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Screening of eltanolone metabolites in dog urine by anion-exchange/reversed-phase liquid chromatography and mass spectrometry

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Abstract

Strong anion-exchange (SAX) chromatography and reversed-phase liquid chromatography (RPLC) followed by different mass spectrometric techniques for the separation and identification of conjugated and unconjugated ^{14}C-labelled eltanolone (5β-Pregnan-3α-ol-20-one) metabolites in biological fluids are presented. Conjugates of estradiol were used as model compounds for the development of a SAX based group separation of neutral steroids, glucuronides, sulfates and di-conjugated steroids. The usefulness of the technique is demonstrated by the analysis of ^{14}C-labelled eltanolone metabolites in dog urine. The analytical SAX column used prior to RPLC improved the capacity to separate the metabolites from each other and from endogenous components, compared to a single reversed-phase system. Liquid chromatography negative ion electrospray-mass spectrometry (LC–ESI-MS) was used for the molecular mass determination of conjugated eltanolone metabolites. Unconjugated metabolites and hydrolysed conjugates were identified using gas chromatography–mass spectrometry with an electron impact ion source (GC–MS) after trimethylsilyl (TMS) derivatization. An unexpected finding in dog urine was the diglucuronide formation of eltanolone (presumably after enolisation of its carbonyl group).

Keywords: Eltanolone

1. Introduction

In drug metabolism studies steroids and their metabolites generate a very complex separation pattern when a single reversed-phase column is used. Separation systems consisting of coupled columns with different separation mechanisms could be useful in these kind of studies. By expanding the separation into two dimensions, sample components which are unresolved in the first dimension can often be separated in the second. The theoretical overall peak capacity is the product of the peak capacity of each of the two systems, provided that the two columns have different separation mechanisms [1,2].

In the present study, a system has been developed where the reversed-phase liquid chromatography (RPLC) is preceded by strong anion-exchange (SAX) chromatography, a very useful technique in steroid screening as it separates different conjugates from each other. Separation techniques using solid-

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phase extraction with anion-exchange columns during sample preparation have previously been reported [3–5]. Here, the separation efficiency has been improved by the use of an analytical SAX column prior to RPLC.

The determination of the molecular mass of the conjugated metabolites by liquid chromatography negative ion electrospray mass spectrometry (LC–ESI-MS) is important since unpredictable elution order in the SAX chromatography has been observed. LC–ESI-MS, an atmospheric pressure ionization technique, has become routine during the last decade in studies of drug metabolism, especially for thermally unstable and ionized compounds of relatively high molecular masses such as drug conjugates [6,7].

Unconjugated and hydrolysed conjugated analytes were identified using gas chromatography electron impact mass spectrometry (GC–MS) after trimethylsilyl (TMS) derivatization. This technique is well established and widely used [8]. Because of its limited capacity to analyse thermally unstable and non-volatile components such as conjugated and highly hydroxylated steroids, LC–ESI-MS is a good complement to GC–MS.

The aim of the present study was to develop a method suitable for the separation and direct identification of conjugated and phase I metabolites of eltanolone in biological fluids. The usefulness of the method is demonstrated by an investigation of the metabolism of eltanolone in the dog.

2. Experimental

2.1. Reference compounds and chemicals

The ^{14}C-eltanolone emulsion (4 mg/ml, specific activity 331 kBq/ml) was from Pharmacia and Upjohn, Stockholm, Sweden. The eltanolone (5β-Pregnan-3α-ol-20-one) used as a reference standard was purchased from Schering AG, Berlin, Germany.

β-Glucuronidase, Type H-1, 345 200 units/g (*Helix Pomatia)*, 5β-Pregnane-3α,20α-diol, 5β-Pregnane-3α,20β-diol, 5β-Pregnane-3α,6α-diol-20-one, 5β-Pregnane-3α,20α-diol glucuronide, 17β-estradiol-3-sulfate, 17β-estradiol-3-(β-D-glucuronide), β-estradiol-3-sulfate-17- glucuronide and β-estradiol-3,17-disulfate were purchased from Sigma Chemical Co, St Louis, MO, USA. 5β-Pregnane-2β,3α-diol-20-one was from SRI International, CA, USA. Deionized water was purified in a Milli-Q plus system from Millipore. Methanol and acetonitrile from Lab-Scan, Ltd., Dublin, Ireland were of HPLC grade. Scintillation fluid Flo Scint IV was from Packard Instrument B.V., Groningen, the Netherlands.

Semicarbazide hydrochloride, acetic acid (glacial), formic acid (98–100%), trifluoroacetic acid (TFA), hydrochloric acid, sodium acetate, sodium sulfate and pyridine were from Merck, Darmstadt, Germany. *N,O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce, Rockford, Ill, USA. Diethylether was from Prolabo, Paris, France.

All other chemicals used were of analytical grade and were obtained from commercial suppliers.

2.2. Instrumentation

2.2.1. Strong anion-exchange (SAX) chromatography

The column (4.6×250 mm) was packed with 5 μm Spherisorb S5SAX particles by Phase Separations Ltd, Deeside, UK. Both mobile phases were prepared from a 60% methanol–water mixture. Ammonium acetate was added to a concentration of 0.02 *M* and 1 *M* for eluents A and B, respectively. The pH was adjusted to 5.0 with acetic acid. The analytes were injected using a Rheodyne 7125 injector with a 100 μl loop. Elution was performed at 1 ml/min with 100% A for 4 min followed by a linear gradient to 100% B for 14 min and thereafter isocratic elution with 100% B for 17 min.

2.2.2. UV detection

A Waters 490E UV detector set to 254 nm was used for method development using the UV-absorbing estradiols as model compounds.

2.2.3. Radioactivity detection

For the monitoring of radioactive compounds from LC, a Radiomatic FLO-ONE/Beta Series A-500 radioactivity detector (Radiomatic Instruments and Chemical Company, Inc., Meriden, USA) was used. The volume of the flow cell was 500 μl. The effluent from the LC column was split, and 20% was mixed with the scintillation fluid Flow Scint IV (4.0 ml/

min) and passed through the detector flow cell. Radioactive fractions were collected from the rest of the effluent (80%).

2.2.4. Reversed-phase liquid chromatography (RPLC)

The columns used were a Kromasil 100-5C18 (4.6 x 250 mm, 5 μm particles) packed by Hichrom Ltd, Berkshire, UK and a precolumn RP-18 Newguard (15×3.2 mm, 7 μm particles) from Applied Biosystems, Foster City, CA, USA. The injector was the same as above. Both eluents contained 0.1% TFA with 10% and 90% methanol in water for eluents A and B, respectively. The analytes from the dog urine were eluted at 1 ml/min with a linear gradient from 30% B to 80% B for 25 min, followed by a gradient from 80% B to 100% B for 2 min and thereafter 100% B for 13 min.

2.2.5. LC–ESI-MS

The mass spectrometer used was a Quattro triple quadrupole (Micromass, Altrincham, UK) fitted with an electrospray interface. The analytes were injected with a Rheodyne injector with a 20 μl loop into a C18 Hypersil column (1×250 mm, 5 μm particles) packed by SGE, Austin, TX, USA. Both mobile phases contained 0.5% acetic acid with 10% and 90% acetonitrile in water for eluents A and B, respectively. The analytes were eluted from 10% to 80% of eluent B for 20 min, followed by isocratic elution for 15 min with 80% B and 5 min with 100% B. The flow-rate was 30 μl/min. The column effluent was split in a Valco tee coupling with 10 μl/min to the mass spectrometer and 20 μl/min to the radioactivity detector. The flow-rate of the scintillation fluid was 1.0 ml/min. The mass spectrometer signal appeared 0.6 min after the radioactivity signal due to longer transfer lines. Electrospray mass spectra were recorded in the negative ion mode in the mass range 300–800 m/z at a scan speed of 4 s/scan. The ion source temperature was set to 70°C.

2.2.6. LC–ESI-MS/MS

For the LC–ESI-MS/MS analyses the RPLC parameters were the same as for LC–ESI-MS. Electrospray MS/MS product ion spectra were recorded in the negative ion mode in the mass range of 20–700 m/z with a scan speed of 4 s/scan. Xenon was used as collision gas giving a collisional energy (E_{CM}) in the center-of-mass frame of 13.1 eV.

2.2.7. GC–MS

An SX-102 mass spectrometer (Jeol, Tokyo, Japan) connected to a Hewlett Packard Model 5890A gas chromatograph was used for the GC–MS analyses. A 25 m×0.2 mm I.D. HP-1 column (cross-linked methyl silicon) with a film thickness of 0.5 μm was used. The injector temperature was 250°C. The split valve was opened 1 min after injection. The column was held at 140°C for 3 min, and then raised to 280°C at a rate of 50°C/min. Mass spectra were recorded in the mass range m/z 25–600 at a cycle time of 1.5 s.

2.3. Analytical procedure

2.3.1. C_{18} solid-phase extraction and SAX-LC

Urine samples from one female and one male dog were collected 0–24 h after single intravenous injections of a ^{14}C-eltanolone emulsion in doses of 14 mg/kg. The samples (20–50 ml) were acidified with formic acid to pH 3 and extracted through 5g (20 ml) C_{18} solid-phase columns, preconditioned with one column volume of methanol and one volume of 0.1% TFA in water. The columns were washed with one volume of 0.1% TFA and the analytes were eluted with 2–3 volumes of methanol. The eluates were evaporated and dissolved in 60% methanol in water to a final volume of 500 μl. The samples were centrifuged at 2000×g for 5 min and then filtered through 0.45 μm nylon filters. Of the filtrate, 100 μl was injected into the SAX column.

Urine fractions containing radioactive material were collected and divided in two aliquots. One part was used for hydrolysis and analysis by GC–MS and the other part for further separation by RPLC and LC–ESI-MS.

2.3.2. RPLC and LC–ESI-MS of SAX fractions

The samples collected in the SAX system were evaporated to 1/3 of the original volume and then concentrated by extraction through 500 mg (3 ml) C_{18} solid-phase columns as described above. The eluates were evaporated and diluted with 40% methanol to a final volume of 100 μl prior to injection into the RPLC system. Fractions were

collected for analysis by LC–ESI-MS. Prior to LC–ESI-MS analysis, the fractions from the RPLC system were liquid/solid extracted and evaporated as described above and dissolved in 30% acetonitrile to a final volume of 20 μl.

2.3.3. Derivatization with semicarbazide

The liquid/solid extracted RPLC fraction was evaporated and dissolved in 20 μl methanol and 100 μl 50 m*M* semicarbazide, buffered with ammonium hydrogen carbonate to pH 4, was added. Derivatization took place in darkness for 15 min. Acetic acid (400 μl, 1% in water) was added and the mixture was liquid/solid extracted as above and evaporated. The residue was dissolved in 30% acetonitrile and injected into the LC–ESI-MS system.

2.3.4. Hydrolysis, TMS-derivatisation and GC–MS

The samples collected in the SAX system, except samples from the non-conjugated fraction, were liquid/solid extracted as described above. The eluates were evaporated and dissolved in 3 ml 0.2 *M* sodium acetate pH 5 containing 14 mg β-glucuronidase/ml. The samples were hydrolysed overnight in a water bath at 37°C. After hydrolysis the mixtures were alkalized with sodium hydroxide (0.5 ml, 1 *M*) and extracted with diethylether (3×4 ml). The organic phase was washed with hydrochloric acid (1 ml, 0.1 *M*), dried with anhydrous sodium sulfate and evaporated to dryness. The dried hydrolysed residues were dissolved in BSTFA (20 μl) and pyridine (3 μl) and heated to 80°C for 1 h in order to complete the TMS derivatization of hydroxyl groups. Samples from the unconjugated fraction were liquid/liquid extracted with diethylether, dried and TMS-derivatized as described above. The samples were then analysed by GC–MS.

3. Results and discussion

3.1. Separation methods and ionization techniques

The estradiol conjugates were used as reference substances since they were commercially available and in contrast to the eltanolone derivatives detectable by UV. The elution order was found to be the following: unconjugated estradiol, estradiol monoglucuronide, estradiol disulfate, estradiol monosulfate and estradiol-3-sulfate-17-glucuronide. The elution order of the monosulfate and the disulfate was unexpected since the conjugates were expected to elute in the order of their acidity and charge (i.e. a divalent ion should have been retained longer than a monovalent ion). Methanol (60%) was added to the ion-exchange eluent in order to improve the solubility of the steroids and to eliminate hydrophobic interactions with the stationary phase, and thereby achieving a separation entirely based on ion-exchange. The elution order of the eltanolone sulfates found in rat urine, as observed by radioactivity detection, was demonstrated to be similar to the elution order of estradiols; the monosulfates eluting after the disulfates. However, the elution order of sulfate-glucuronides was in this case different as they were coeluting with the disulfates. In dog urine, where mono- and di-glucuronides of eltanolone were found, the divalent ions were retained longer than the monovalent ions in contrast with the case of sulfates. This unpredictable elution order of conjugates, demonstrated above, shows the need for direct molecular mass determination of the conjugated metabolites preferably by LC–ESI-MS, a technique offering sensitivities at the 15 pg level of steroid conjugates [7].

In the analysis of eltanolone metabolites in dog urine, an RPLC separation step, after the SAX chromatography and prior to LC–ESI-MS, was considered necessary in order to obtain samples clean enough for good quality LC–ESI-MS analyses.

An important benefit with LC–ESI-MS is the access to retention times from the radioactivity signal in parallel with MS, enabling radiolabelled metabolites to be distinguished from endogenous substances. A limitation, however, is that LC–ESI-MS only provides the molecular masses of the compounds, while further analyses by LC–ESI-MS/MS or by GC–MS with electron impact (EI) are necessary to obtain more structural information.

GC–MS was used for the identification of unconjugates and of the aglycones of conjugates after hydrolysis, liquid/liquid extraction and TMS-derivatization of the SAX fractions. No RPLC step was necessary prior to GC–MS due to an effective sample clean-up with liquid/liquid extraction. In GC–MS commercial available reference standards

are usually required for an accurate identification. In the present study of eltanolone metabolites in dog urine reference substances were available for all phase I metabolites except for one. The access to reference substances of conjugated eltanolone derivatives is limited. For glucuronides, only eltanolone-glucuronide and 5β- pregnane-3α,20α-diol-glucuronide were commercially available.

In the analysis of uncharged polyhydroxylated eltanolone metabolites having three or more hydroxyl substituents, GC–MS cannot be used, as the compounds become too polar to evaporate into the gas phase without decomposition. ESI is best suited for compounds ionized in solution, but by monitoring an adduct ion of the uncharged compound, such as $[M+Na]^+$, the sensitivity of ESI increases. However, a rapid loss of sensitivity occurs when monitoring the $[M+Na]^+$ ion on steroids containing only hydroxy groups and no ketone groups in their structure compared to other types of steroids as demonstrated by Ma et al [9]. We suggest that this loss of sensitivity proceeds with increasing amounts of hydroxy groups on the eltanolone metabolites. Atmospheric pressure chemical ionization (APCI) mass spectrometry, was found to be a better choice than ESI in the case of polyhydroxylated eltanolone metabolites. With APCI, the molecular masses and the number of hydroxyls and keto groups (indicated by the loss of H_2O) could be determined. This technique was used for the identification of hydroxylated eltanolone metabolites in the rat (to be published). A difficulty was to determine whether the ion with the highest molecular mass was the $[M+H]^+$ ion or the $[(M+H)–H_2O]^+$ ion. In most spectra, however, the $[M+H]^+$ ion was small but visible. To estimate the polarity of a compound, the elution order in the RPLC chromatogram was also a good help. The fragmentation of steroids using APCI has been investigated by Kobayashi et al [10]. The results from that study suggested that APCI-MS is a suitable tool for the determination of the molecular mass of polar, nonvolatile and thermolabile steroids.

3.2. Identification of metabolites in dog urine

The example below shows how the major ^{14}C-eltanolone metabolites found in dog urine were identified. A flow scheme of the methods used is shown in Fig. 1.

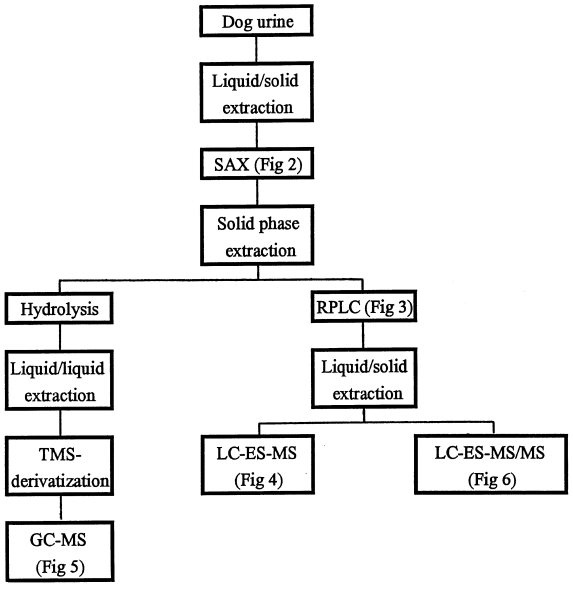

Fig. 1. Flow scheme of the analytical procedure.

The SAX chromatograms from male and female dog urine detected with a radioactivity detector are shown in Fig. 2. The sample from the male dog urine separated into three peaks in the SAX column (Fig. 2a). The first corresponded to unconjugated metabolites, while the second and third peak corresponded to monoglucuronide conjugates. The female dog urine sample separated by SAX chromatography into four peaks (Fig. 2b), the first peak corresponded to unconjugated metabolites, the following two peaks were monoglucuronides and the last peak corresponded to diglucuronidated metabolites. The collected SAX fractions were concentrated and further separated by RPLC. The RPLC chromatograms of the four SAX fractions from the female dog urine demonstrate the need for more than one separation system (Fig. 3a–d); Several compounds which were separated according to their type of conjugation in the SAX chromatography had the same retention time on the reversed-phase system, and should have coeluted if the urine sample had been injected directly into the RPLC system.

The radioactive fractions separated by RPLC, containing the major components of the different conjugates, were collected for further separation and

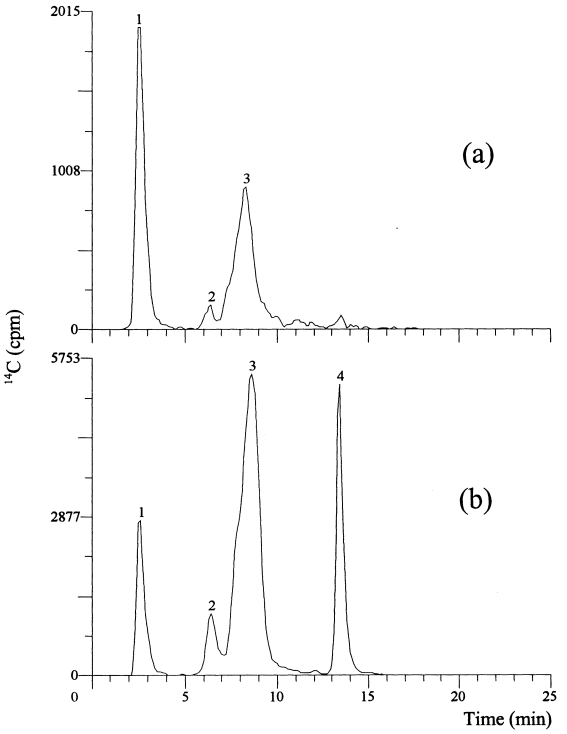

Fig. 2. SAX chromatography–radioactivity detection of ^{14}C-eltanolone metabolites in male (a) and female (b) dog urine. Peaks: (a) 1=unconjugates; 2, 3=monoglucuronides. (b) 1= unconjugates; 2,3=monoglucuronides and 4=diglucuronides. Samples were eluted with 100% A (0.02 *M* ammonium acetate, 60% methanol in water, pH 5) for 4 min, followed by a linear gradient to 100% B (1 *M* ammonium acetate, 60% methanol in water, pH 5) for 14 min and then isocratic elution with 100% B for 17 min. flow-rate: 1 ml/min. The effluent from the LC column was split, 20% was mixed with the scintillation fluid Flow Scint IV (4.0 ml/min) and used for radioactivity detection. Radioactive fractions were collected from the rest of the effluent (80%).

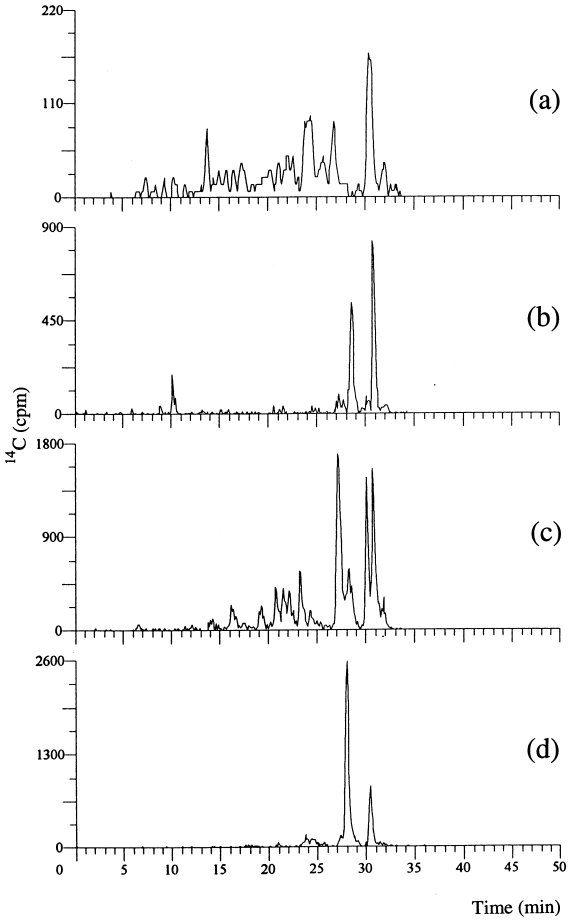

Fig. 3. RPLC radioactivity detection of four SAX fractions from the dog urine sample. (a) unconjugates, (b) and (c) monoglucuronides and (d) diglucuronides. The columns used were a Kromasil 100-5C18 (4.6×250 mm) and a precolumn RP-18 Newguard (15×3.2 mm). Both eluents contained 0.1% TFA with 10% and 90% methanol for eluent A and B, respectively. Samples were eluted with a gradient from 30% B to 80% B for 25 min, then a gradient from 80% B to 100% B for 2 min and thereafter 100% B for 13 min. Flow-rate: 1 ml/min. Detection conditions as given in Fig. 2.

analysis by LC–ESI-MS together with radioactivity detection. Molecular masses of all major conjugated metabolites were obtained by LC–ESI-MS. LC–ESI-MS for a pooled fraction of the two major components in the RPLC chromatogram in Fig. 3d, corresponding to two different diglucuronidated metabolites, is shown in Fig. 4. The radioactive trace is shown in Fig. 4a and the base peak intensity (BPI) chromatogram is shown in Fig. 4b. The two metabolite peaks are labelled with arrows. The most intense peak, with a molecular mass of 670 Da ($[M–H]^-$ 669), corresponded to diglucuronidated ‘eltanolone’. The smaller peak, with a molecular mass of 672 Da ($[M–H]^-$ 671), corresponded to diglucuronidated pregnan-diol (Fig. 4c and d). The finding of a diglucuronidated ‘eltanolone’ was unexpected since this steroid has only one potential hydroxy-group (3-OH) available for conjugation.

GC–MS analyses were performed directly on the SAX fractions after hydrolysis, liquid–liquid extraction and TMS-derivatization. EI mass spectra of eltanolone and its major phase I metabolites in dog

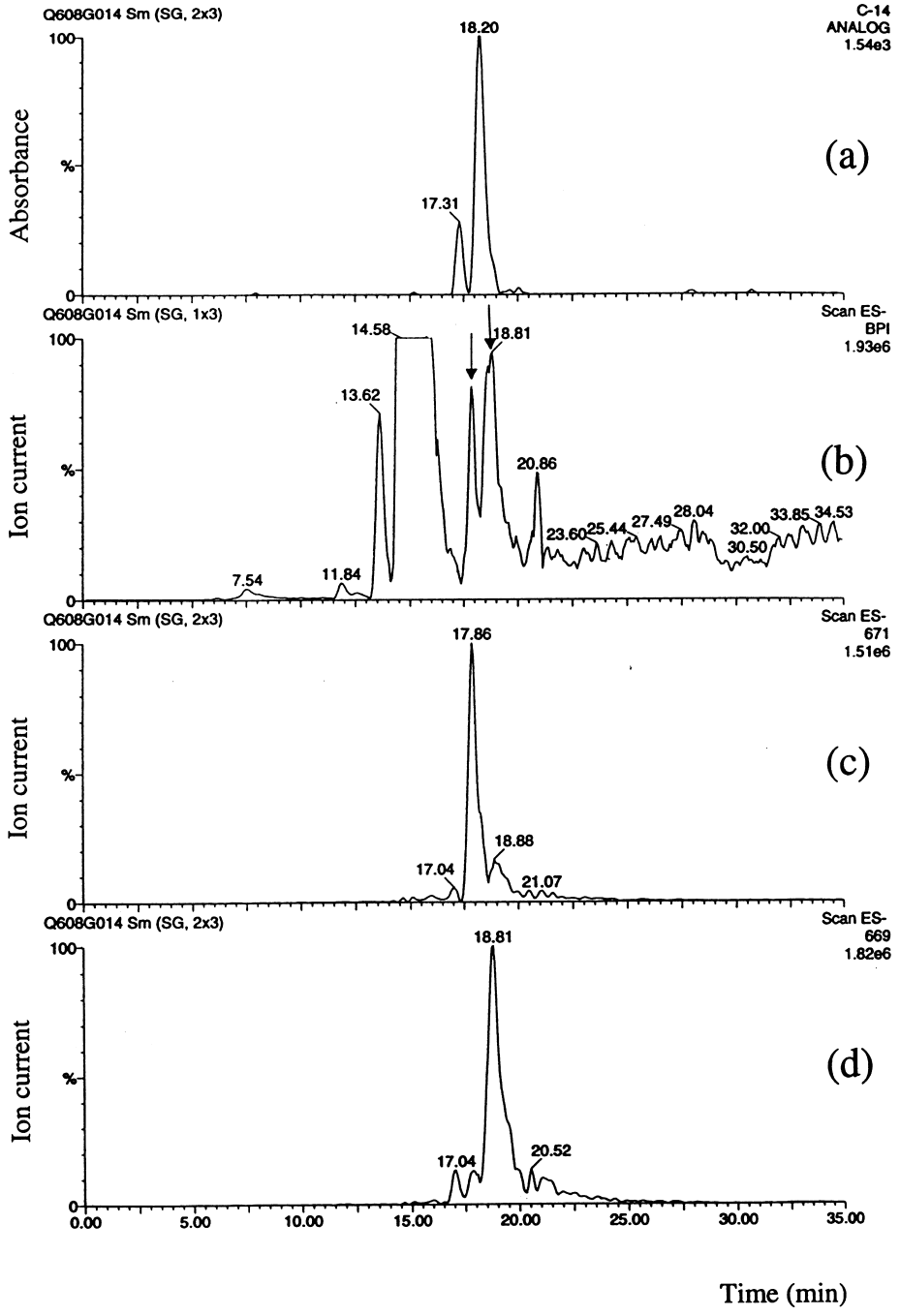

Fig. 4. LC–ESI-MS of the RPLC fraction containing the two diglucuronidated metabolites shown in Fig. 3d. (a) ^{14}C-trace (b) base peak intensity (BPI) (c) ion chromatogram of $[M-H]^-$ 671 Da and (d) ion chromatogram of $[M-H]^-$ 669 Da. The column used was a C18 Hypersil (1×250 mm). Both eluents contained 0.5% acetic acid with 10% and 90% acetonitrile for eluent A and B, respectively. Samples were eluted with a gradient from 10 to 80% B for 20 min, followed by isocratic elution for 15 min with 80% B and 5 min with 100% B. flow-rate: 30 μl /min. MS conditions, see text.

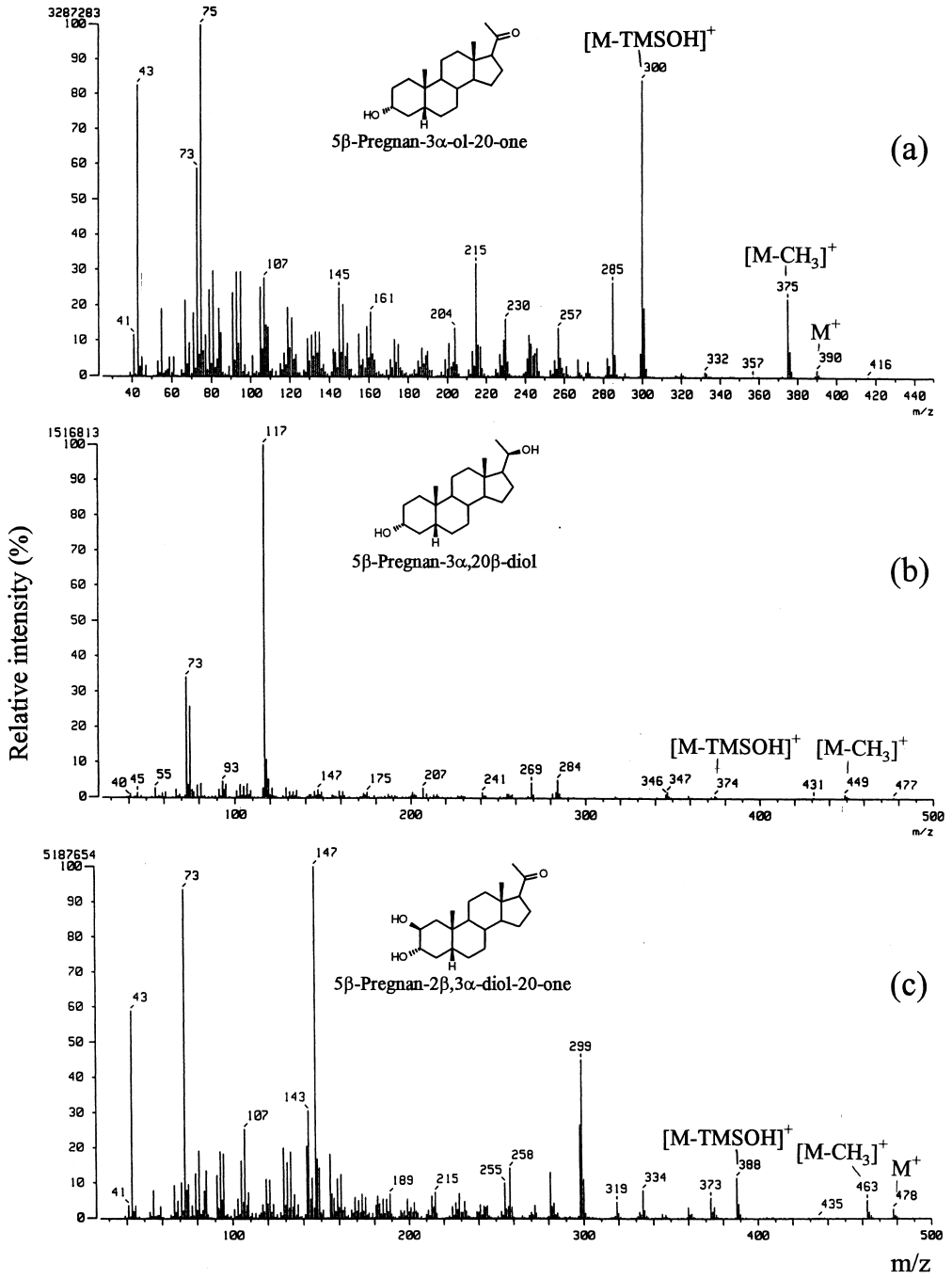

Fig. 5. GC–MS spectra of eltanolone and its main phase I metabolites found in dog urine. (a) eltanolone, (b) 5β-pregnan-3α,20β-diol, (c) 5β-pregnan-2β,3α-diol-20-one, (d) 5β-pregnan- 3α,6α-diol-20-one, (e) 5β-pregnan-3α,20α-diol, and (f) 5β-pregnan-2β,3α,20β-triol. A HP-1 (25 m×0.2 mm) column was used. The column temperature program was 140°C for 3 min, then raised to 280°C at a rate of 50°C/min. Injector temperature: 250°C. Mass spectra were recorded in the mass range m/z 25–600 at a cycle time of 1.5 s.

urine are shown in Fig. 5. Reference substances were available for all phase I metabolites except for one, which has only been tentatively identified. Isomers were distinguished from each other by the difference in their retention times on the GC column.

The main metabolic pathway for eltanolone in the

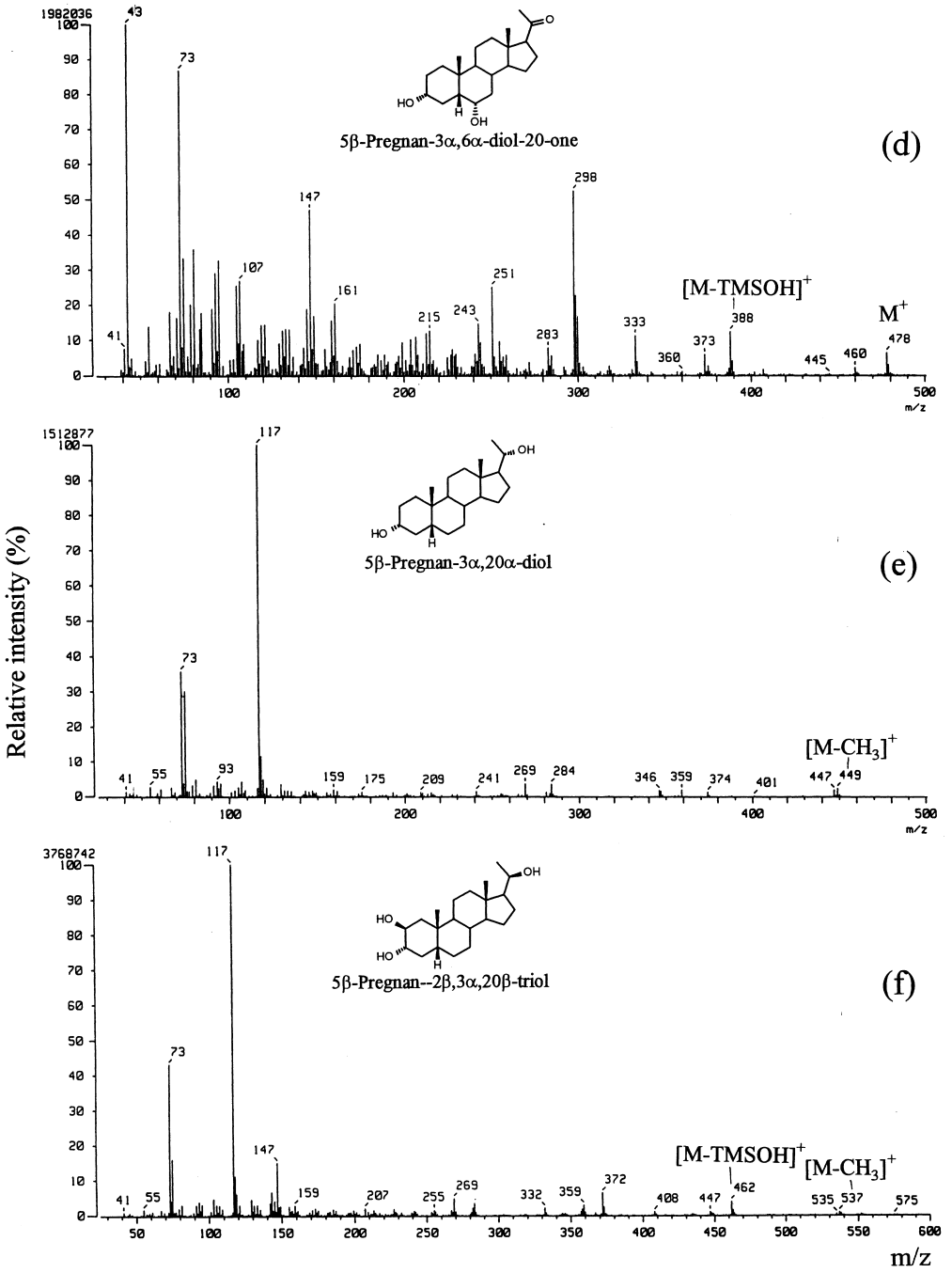

Fig. 5. (*continued*)

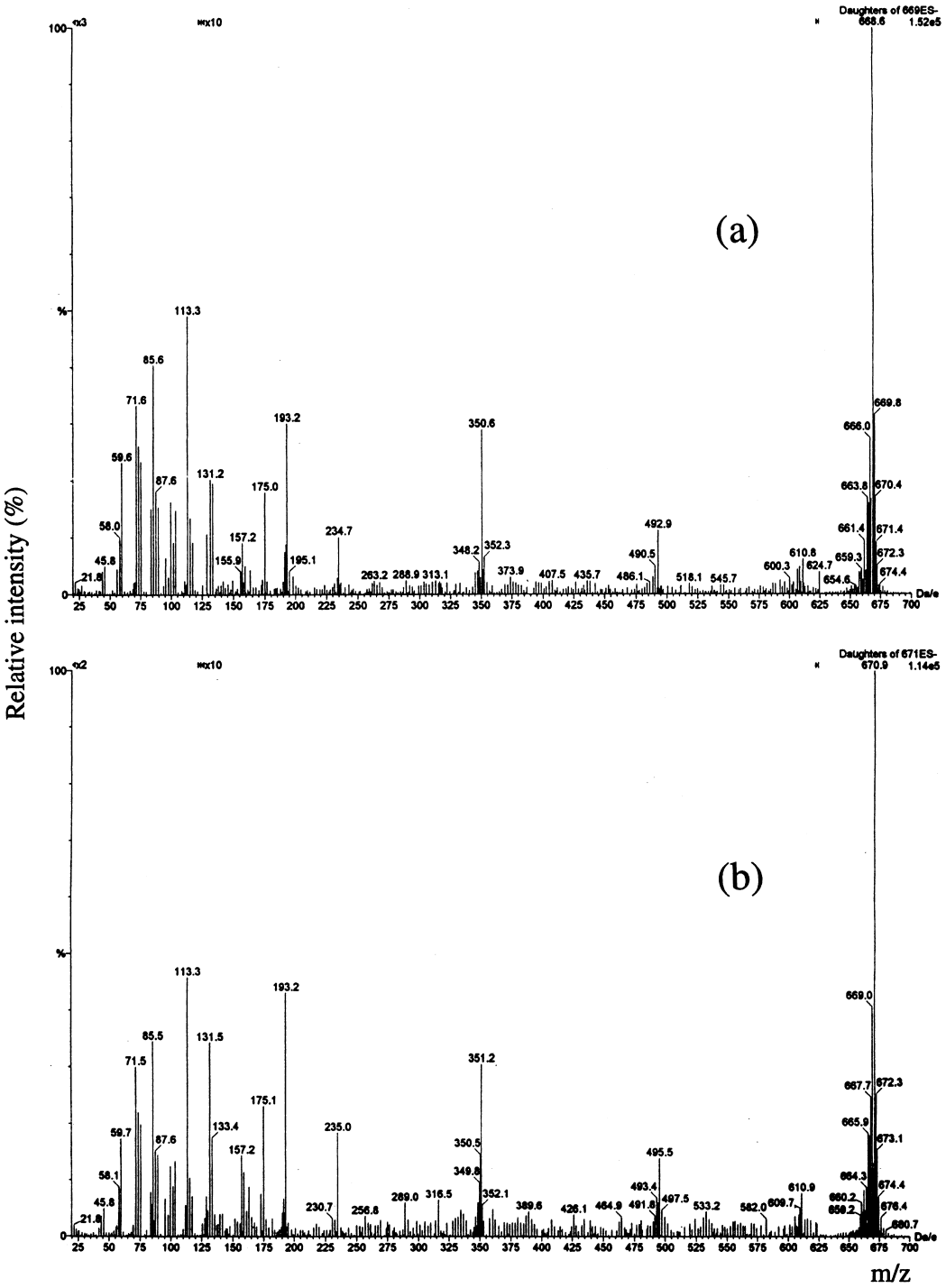

Fig. 6. Product ion spectra of (a) $[M-H]^-$ 669 Da, corresponding to diglucuronidated 'eltanolone' and (b) $[M-H]^-$ 671 Da, corresponding to diglucuronidated 5β-Pregnan-3α,20β-diol. HPLC conditions as in Fig. 4. MS conditions, see text.

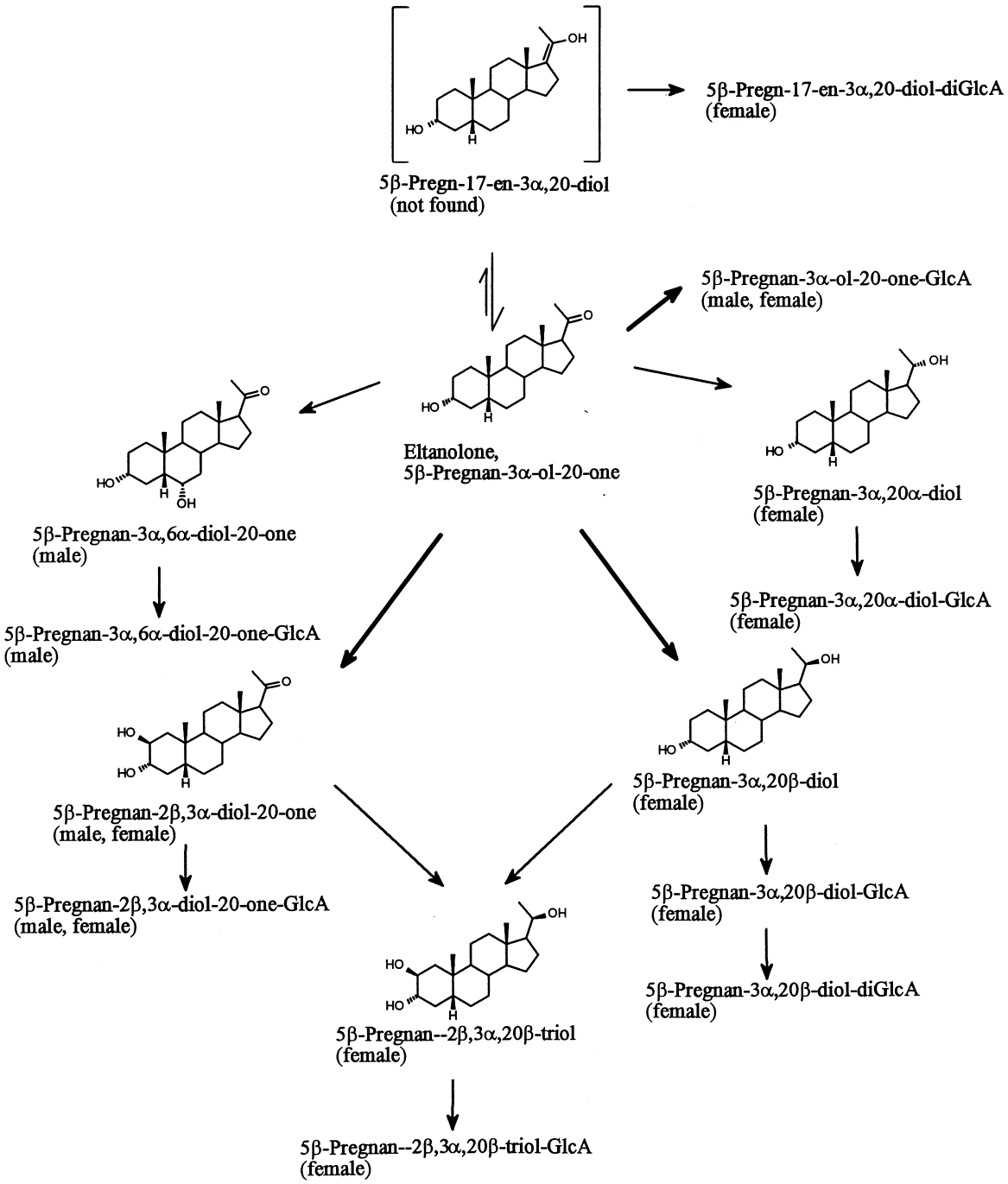

Fig. 7. Metabolic scheme for all major eltanolone metabolites found in dog urine including the proposed pathway for diglucuronidation of eltanolone forming the 5β-Pregn-17-ene-3α,20-diol-diglucuronide.

male dog urine was the oxidations in position C-2 and C-6. The major metabolites in the male dog were the 5β-pregnan-2β,3α-diol-20-one (Fig. 5c) and 5β-pregnan-3α,6α-diol-20-one (Fig. 5d), and their corresponding glucuronides. In the female dog urine, the main metabolic pathway was the reduction of the side chain carbonyl primary yielding the 20β-OH isomer and secondly the 20α-OH isomer. Oxidations occured as well, mainly in position C-2. The major metabolites in the female dog urine were the 5β-pregnan- 3α,20β-diol (Fig. 5b), 5β-pregnan-2β,3α-diol-20-one (Fig. 5c), 5β-pregnan-3α,20α-diol (Fig. 5e), 5β-pregnan-2β,3α,20β-triol (Fig. 5f), the last one only tentatively identified, and their corresponding glucuronides.

The spectrum of the tentatively identified metabolite 5β-pregnan-2β,3α,20β-triol (Fig. 5f), shows a small $[M-CH_3]^+$ ion at m/z 537 and a base peak at m/z 117, corresponding to a silylated reduced side-chain $[CH(OTMS)CH_3]^+$. Ions at m/z 462, 372, and 282/283 are formed from consecutive losses of two trimethylsilyl–OH and either another trimethylsilyl–OH or a trimethylsilyl–O radical. The loss of the trimethylsilyl–O radical indicates that a 2,3-diol has formed. This metabolite is probably due to a second metabolic hydroxylation of either 5β-pregnan-2β,3α-diol- 20-one or 5β-pregnan-3α,20β-diol or both. The structure is most likely the 5β-pregnan- 2β,3α,20β-triol.

Two components were seen in the GC–MS analysis of the hydrolysed SAX fraction containing the two diglucuronides. The largest was identified as eltanolone, indicating that the largest peak in the LC–ESI-MS chromatogram (Fig. 4a) really was diglucuronidated 'eltanolone'. The other peak was identified as 5β-Pregnane-3α,20β-diol, corresponding to the conjugated metabolite with a molecular mass of 672 Da. Diglucuronides were only found in female urine.

To confirm the unexpected finding of diglucuronidated 'eltanolone' the presence of the two diglucuronides in the female dog urine was also verified by LC–ESI-MS/MS. Product ion spectra of the $[M-H]^-$ ion 669 Da (Fig. 6a) and the $[M-H]^-$ ion 671 Da (Fig. 6b) showed fragments at m/z 493 Da and m/z 495 Da, respectively, corresponding to a loss of one glucuronic acid. The presence of the glucuronic acid was also confirmed by the ions $[Gluc-H]^-$ and $[Gluc-H-H_2O]^-$ at m/z 193 and 175 Da, respectively.

To establish the configuration of the two glucuronides on eltanolone, the subfraction from the RPLC was derivatized with semicarbazide. This reagent would form a semicarbazone with a free carbonyl group. An LC–ESI-MS spectrum of the derivatized subfraction showed that no semicarbazone was formed from the diglucuronidated eltanolone. This result confirmed that the glucuronidation involved the carbonyl group, but in its enolic form, i.e. 5β-Pregn-17-ene-3α-20α-diol or 5β-Pregn-17-ene-3α-20β-diol. Derivatization of another RPLC fraction containing the monoglucuronidated eltanolone with a free carbonyl, demonstrated that the derivatization had a yield of approximately 96%.

The metabolic scheme for all major eltanolone metabolites found in dog urine including the proposed pathway for diglucuronidation of eltanolone forming the 5β-Pregn-17-ene-3α-20-diol- diglucuronide is shown in Fig. 7.

4. Conclusion

Gradient elution used in several dimensions provided a large difference in selectivity, which allowed efficient isolation and identification of eltanolone metabolites in dog urine, with a minimum of separation optimization. The separation technique described has with individual modifications been proved useful in metabolism studies of eltanolone in rat, dog, monkey and man. The separation system is flexible, which is necessary as the degree of hydroxylation and conjugation of the metabolites varies between different species. This flexibility could make the technique applicable for metabolite studies of other steroids than eltanolone.

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