphates and bile acid sulphates are much simpler than the corresponding thermospray mass spectra, which are dependent on vaporiser temperature and cationisation [8], and hence are more useful for compound identification. Negative ion electrospray mass spectra of

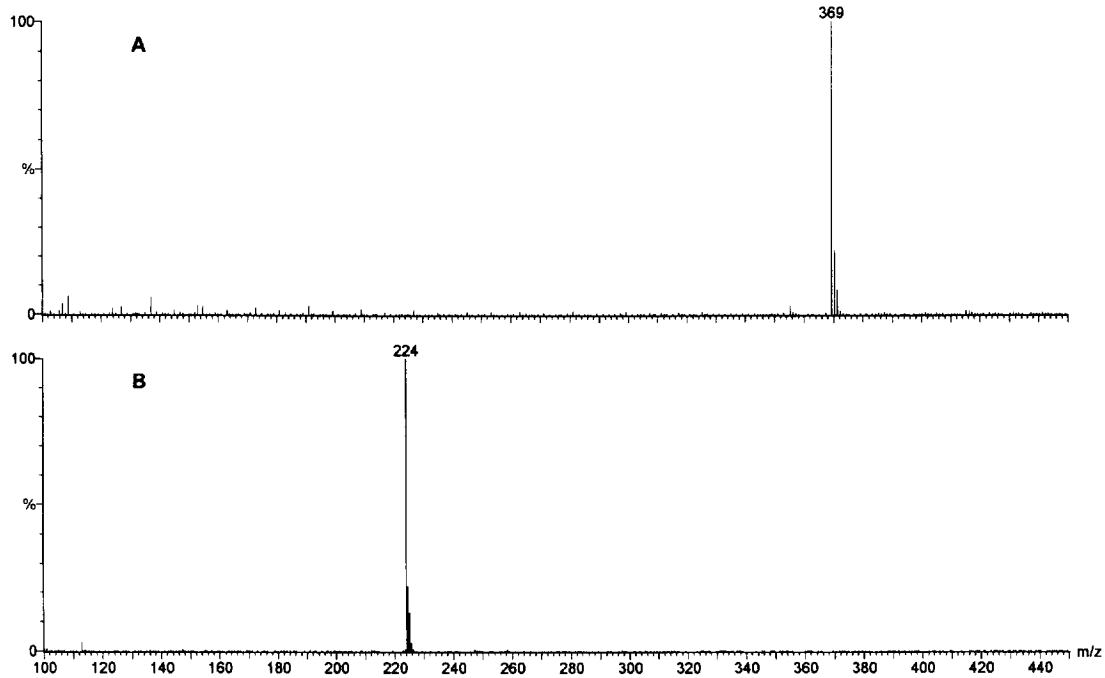

Fig. 1. Electrospray negative ion mass spectra of (A) androst-5-en-3β,17β-diol 17-sulphate and (B) androst-5-en-3β,17β-diol disulphate.

representative steroid mono- and disulphates are shown in Fig. 1.

The daughter ion mass spectra of the major ions of each steroid and bile acid sulphate (listed in Table 1 and Table 2) are shown in Table 3. The spectra fall into two categories depending on the location of the sulphate group or groups within the molecule. When sulphate is attached to an alicyclic ring, the base peak in the mass spectrum is at m/z 97 (corresponding to HSO_4^-). For six of the compounds, the ion m/z 80 (corresponding to SO_3^-) is also present and for one compound, 3β,19-dihydroxyandrost-5-en-17-one 19-sulphate, m/z 80 is the base peak in the mass spectrum. The daughter ion mass spectrum of 17β-estradiol 17-sulphate, where the sulphate group is attached to the alicyclic D ring of the molecule, is shown in Fig. 2A. When the sulphate group is attached to an aromatic ring, m/z 97 is absent and more high mass fragment ions, together with m/z 80, are observed in these mass spectra. This is exemplified in the spectrum of 17β-estradiol 3-sulphate, where the sulphate group is attached to the aromatic A ring of the molecule (figure 2B). The response factor for aromatic sulphates is reduced compared to alicyclic sulphates. Weidolf et al. [12] have discussed the differences between the daughter ion mass spectra of alicyclic and aromatic steroid sulphates and have suggested that the lack of an available hydrogen atom at the 3-position of the aromatic ring is why HSO_4^- is not formed. Our data supports their suggestion that the clear differences between the daughter ion mass spectra of alicyclic and aromatic steroid sulphates may be of diagnostic value in the identification of unknown conjugates. The daughter ion mass spectra of steroid disulphates and bile acid sulphates also appear to be dependent

Table 3
Daughter ion mass spectra[a] of steroid and bile acid sulphates

Compound	Base peak (m/z)	Other ions (>5% base peak)
Alicyclic steroids[b]		
5α-Androstan-17β-ol sulphate TEA salt	97	80(8)
Androst-5-en-3β,17β-diol 17-sulphate K salt	97	80(13)
Testosterone 17-sulphate K salt	97	177(6), 96(18), 80(16)
3β,19-Dihydroxyandrost-5-en-17-one 19-sulphate K salt	80	97(13)
Cortisol 21-sulphate Na salt	97	81(70), 80(43)
Tetrahydrocortisone 21-sulphate Na salt	97	81(17), 80(12)
Aromatic steroids		
17β-Estradiol 3-sulphate TEA salt	271	183(19), 171(8), 158(12), 145(19), 80(81)
17β-Estradiol 17-sulphate Na salt	97	80(10)
Estriol 3-sulphate Na salt	287	183(10), 171(43), 159(12), 145(17), 80(89)
Estrone 3-sulphate Na salt	145	269(82), 183(17), 159(20), 123(8), 80(73)
Steroid disulphates and bile acid sulphates		
Androst-5-en-3β,17β-diol disulphate di K salt	97	
Androst-5-en-3β,17α-diol disulphate di K salt	97	
3β,19-Dihydroxyandrost-5-en-17-one disulphate di K salt	80	123(6), 110(6), 97(87)
20-Oxopregn-5-en-3β,17α-diol disulphate di K salt	97	
17β-Estradiol-3,17-disulphate di K salt	80	175(7), 97(65), 96(33)
3β-Hydroxycholest-5-en-24-oic acid 3-sulphate Na salt	97	
Lithocholic acid 3-sulphate Na salt	97	
Taurolithocholic acid 3-sulphate di Na salt	97	124(5), 107(7), 80(23)
Glycolithocholic acid 3-sulphate di Na salt	97	80(7), 74(78)

TEA, tetraethylammonium.

[a] The ions chosen for collision induced dissociation were the base peaks in the mass spectra listed in Table 1 and Table 2.

[b] The daughter ion mass spectra of alicyclic steroid sulphates consisted of a single ion of m/z 97, except for those compounds listed below.

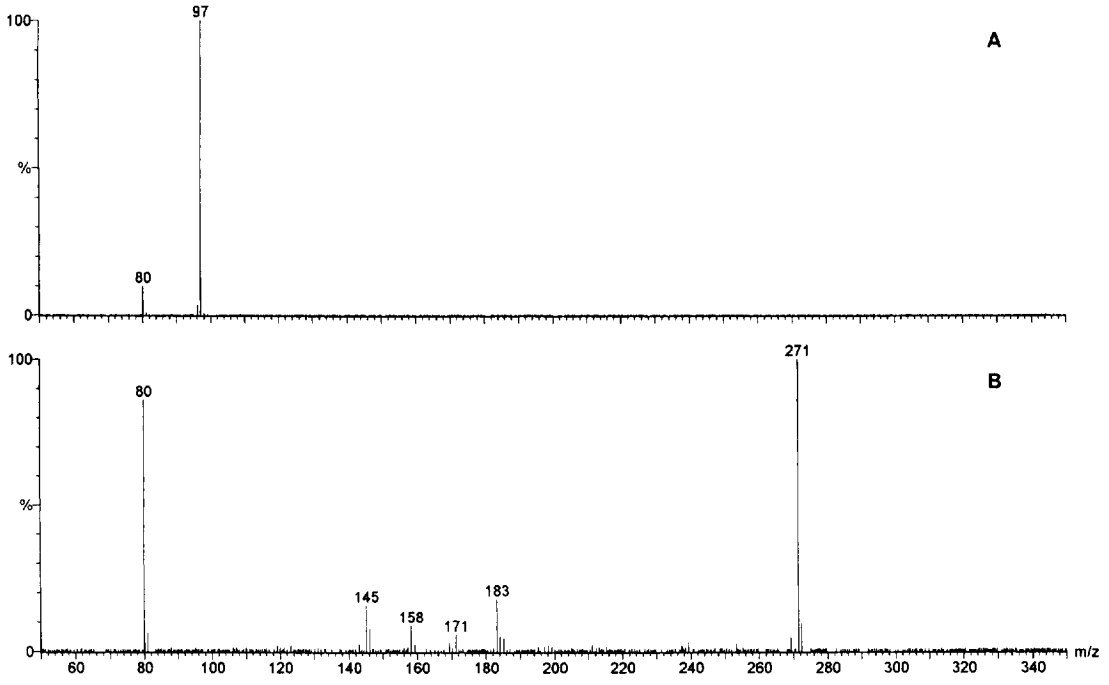

Fig. 2. Daughter ion mass spectra of (A) 17β-estradiol 17-sulphate and (B) 17β-estradiol 3-sulphate.

on the position of sulphate groups within the molecule. Alicyclic steroids with sulphate groups located at C_3 and C_{17} exhibit simple daughter ion spectra with m/z 97 often the only ion present. For 3β,19-dihydroxyandrost-5-en-17-one disulphate and 17β-estradiol-3,17-disulphate, ions of m/z 97 are present but the base peaks in the mass spectra are ions of m/z 80.

Electrospray MS is a concentration (and not a mass) dependent technique and the mass spectrometric response is limited by the absolute concentration of the analyte. Using flow injection analysis, full mass spectra could be obtained for testosterone 17-sulphate ($[M-H]^-$: m/z 367) and estrone 3-sulphate ($[M-H]^-$: m/z 349) at the 1 pg/μl (3 nM) level with signal-to-noise ratios of 35 to 1 and 10 to 1 respectively. Here, using flow-rates of 10 μl/min and MS scan rates of 325 u/s, it was possible to obtain good quality spectra, with a minimum of 10 scans, by injecting 5 pg into the mass spectrometer ion source. The mass spectrometric response post HPLC will be limited by dilution of the analyte across the HPLC peak width. Consequently HPLC–MS will not achieve the same absolute sensitivity (in terms of mass) as obtained by flow injection analysis, but the invariant retention times of HPLC do offer a further degree of specificity in sample screening.

There are a number of advantages to the HPLC–MS analysis of polar conjugates by electrospray-MS, particularly if the added benefits of increased sensitivity of microbore HPLC columns are included. The optimal solvent flow-rate of 50 μl/min through a 1 mm I.D. microbore HPLC column is fully compatible with the electrospray interface, removing the need for solvent splitting post HPLC. In contrast to our observations with more lipophilic species, there was no evidence of electrically mediated retardation of steroid sulphates in the PEEK tubing used to connect the HPLC column and the high voltage electrospray interface. Isocratic HPLC conditions were optimised on a C_{18} microbore HPLC column for the separation of a mixture of seven steroid monosulphates. Five of the components in the mixture were fully resolved by HPLC and detected in the relevant ion channel with a signal-to-noise ratio of >10 to 1: estriol 3-sulphate (m/z 367, 2.13

min), cortisol 21-sulphate, (m/z 441, 4.40 min), 17β-estradiol 17-sulphate (m/z 351, 5.89 min), dehydroisoandrosterone sulphate (m/z 367, 13.17 min) and 17α-hydroxy-5α-androstan-3-one 17-sulphate (m/z 369, 16.30 min). The two unresolved compounds, testosterone 17-sulphate (m/z 367, 8.60 min) and estrone 3-sulphate, (m/z 349, 8.74 min) could be readily differentiated by differences in their mass spectra. The ion chromatograms shown in Fig. 3 were obtained on 100 pg of each analyte in the mixture injected on column. The analytes eluted from the column with peak widths of 0.8–1.8 min (40–90 μl) which allowed the collection of sufficient scans to generate good quality full scan mass spectra for each steroid sulphate. MRM was also carried out post HPLC using the strong

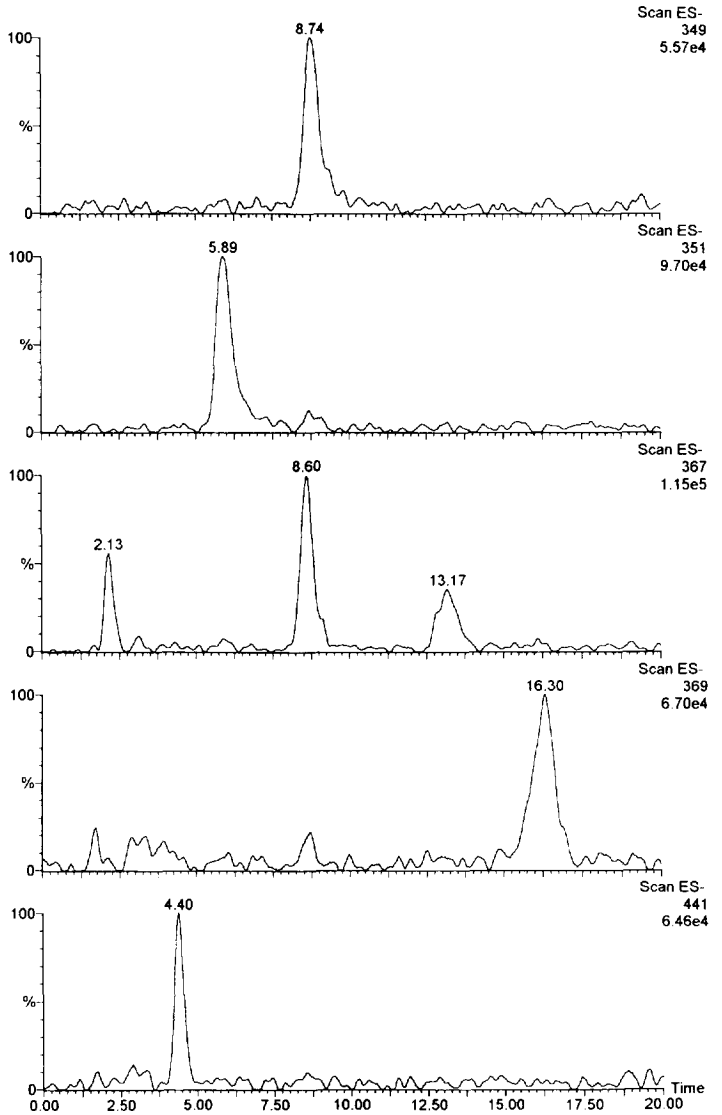

Fig. 3. Reversed-phase microbore HPLC–electrospray MS profiles of a mixture of seven steroid monosulphates (100 pg of each on column). Estrone 3-sulphate (m/z 349, 8.74 min), 17β-estradiol 17-sulphate (m/z 351, 5.89 min), estriol 3-sulphate (m/z 367, 2.13 min), testosterone 17-sulphate (m/z 367, 8.60 min), dehydroisoandrosterone sulphate (m/z 367, 13.17 min), 17α-hydroxy-5α-androstan-3-one 17-sulphate (m/z 369, 16.30 min) and cortisol 21-sulphate (m/z 441, 4.40 min).

$[M-H]^-$:HSO_4^- transitions in the argon CID spectra (Table 3). The alicyclic monosulphates could be readily monitored at the 50 ng level (Fig. 4).

The studies described above are in agreement with the work of other groups [12,13] and indicate that combined HPLC electrospray ionisation MS can be successfully applied to the analysis of steroid sulphates. For these compounds, electrospray ionisation gives high sensitivity. Further, for steroid disulphates and bile acid sulphates, electrospray mass spectra are much simpler and more reproducible than thermospray mass spectra. The production of daughter ions, and the use of multiple reaction monitoring, gives the methodology even greater specificity and this

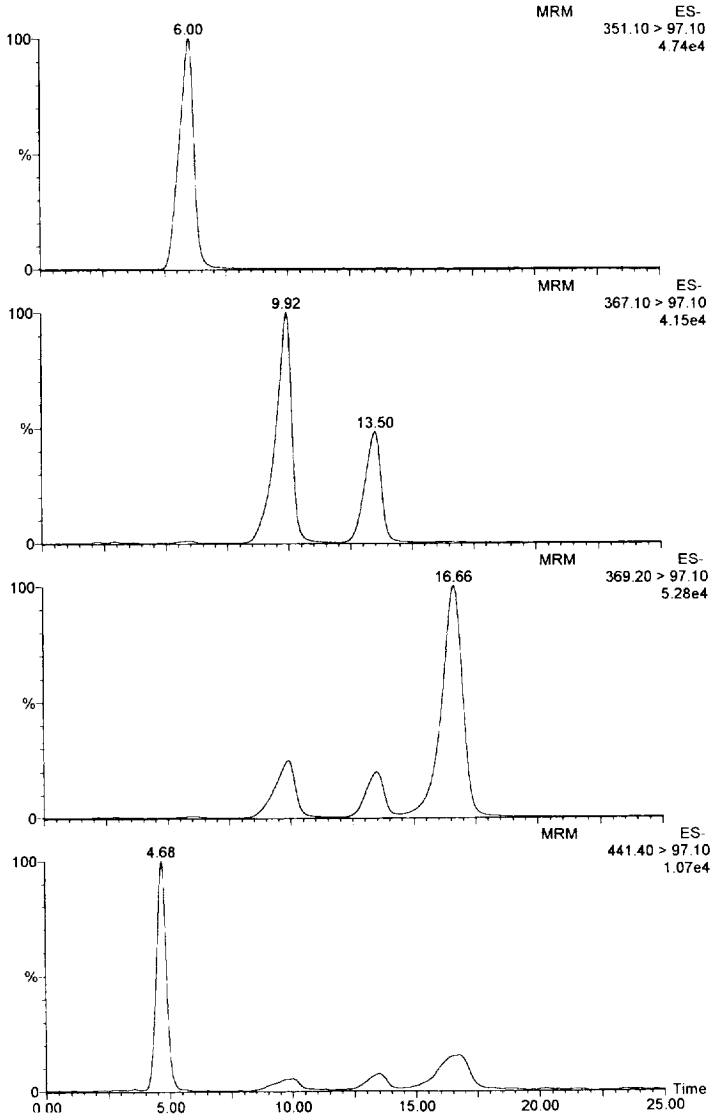

Fig. 4. Microbore HPLC–electrospray MS with multiple reaction monitoring of the $[M-H]^-$:HSO_4^- transitions for 17β-estradiol 17-sulphate (m/z 351/97, 6.00 min), testosterone 17-sulphate (m/z 367/97, 9.92 min), dehydroisoandrosterone sulphate (m/z 367/97, 13.50 min), 17α-hydroxy-5α-androstan-3-one 17-sulphate (m/z 369/97, 16.66 min) and cortisol 21-sulphate (m/z 441/97, 4.68 min).

could well be important in the identification and measurement of unknown steroid sulphates isolated from biological sources.

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