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Journal of Chromatography B, 833 (2006) 245–256
JOURNAL OF CHROMATOGRAPHY B
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Detection of altrenogest and its metabolites in post administration horse urine using liquid chromatography tandem mass spectrometry—increased sensitivity by chemical derivatisation of the glucuronic acid conjugate

Matilda Lampinen-Salomonsson [a], Elin Beckman [a], Ulf Bondesson [a,b], Mikael Hedeland [b,*]

[a] *Division of Analytical Pharmaceutical Chemistry, Department of Medicinal Chemistry, Uppsala University, Biomedical Centre, P.O. Box 574, SE-751 23 Uppsala, Sweden*
[b] *Department of Chemistry, National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden*

Received 23 December 2005; accepted 3 February 2006
Available online 7 March 2006

Abstract

Altrenogest (17α-allyl-17β-hydroxyestra-4,9,11-trien-3-one) is a steroid used for the control of estrus in horses. This drug can potentially be abused in racehorses as the occurrence of estrus can alter their performance. This work describes an analytical method based on liquid chromatography–tandem mass spectrometry for the detection of altrenogest in horse urine down to a concentration of 13 pg/mL (0.042 nM). Furthermore, the qualitative aspect of metabolism of altrenogest in the horse has been studied. The main transformations that were found for this species were conjugation with glucuronic acid and sulfate. These phase II metabolites were identified by molecular mass and by comparison of their collision-induced dissociation product ion spectra with that of the synthetic aglycone at positive and negative potential, respectively. No phase I metabolites were discovered. In order to increase the ionisation in positive electrospray, a derivatisation procedure forming a basic oxime was tested. This process significantly increased the detection sensitivity for altrenogest glucuronide in horse urine.

Keywords: Altrenogest; Horse urine; LC–MS/MS; Derivatisation; Metabolism; Glucuronide; Sulfate; Conjugate; Doping control

1. Introduction

Altrenogest (17α-allyl-17β-hydroxyestra-4,9,11-trien-3-one; Fig. 1) is a progestational drug, used primarily in mares to control the occurrence of estrus [1]. Its pharmacological effect is of great importance in breeding, but it can be misused in racehorses, since estrus often has a negative effect on the performance.

Within the rules of horseracing, it is stated that it is forbidden to compete with a horse that is under the influence of any kind of medication [2]. In order to maintain animal welfare, as well as fairness of competition and gambling, it is thus of outmost importance to have a safe and efficient control system that can detect prohibited administration of drugs. A finding of a prohibited substance is defined as the identification of either the drug itself in a biological fluid, a metabolite of the drug or an isomer of the substance [2]. This means that qualitative analytical methods are sufficient for the search for most drugs, including altrenogest, as there are no established threshold values.

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has developed during the past decade to be one of the most important techniques for bioanalysis of drugs [3]. However, when it comes to analysis of steroids, a problem that is often encountered is low sensitivity [4]. This is due to the fact that this class of substances generally lack basic or acidic functions, i.e., they are not easily ionisable in different kinds of LC–MS interfaces. In order to overcome this drawback, several derivatisation techniques have been applied for the purpose of facilitating ionisation by introduction of basic or acidic groups or quaternary ammonium functions [4–11].

To the very best of our knowledge, there are no publications on the detection of altrenogest or its metabolites in horse urine. The aim of the present study was thus to develop a qualitative analytical method based on LC–MS/MS for screening and identification of this substance and its metabolites in this matrix post administration. Furthermore, a chemical derivatisa-

* Corresponding author. Tel.: +46 18 674209; fax: +46 18 674099.
E-mail address: Mikael.Hedeland@sva.se (M. Hedeland).

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doi:10.1016/j.jchromb.2006.02.014

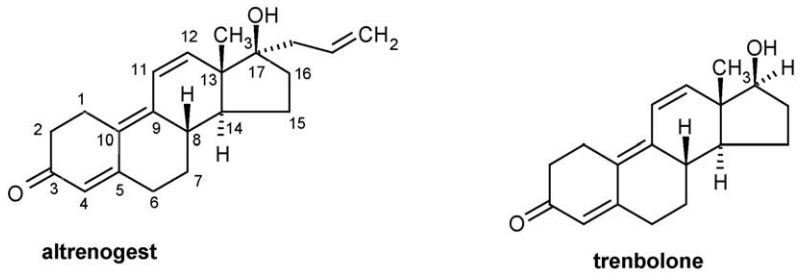

Fig. 1. Structures of altrenogest and trenbolone with carbon numbering of the steroid skeleton.

tion technique introducing a basic oxime group was tested for the purpose of improving the ionisation efficiency in the electrospray interface.

2. Experimental

2.1. Chemicals and reagents

Altrenogest was a gift from InterVet Pharma R&D (Beaucouzé, France). β-Glucuronidase (from *Escherichia coli* K12, according to the manufacturer's instructions, a specific activity towards 4-nitrophenyl-β-D-glucuronide of approx. 140 U/mg at 37 °C) was obtained from Roche (Mannheim, Germany). Protease (from Bovine Pancreas, according to the manufacturer, with 7.3 units/mg solid, with the definition that one unit will hydrolyse casein to produce colour equivalent to 1.0 μmol (181 μg) of tyrosine per min at pH 7.5 at 37 °C (colour by Folin-Ciocalteu reagent)), and trenbolone were purchased from Sigma (St. Louis, MO, USA). Hydroxyammonium chloride was obtained from Fluka (Buchs, Switzerland). Hexane, dichloromethane, formic acid and methanol were obtained from Merck (Darmstadt, Germany). Ammonium formate was obtained from BDH Laboratory Supplies (Poole, UK). All other chemicals were of analytical reagent grade and used without further purification. The water was purified using a Milli-Q-water purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of solutions

Altrenogest and trenbolone were weighed and dissolved in methanol, and for each new experiment fresh stock solutions were prepared. The stock solutions were used to prepare standards at different concentrations in methanol/water (1:1). The 2.0 mM ammonium formate buffer was prepared by dissolving ammonium formate (0.13 g in 1.00 L) in Milli-Q water and adjusting the pH to 3.4 with 10% formic acid.

2.3. Sample pre-treatment

2.3.1. Liquid–liquid extraction (LLE)

To 6.0 mL of horse urine (blank for addition of standards or collected from horses given altrenogest), 3.0 mL of 0.1 M potassium phosphate buffer (pH 6.05) was added to adjust pH to 6–6.5. One set of samples were hydrolysed by adding 130 μL of β-glucuronidase and 60 μL of protease to each extraction tube (incubation for 2 h at 50 °C). All samples were at room temperature before the organic phase used in LLE was added. As organic phase, 10 mL of hexane/dichloromethane (1:1) was used. This composition was chosen on the basis of an experiment with different compositions of hexane/dichloromethane. The mixture of urine and organic phase was put on a blender for 25 min and was centrifuged for 10 min at 3600 rpm, 20 °C. The organic layer was transferred to a new glass tube and was centrifuged for another 10 min at the same speed as before. Once again the upper layer was transferred to a new glass tube and the organic phase was evaporated to dryness at 50 °C. The residue was dissolved in 60 μL methanol/water (1:1) and was vortexed for 30 s. The solutions were transferred to vials and analysed using LC–MS/MS.

2.3.2. Solid-phase extraction (SPE)

In the search for phase II metabolites, a SPE method was used. To 6 mL of urine 2 g of ammonium sulphate was added and the sample was vortexed and centrifuged for 10 min at 3500 rpm. This was done to achieve a clean-up of the urine by salting out high molecular-weight compounds. The SPE column, Bond Elut C18 100 mg (Varian, Palo Alto, USA) was coupled to a vacuum manifold. The supernatant was decanted onto the SPE column (previously conditioned with 5 mL methanol and 2 × 5 mL water). The column was washed with 5 mL water and dried at −50 kPa for 5 min. The sample was eluted with 5 mL methanol and evaporated to dryness (65 °C) under nitrogen flow. The residue was dissolved in 200 μL methanol/water (1:1) and vortex shaken. The solution was transferred to vials and analysed using LC–MS/MS.

2.3.3. Derivatisation with hydroxyammonium chloride

The derivatisation procedure was slightly modified from a method developed by Liu et al. [7]. For the derivatisation, the dry extract was dissolved in 1.0 mL methanol (30%, v/v) in Milli-Q water and vortex shaken. The derivatisation reagent hydroxyammonium chloride (50 mg) was added to the tube which was vortex shaken and placed in a heating block at 70 °C for 60 min. The sample was evaporated (65 °C) practically to dryness under nitrogen flow. In order to remove excess reagent and to avoid interferences with the analytical procedure, a liquid–liquid washing step was included. Milli-Q water (200 μL) and 800 μL organic phase (isopropanol (15%, v/v) in dichloromethane) were added and the sample was shaken for 5 min and centrifuged for 5 min at 3500 rpm. The organic

phase was transferred to a new tube and evaporated to dryness under nitrogen flow. The residue was dissolved in 200 μL methanol/water (1:1) and vortex shaken. The solution was transferred to vials and analysed using LC–MS/MS.

2.4. LC–MS/MS system and chromatographic conditions

A Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Electron Corporation, San José, CA, USA), with a Surveyor MS pump carried out the LC–MS/MS analyses. A gradient program (Table 1) controlled the flow rate (300–400 μL/min) and the composition of the mobile phase consisting of ammonium formate buffer (2.0 mM, pH 3.4) and methanol. The separation column was a Luna C_8 50 mm × 2.00 mm (length × I.D., particle diameter 5 μm) equipped with a guard column, C_{18} packed with a filter, 4.0 mm × 2.0 mm (length × I.D.) (Phenomenex, Torrance, CA, USA). The injector had a sample loop of 10 μL. A divert valve was used for 1 min to minimize the introduction of impurities into the mass spectrometer. The ionisation technique was electrospray (ESI) in positive or negative mode. Syringe pump infusion of a standard working solution of altrenogest (1.57 μg/mL = 5.0 μM) dissolved in methanol in a T coupling with the LC pump flow of 300 μL/min was used for tuning. The ESI source voltage was set at 3.5 kV and sheath gas flow rate and auxiliary gas were 50 and 2 arbitrary units, respectively. The Q1 and Q3 peak widths were set to 0.7 FWHM and the scan time was 1 s. For full MS^1 the scan range was varied in between *m/z* 50 and 700. When running collision-induced dissociation (CID), argon was used as the collision gas at a pressure of 1.5 mTorr. For Selected Reaction Monitoring (SRM) mode, the following transitions were recorded: altrenogest *m/z* 311 $[M+H]^+ \rightarrow$ *m/z* 227, altrenogest oxime *m/z* 326 $[M+H]^+ \rightarrow$ *m/z* 225 and altrenogest glucuronide *m/z* 487 $[M+H]^+ \rightarrow$ *m/z* 311 using collision energy 30, 27 and 30 V, respectively. Signal-to-noise ratios in SRM mass chromatograms were calculated using the software Xcalibur v.1.3 with the peak identification program Genesis. The signal was base-line corrected and the noise was calculated as the residual difference from a linear regression line of the base-line noise.

2.5. Validation of the qualitative method

To determine the recovery of the LLE method, unhydrolysed and hydrolysed extracted samples (with the concentration 0.54 ng/mL altrenogest in blank horse urine = 1.7 nM) were compared to calibration standard solutions (altrenogest dissolved in methanol/water) in a calibration curve, using LC–MS/MS in positive SRM mode.

A pool of urine was spiked with altrenogest to a concentration of 1.0 μg/mL (3.2 μM). This pool was divided into six aliquots and used to determine the repeatability in peak area. Three of these were hydrolysed, extracted and analysed on day 1 with the described LLE–LC–MS/MS method, and the remaining three were hydrolysed, extracted and analysed on day 2, after storage in the freezer.

In order to determine the stability of altrenogest in the extracts, six hydrolysed samples (1.0 μg/mL altrenogest in urine) were prepared using the LLE method. The dried extracts were dissolved in methanol/water (1:1). Three of these solutions were put in vials that were left on the bench at room temperature and three were put in vials placed in the freezer. The LC–MS/MS analysis was performed 5 days later.

Table 1
The LC gradient program

Time (min)	Flow (μL/min)	Buffer (%)	Methanol (%)
0	300	40	60
4.00	300	40	60
8.00	300	10	90
8.10	300	40	60
8.20	400	40	60
10.40	400	40	60
10.50	300	40	60

2.6. Administration of altrenogest to horses and ethic permission

Regu-Mate® (altrenogest 0.044 mg/kg bodyweight) was given orally together with food to three different horses (standardbred mares) for 10 consecutive days. Urine samples were taken on different occasions during the administration period. The study protocol was approved by the Local Ethics Committee for Animal Experiments, Uppsala, Sweden.

3. Results and discussion

3.1. LC–MS/MS of altrenogest and trenbolone

The electrospray-MS/MS characteristics of altrenogest were investigated in order to select appropriate conditions for high sensitivity screening as well as to obtain a reliable reference product ion spectrum for unequivocal identification of the substance in biological samples. Furthermore, to assist the elucidation of the fragment structures, the closely related steroid trenbolone (Fig. 1) has been studied as well. The positive ion MS^1 spectra were dominated by $[M+H]^+$. Full product ion spectra in positive mode of $[M+H]^+$ of altrenogest and trenbolone, together with suggested cleavages are shown in Fig. 2. Both were in agreement with previous reports [12]. Both altrenogest and trenbolone initially lose 18 u (H_2O), giving the fragments *m/z* 293 and 253, respectively. One fragment that was characteristic for altrenogest was *m/z* 269, which originated from the loss of propene from the C-17 position in the molecular structure. The major fragment ion for the respective compound, i.e., *m/z* 227 for altrenogest and *m/z* 199 for trenbolone, also existed in the spectrum of the other steroid. This indicated that these ions were formed by the loss of C-17, as the substituent in this position gives the only structural difference between the two compounds. It has earlier been suggested that *m/z* 227 was a primary fragment from the protonated molecule and that *m/z* 199 was secondarily formed by the subsequent neutral loss of ethane (28 u) involvning a methyl migration [12]. Apart from the above-discussed ions, both spectra displayed a low mass end rich in fragments. There were no obvious differences between the compounds in

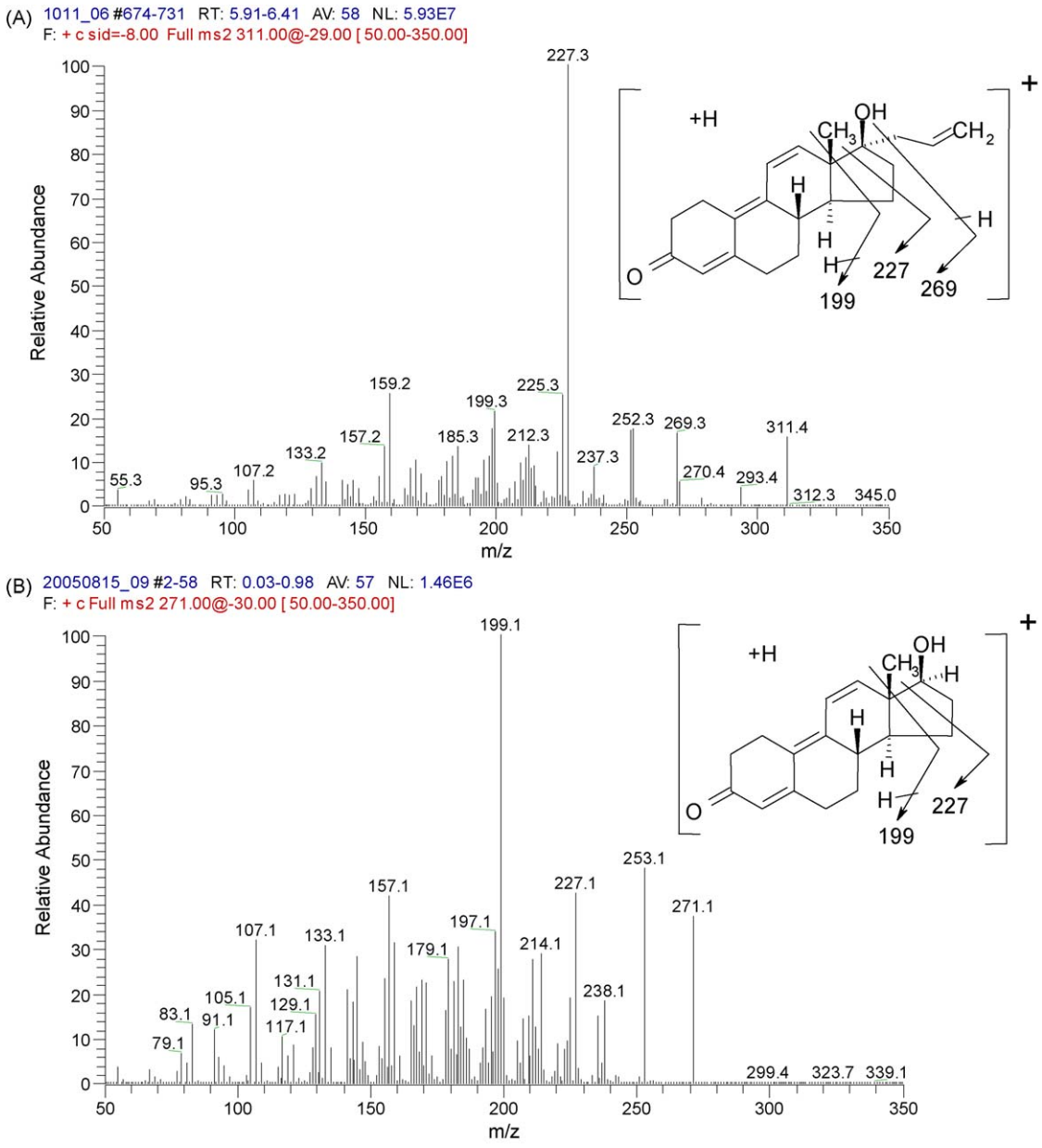

Fig. 2. (A) Positive product ion spectrum and suggested cleavage sites of altrenogest, precursor $[M+H]^+$ *m/z* 311, collision energy 29 V. (B) Positive product ion spectrum and suggested cleavage sites of trenbolone, precursor $[M+H]^+$ *m/z* 271, collision energy 30 V.

this region, suggesting that the obtained fragments were contained in the A and/or B rings of the steroids.

The negative ion MS^1 spectra were dominated by $[M-H]^-$. The MS/MS fragmentation of $[M-H]^-$ showed similarities as well as differences compared with positive mode (Fig. 3). Neutral losses of water (18 u) also occurred in negative mode for both compounds, giving *m/z* 291 and 251 for altrenogest and trenbolone, respectively. Furthermore, altrenogest lost 42 u (propene) from $[M-H]^-$ to yield the characteristic fragment *m/z* 267. The product ion spectrum for trenbolone contained the ion *m/z* 241 (loss of 28 u from *m/z* 269), which corresponds to the loss of CO, but there were no signs of a corresponding loss for altrenogest. All the other major fragment ions, *m/z* 225, 213, 211, 197 and 171, were produced from both compounds, indicating that they did not contain C-17 (see Fig. 3 for suggested cleavages). Loss of ethane (28 u) from *m/z* 225 gave rise to the fragment *m/z* 197, in the same manner as *m/z* 227 → *m/z* 199 in positive ion mode. A fragmentation that was not obvious in positive mode was the formation of *m/z* 211/213. These fragments were probably produced by loss of the D ring with and without formation of an unsaturation between C-13 and C-14. Another cleavage only observed in the negative mode was the formation of *m/z* 171, corresponding to a dissociation of the C ring.

The electrospray sensitivity, using full scan MS^1, was higher for both compounds in positive than in negative mode in accordance with previous reports [13]. This fact has been ascribed to a relatively high proton affinity of the 3-keto function, caused by the large conjugated π-electron system of 4,9,11-triene

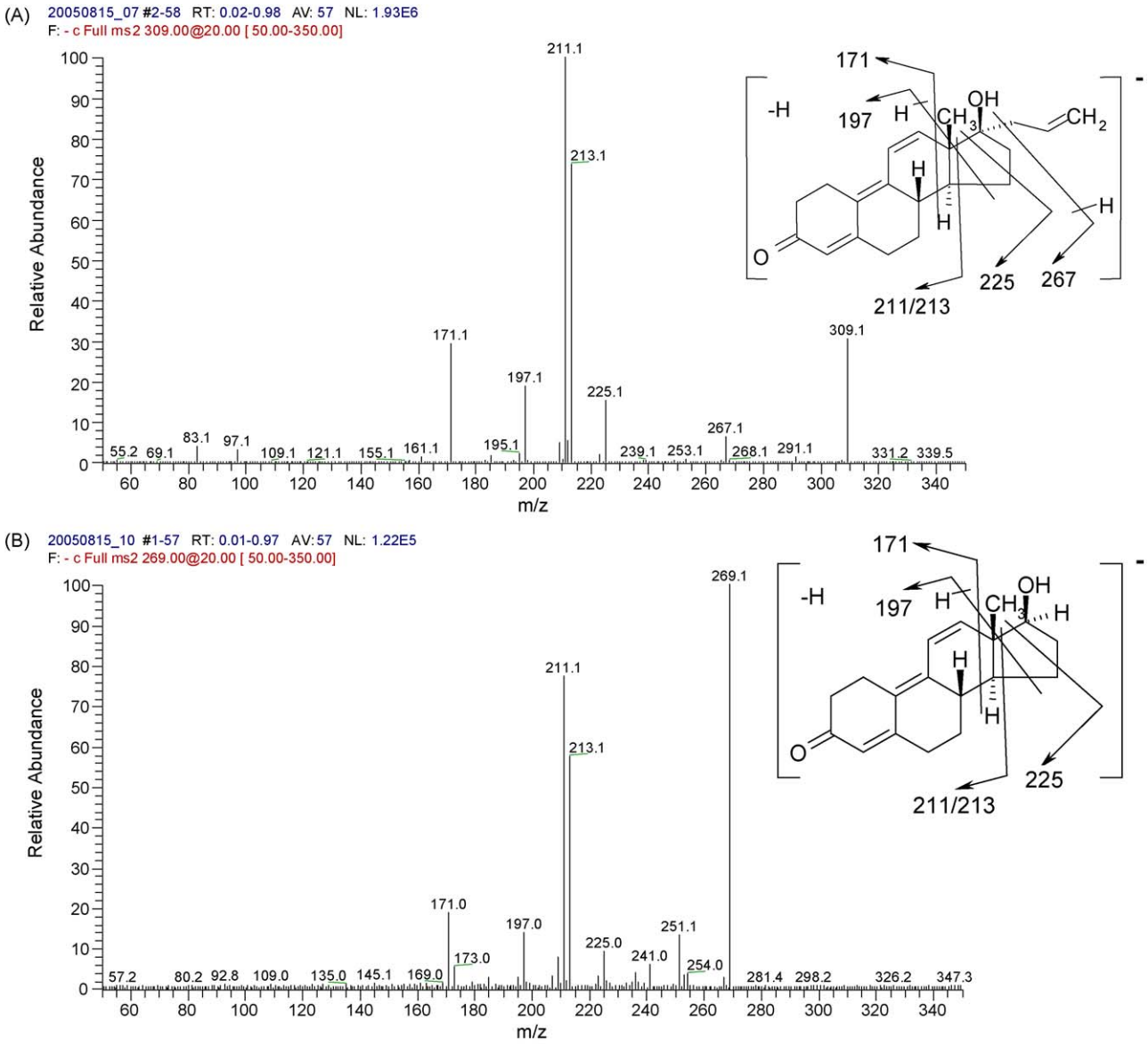

Fig. 3. (A) Negative product ion spectrum and suggested cleavage sites of altrenogest, precursor $[M-H]^-$ *m/z* 309, collision energy 20 V. (B) Negative product ion spectrum and suggested cleavage sites of trenbolone, precursor $[M-H]^-$ *m/z* 269, collision energy 20 V.

steroids, which is believed to stabilise the protonated form. On the basis of the above-mentioned results, an LC-positive–ESI-MS/MS screening procedure could be developed for altrenogest in horse urine samples, using SRM with the transition *m/z* 311 $[M+H]^+ \rightarrow$ *m/z* 227. Furthermore, the product ion spectrum was selective enough to be used as a fingerprint for unambiguous identification of the compound.

3.2. Validation of a qualitative method for altrenogest in horse urine

A liquid–liquid extraction (LLE) method was applied for pre-treatment of horse urine samples in the qualitative analysis of altrenogest. An organic phase based on mixtures of different proportions of dichloromethane and hexane was evaluated. A slight increase in extraction recovery was observed in the range of 0–50% of dichloromethane, whereas no significant change was observed at higher proportions of this solvent. A mixture of 50/50 (v/v) dichloromethane/hexane was chosen for the rest of the experiments. It gave a recovery of 72%, which was considered enough for qualitative testing of altrenogest misuse in the horse.

Altrenogest could be detected post administration in horse urine using this extraction method and analysis with LC–MS/MS in the SRM mode (Fig. 4A). However, the peak area for the same sample increased about 400 times if the sample was treated with β-glucuronidase prior to extraction (Fig. 4B). This finding indicated that a great part of altrenogest was renally excreted in a conjugated form and that hydrolysis would be an important step in a screening method for this substance. In the validation process, it was found that treatment with β-glucuronidase on urine spiked with altrenogest caused a 50% drop in signal intensity.

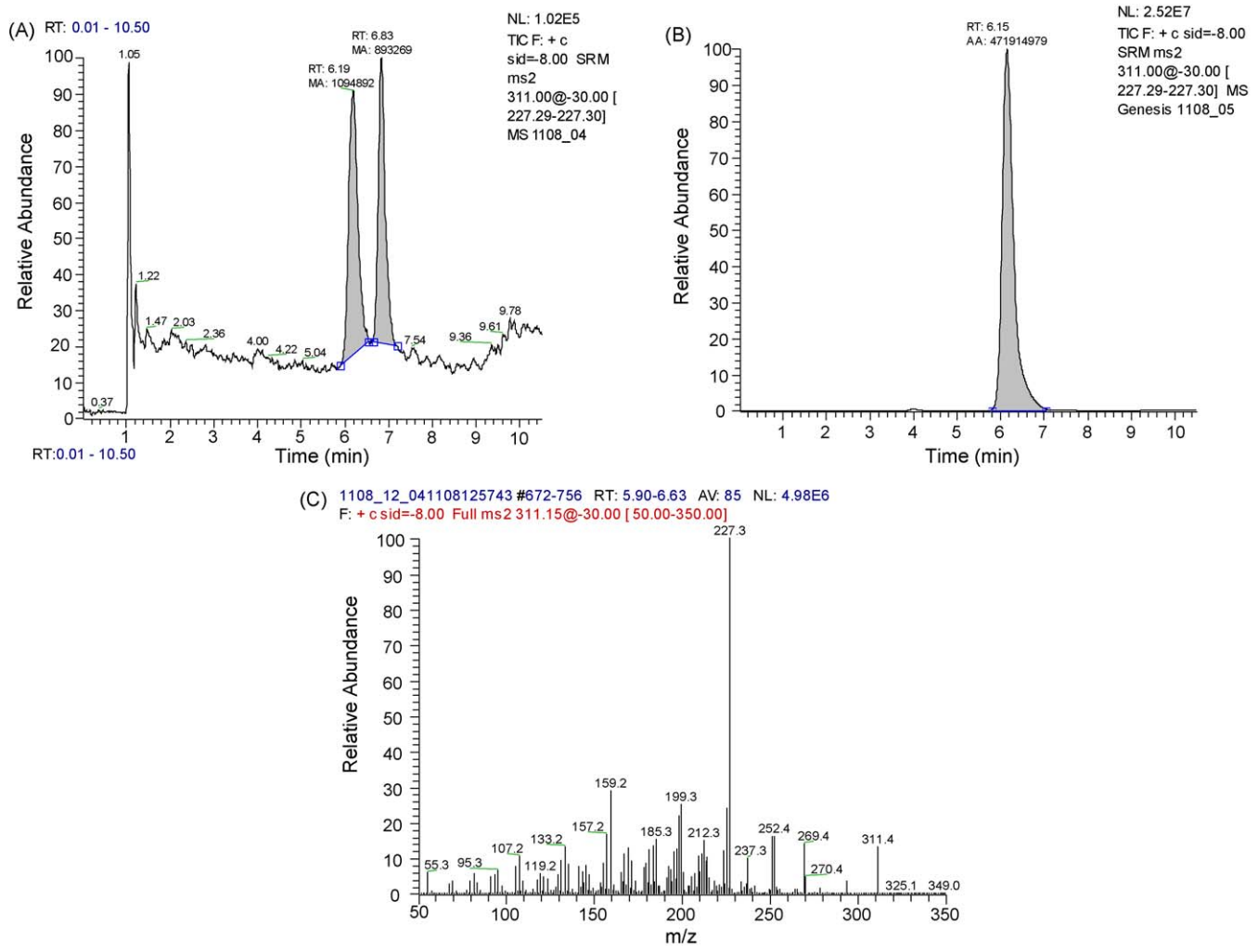

Fig. 4. (A) Mass chromatogram from an LC–MS/MS analysis in the SRM mode (transition *m/z* 311 → 227) of an LLE extract of unhydrolysed horse urine (sample taken on the 8th day of Regu-Mate administration). The peak corresponding to altrenogest eluted at 6.2 min. (B) Mass chromatogram from an LC–MS/MS analysis in the SRM mode (transition *m/z* 311 → 227) of the same sample as in the top chomatogram after β-glucuronidase hydrolysis. (C) Full product ion scan of the peak at 6.2 min (precursor ion *m/z* 311) after β-glucuronidase hydrolysis (collision energy 30 V).

This phenomenon might be due to increased ion suppression by hydrolysed matrix components. However, the net gain in sensitivity by hydrolysis of real samples greatly compensated for this effect. A product ion spectrum good enough for confirmatory purposes could also be obtained (Fig. 4C; cf. Fig. 2A).

The sensitivity of the method was evaluated by spiking blank horse urine with different amounts of altrenogest. A level of 13 pg/mL altrenogest (0.042 nM) added to urine gave a signal-to-noise ratio of about 10 with analysis in the SRM mode. It is however difficult to establish an absolute limit of detection (LOD) in this type of matrix as horse urine from different individuals varies considerably regarding analytical interferences. This sensitivity was better than those previously reported using LC–MS/MS for trenbolone and other steroids in horse [14] or cow urine [15–17].

Evaluation of the repeatability of a qualitative method is also of interest, since a great variation in extraction recovery and mass spectrometric performance from sample to sample also means a variation in the LOD. Six portions of horse urine were spiked with altrenogest at a level of 1.0 μg/mL and three of these were hydrolysed, extracted and analysed in parallel per day. The within-day relative standard deviations in peak area were only 6.0% on day 1 and 1.9% on day 2.

Another parameter that is practically important for a routine method is the stability of the substance in the extract prior to analysis. The peak area obtained from an extract that had been left at room temperature for 5 days was only about 3% of the area from an identical extract kept in the freezer for the same period of time. Thus, the vials should be frozen if there is a delay between extraction and LC–MS/MS analysis.

In summary, these validation results showed that the developed method is fit for use for screening for altrenogest abuse in horse urine by LC–MS/MS in the SRM mode and that confirmations can be performed using full scan product ion mass spectrometry.

3.3. Metabolism of altrenogest in the horse

There were no signs of phase I metabolites of altrenogest in the tested urines after treatment with β-glucuronidase, LLE

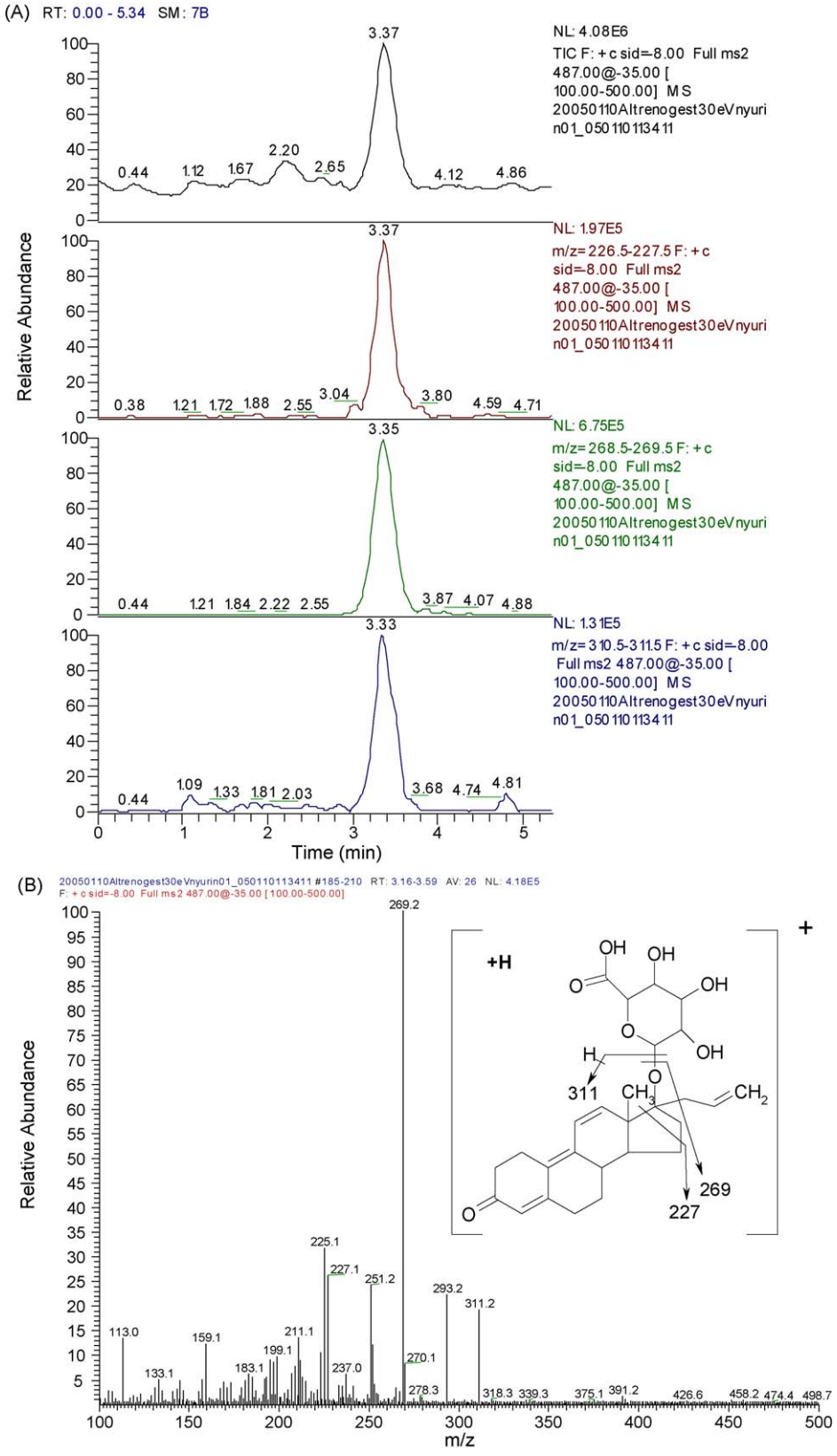

Fig. 5. (A) Extracted ion chromatograms of a directly injected urine sample taken on the 8th day of Regu-Mate administration. Full scan product ion, precursor *m/z* 487, collision energy 35 V. (B) Full scan product ion spectrum of the peak eluting at 3.37 min.

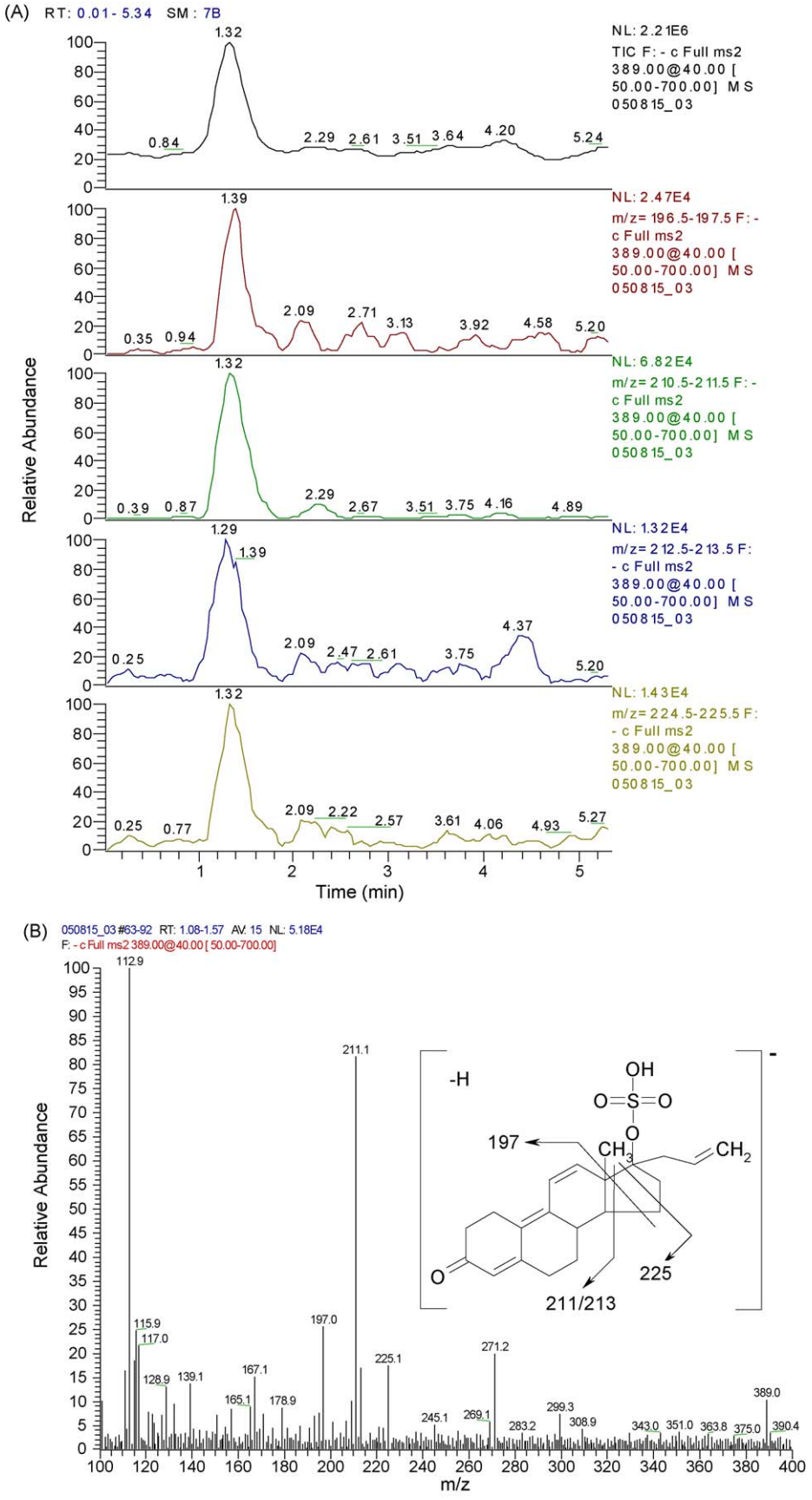

Fig. 6. (A) Extracted ion chromatograms of significant peaks of altrenogest in negative mode. Full scan product ion, precursor ion *m/z* 389 corresponding to altrenogest sulfate collision energy 40 V. Sample taken on the 5th day of Regu-Mate administration. (B) Full scan product ion spectrum of the peak eluting at 1.3 min.

and analysis with LC–MS and LC–MS/MS. The structurally similar trenbolone has earlier been found have a quite differing metabolic pattern in different species [18–20]. Hydroxylation in the 16-position and oxidation of the 17β-hydroxyl to a ketone were the major pathways in rats, and stereo inversion of the 17-hydroxy group (β → α) was the main metabolic reaction in cows. In both species, the major part of the administered dose was excreted as glucuronides and sulfates in the bile. In humans, trenbolone was found to be both directly conjugated and metabolised to the 17α-hydroxy form prior to conjugation with glucuronid acid [21]. A similar metabolic pattern has also been described in the horse, with the major part stereo inverted followed by glucuronidation and a minor part directly sulfated [22,23]. To the best of our knowledge, there are no reports on the metabolism of altrenogest in horse or any other species. The mechanism of stereo inversion suggested for trenbolone was believed to involve an initial oxidation to a 17-keto compound and a subsequent reduction to 17α-trenbolone [18]. This route of reactions would not be possible for altrenogest as the 17-allyl group prevents the formation of a 17-keto intermediate, a fact that might explain the lack of this type of phase I metabolites for this steroid.

A glucuronide conjugate of altrenogest could be detected in horse urine after administration of altrenogest, as expected from the results with β-glucuronidase treatment. The sensitivity was not high enough to detect the protonated molecular ion *m/z* 487 ($[M+H]^+$) directly from an MS^1 scan experiment, but identification by CID could be performed in both directly injected urine and SPE extracts. The product ion spectrum of the precursor ion *m/z* 487 from the suspected glucuronide was compared with the results obtained from the altrenogest standard (Fig. 5; cf. Fig. 2A). After the classical neutral loss of 176 u, giving *m/z* 311, the remaining major *m/z* values were in agreement with those found for altrenogest, a fact that strongly suggested the presence of altrenogest glucuronide. The sensitivity for the glucuronic acid conjugate was lower in negative electrospray mode, although an acidic group had been introduced into the molecule.

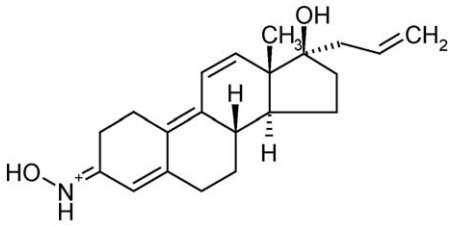

Fig. 7. Structure of protonated altrenogest oxime.

Searches for a sulfate conjugate were performed both in negative and in positive mode. In negative electrospray, there was a peak with *m/z* 389, corresponding to $[M-H]^-$ of altrenogest

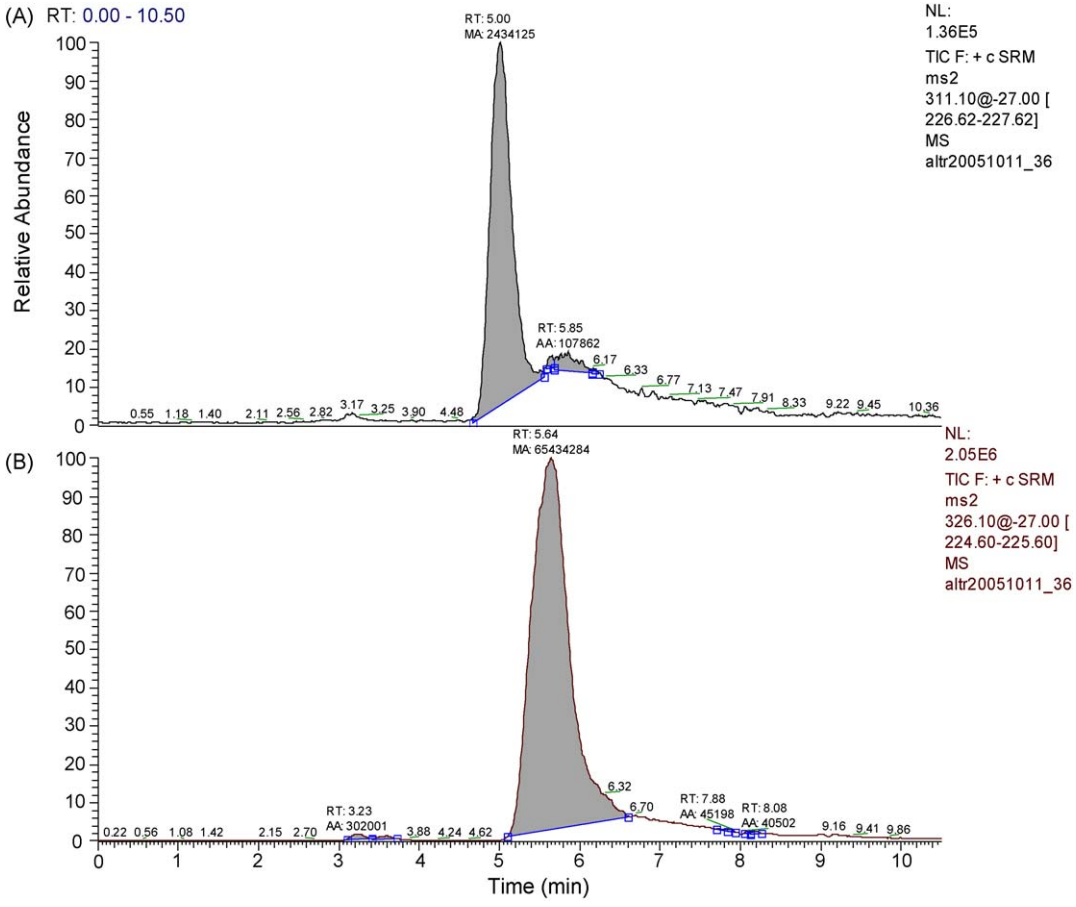

Fig. 8. Mass chromatograms (SRM mode) from an extract of horse urine spiked with altrenogest at 3.3 μg/mL (10.6 μM) and treated with hydroxyammonium chloride. (A) Remaining underivatized altrenogest (transition *m/z* 311 → 227). (B) Altrenogest oxime (transition *m/z* 326 → 225) collected in the same run.

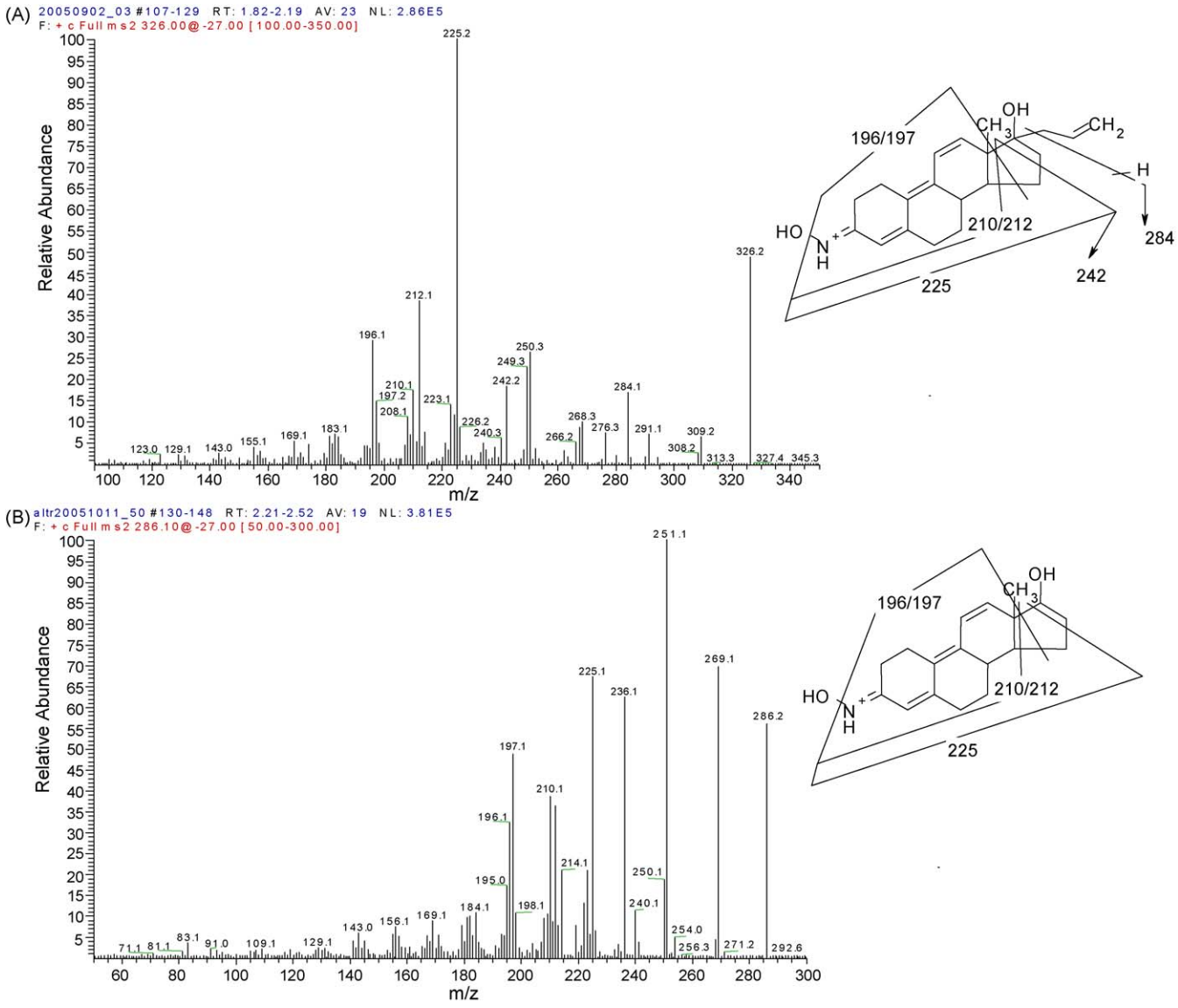

Fig. 9. (A) Positive product ion spectrum and suggested cleavage sites of altrenogest oxime, precursor $[M+H]^+$ *m/z* 326, collision energy 27 V. (B) Positive product ion spectrum and suggested cleavage sites of trenbolone oxime, precursor $[M+H]^+$ *m/z* 286, collision energy 27 V.

sulfate, giving several MS/MS fragments with the same *m/z* as the altrenogest standard, the most important being *m/z* 225, 213, 211 and 197 (Fig. 6; cf. Fig. 3A). The classical neutral loss of 80 u, giving *m/z* 309, was not very obvious in the spectrum, but the good match of the other product ions gave enough evidence to draw the conclusion of the presence of a sulfate conjugate of altrenogest. There were no obvious signs of this metabolite in positive electrospray, probably due the stronger acidity of the sulfate group compared to the carboxylic acid of the glucuronide.

3.4. Chemical derivatisation as a means of increasing ESI sensitivity

The possibility of increasing the electrospray sensitivity of altrenogest and its phase II metabolites by introduction of an alkaline function was investigated. The aim was to produce an oxime (Fig. 7) using the well-known reagent hydroxyammonium chloride [7]. Derivatisation of a standard solution of altrenogest gave the expected product, *m/z* 326 $[M+H]^+$. The absolute yield of the reaction in an extract of spiked horse urine was difficult to calculate as the difference in response between altrenogest oxime and native altrenogest was unknown. However, the peak area for native altrenogest was only about 4% of the peak for the derivative (Fig. 8), indicating that the majority of altrenogest molecules had been transformed to the oxime.

Collision-induced dissociation of altrenogest oxime gave the product ion spectrum shown in Fig. 9A. In order to simplify the interpretation of the fragmentation pattern, the corresponding trenbolone oxime was also produced (Fig. 9B). Both compounds gave peaks corresponding to the loss of an OH radical (17 u) from $[M+H]^+$ (*m/z* 309 and 269 for altrenogest and trenbolone, respectively). This loss has earlier been reported for steroid oximes [7]. A further loss of H_2O from C-17, gave the fragments *m/z* 291 and 251. A major peak in the mass spec-

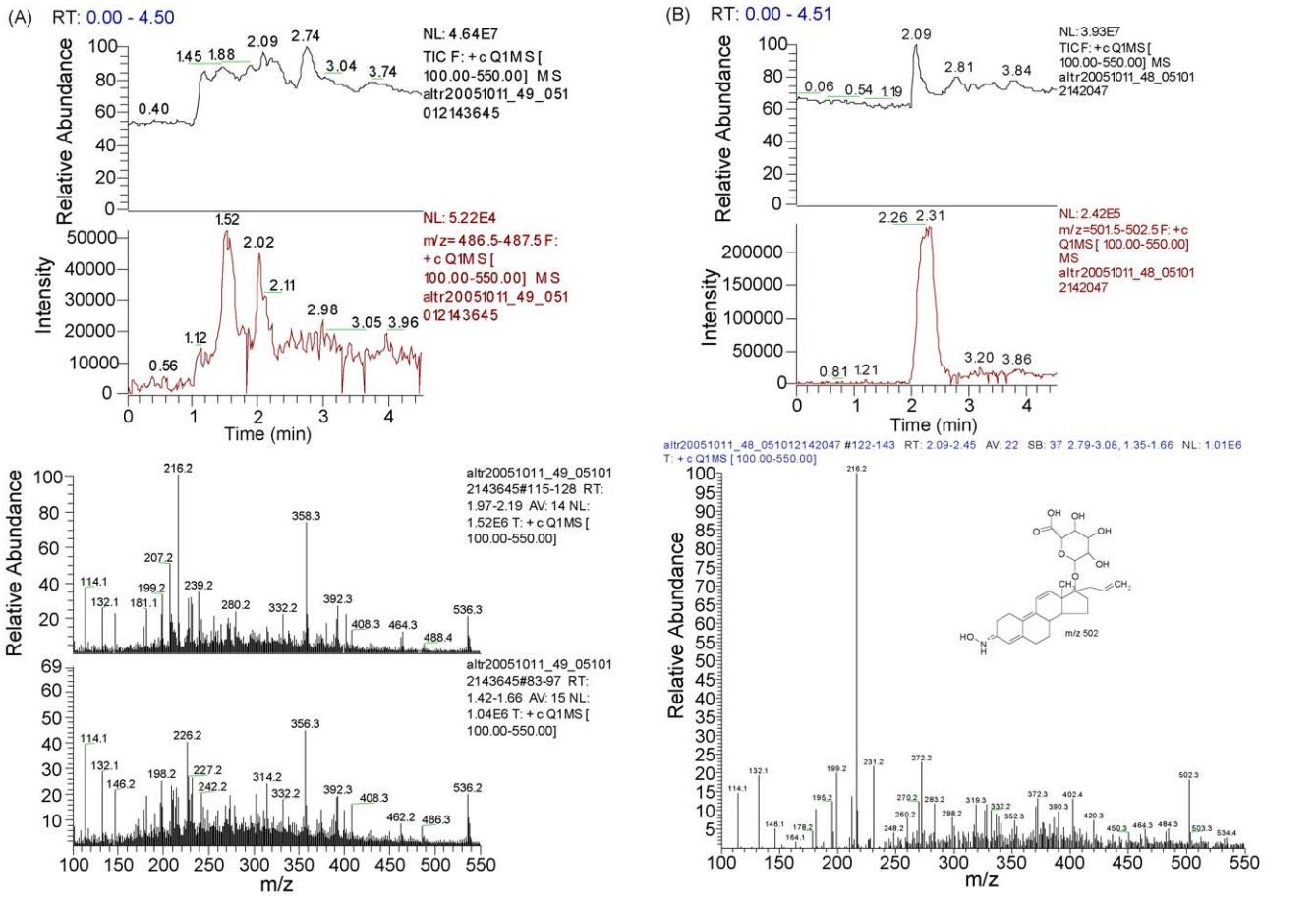

Fig. 10. (A) Positive full scan MS^1 of an SPE extract of unhydrolysed urine (sample taken on the 5th day of Regu-Mate administration) not treated with derivatisation reagent. Extracted ion chromatogram of *m/z* 487 corresponding to $[M+H]^+$ of altrenogest glucuronide and mass spectra at 1.5 and 2.0 min, respectively. (B) Positive full scan MS^1 of the same extract treated with hydroxyammonium chloride. Extracted ion chromatogram of *m/z* 502 corresponding to $[M+H]^+$ of altrenogest glucuronide oxime and mass spectrum at 2.3 min.

trum for trenbolone oxime was *m/z* 236, a loss of 15 ($CH_3^\bullet$) from *m/z* 251. This type of fragmentation was also observed for altrenogest as *m/z* 276 (loss of 15 from *m/z* 291). The subsequent loss of a methyl radical from the $[M+H–OH]^{\bullet+}$ has earlier been observed for testosterone oxime [7]. Release of alkyl radicals has been described for underivatised 4,9,11-triene steroids, where the large conjugated π-electron system has been believed to stabilise the formed cations [12]. The fragment *m/z* 242 for altrenogest, which was not seen for trenbolone, was probably formed from the same pathway as *m/z* 227 for the underivatized ones (see Fig. 9 for suggested cleavage). *m/z* 225, a fragment formed from both steroids, corresponds to same cleavage pre-

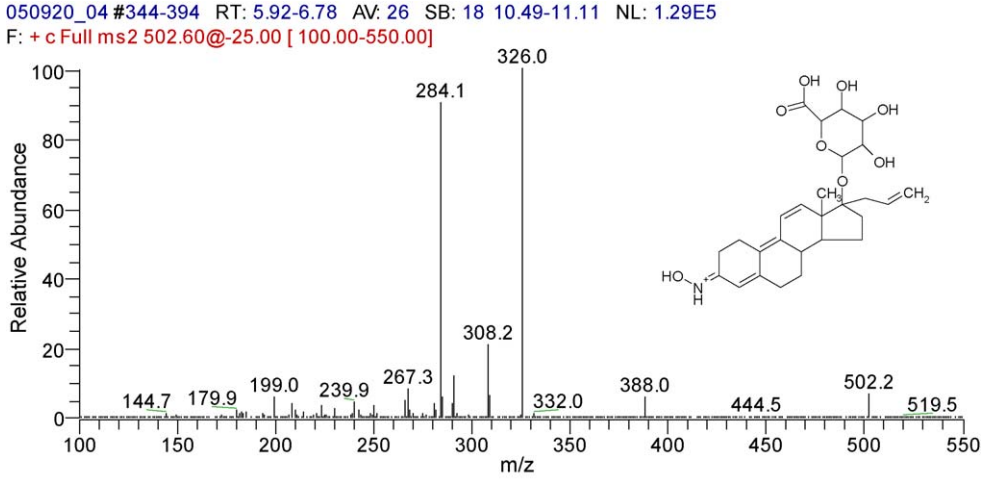

Fig. 11. Full scan product ion spectrum of altrenogest glucuronide oxime $[M+H]^+$ (precursor ion *m/z* 502) collision energy 25 V.

ceded by the loss of the OH radical. Further dissociations of the D ring probably gave rise to the fragments *m/z* 212 and 210, differing in one unsaturation and *m/z* 197/196. The ions with an even *m/z* must be even electron ions according to the nitrogen rule, unless the nitrogen had been lost. One characteristic fragment for altrenogest was *m/z* 284, corresponding to a neutral loss of propene (42 u) from $[M+H]^+$.

The detection sensitivity of altrenogest oxime in horse urine was investigated using SRM with the transition *m/z* 326 $[M+H]^+ \rightarrow$ *m/z* 225. However, there was no apparent gain in sensitivity compared with underivatized altrenogest. It could not be elucidated whether this was a result of losses in the derivatisation/clean-up process or whether it was impossible to increase the electrospray ionisation efficiency of the high proton affinity substance altrenogest. An increase in ion suppression caused by the derivatisation procedure might also be a contributing factor.

In order to investigate how this derivatisation procedure affected phase II metabolites, an extract of an SPE process was also subjected to reaction with hydroxyammonium chloride. There were no signs of an altrenogest sulfate oxime in positive ESI, but interestingly, a peak corresponding to altrenogest glucuronide oxime was easily detected and its electrospray response was significantly higher than for the native metabolite. It was even possible to detect the protonated molecule of the oxime (*m/z* 502) in an extracted ion chromatogram of an MS^1 scan (Fig. 10), something that was impossible for the underivatized altrenogest glucuronide (*m/z* 487). The minor peaks in the extracted ion chromatogram for *m/z* 487 rather seemed to originate from *m/z* 486 and 488 as observed in the mass spectra (Fig. 10A). The identity of the derivatized glucuronide was confirmed by collision-induced dissociation, yielding a product ion spectrum as shown in Fig. 11 (cf. Fig. 9A). The major product ion was *m/z* 326, corresponding to the altrenogest oxime aglycone. The peak at *m/z* 308 suggests the loss of H_2O (18 u) from *m/z* 326 or a loss of glucuronic acid (194 u) from the protonated molecule *m/z* 502. The altrenogest characteristic loss of 42 u (propene) from the algycon, yielding *m/z* 284 was also present. *m/z* 291 and 267 correspond to loss of OH radicals (17 u) from *m/z* 308 and 284, respectively. The appearance of these characteristic product ions of *m/z* 502 was considered as enough evidence for the presence of altrenogest oxime glucuronide.

These results thus demonstrate that introduction of a basic oxime group into an oxo-steroid metabolite can be used to improve the detection sensitivity of biological extracts in electrospray MS and thereby simplify the identification.

4. Conclusions

A useful qualitative analytical method for altrenogest in horse urine has been developed. Altrenogest could be detected in horse urine down to at least 13 pg/mL using LC–MS/MS in the SRM mode. The main metabolic route in the horse for this compound seemed to be through conjugation with glucuronic acid and sulfate. Chemical derivatisation of the altrenogest glucuronide into an oxime significantly increased its detection sensitivity and facilitated the identification of this metabolite.

Acknowledgements

The authors express their gratitude to Mrs. Elisabeth Fredriksson, National Veterinary Institute, Uppsala for experimental assistance and to Associate Professor Anne-Marie Dahlin at the Swedish University of Agricultural Sciences for drug administration and sampling.

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