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High-throughput screening of corticosteroids and basic drugs in horse urine by liquid chromatography-tandem mass spectrometry

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Abstract

This paper describes two high-throughput liquid chromatography-tandem mass spectrometry (LC-MS-MS) methods for the screening of two important classes of drugs in equine sports, namely corticosteroids and basic drugs, at low ppb levels in horse urine. The method utilized a high efficiency reversed-phase LC column (3.3 cm $L \times 2.1$ mm i.d. with 3 μm particles) to provide fast turnaround times. The overall turnaround time for the corticosteroid screen was 5 min and that for the basic drug screen was 8 min, inclusive of post-run and equilibration times. Method specificity was assessed by analysing a total of 35 negative post-race horse urine samples. No interference from the matrices at the expected retention times of the targeted masses was observed. Inter-day precision for the screening of 19 corticosteroids and 48 basic drugs were evaluated by replicate analyses ($n = 10$) of a spiked sample on 4 consecutive days. The results demonstrated that both methods have acceptable precision to be used on a routine basis. The performance of these two methods on real samples was demonstrated by their applications to drug administration and positive post-race urine samples.
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Keywords: Basic drugs; Corticosteroids; Horse urine

1. Introduction

Gas chromatography-mass spectrometry (GC-MS) has long been accepted as a powerful technique for the screening and confirmation of the presence of prohibited substances in biological samples from human and animal athletes. Over the past decade, liquid chromatography-mass spectrometry (LC-MS) has evolved into a mature technique and is gaining wide acceptance in many doping control laboratories. LC-MS or LC-MS^n is particularly suited for the analyses of polar, non-volatile and heat-labile drugs that cannot be adequately handled by GC-MS. In addition, tedious derivatization steps can often be omitted.

Corticosteroids and basic drugs have been known to be abused in both human and equine sports. A number of LC-MS methods have been reported previously to screen for these two classes of compounds in human and equine urine [1–4]; however, the drug coverage was limited and the overall LC-MS turnaround times were generally long.

LC-MS or LC-MS^n has been successfully used in the authors' laboratory for the screening and confirmation of prohibited substances in horse biological samples since 1998. The prohibited substances tested include corticosteroids [5], basic drugs [6], anti-ulcer drugs [7], quaternary ammonium drugs [8], anti-diabetics [9], and anabolic steroids [10]. The current LC-MS screening methods for corticosteroids and basic drugs in horse urine are performed using a C18 LC column (7.5 cm $L \times 3.0$ mm i.d. with 3 μm particles) coupled to an ion-trap MS. A major drawback of ion-trap MS is that there is only a limited number of MS/MS events that can be monitored at one time due to the relatively long duty

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cycle time. For the screening of large number of analytes, it often requires the segregation of the analytes into separate retention time groups or segments in order to cover all the analytes in a single LC-MS run. For analytes that elute very close to the segment interchange times, it is often necessary to include the respective MS/MS events in both contiguous segments to make allowance for possible retention time drift, further reducing the number of analytes that can be monitored in each run. In extreme cases where a large number of analytes are tested (like our basic drug screen which covers at least 48 targets), it is often necessary to separate the analytes monitored into two different LC-MS runs in order to provide effective detection. Another drawback of our current LC-MS methods is that the turnaround times are often long (about 20 min) which have a negative impact on productivity. In 2001, Thevis et al. published a high speed LC-MRM method for the screening of 32 beta-receptor blocking agents in human urine within a 7-min LC run [11]. However, to the best of our knowledge, no high-throughput method capable of detecting multiple corticosteroids or basic drugs in horse urine, which is a much more complicated and viscous matrix than human urine, has been developed for routine application. This study describes the development of two high-throughput methods for the screening of corticosteroids and basic drugs using a short LC column coupled to a triple-quadrupole MS. The new methods took advantage of the fast turnaround time of the short LC column (3.3 cm $L \times 2.1$ mm i.d. with 3 μm particles) and the ability of the triple-quadrupole MS to cover a large number of target analytes in multiple-reaction-monitoring mode (MRM). Using the new methods, 19 corticosteroids and 48 basic drugs can be covered in 5- and 8-min LC-MS runs respectively.

2. Experimental

2.1. Materials

Anileridine hydrochloride, butorphanol tartrate, cimetidine, cocaine hydrochloride, desipramine hydrochloride, dexamethasone, droperidol, famotidine, fluocinonide, fluorometholone, fluprednisolone, guanabenz acetate, labetalol hydrochloride, mazindol, methylprednisolone, morphine glucuronide, nadolol, nizatidine, nylidrin hydrochloride, oxycodone hydrochloride, oxymorphone, pindolol, prazosin hydrochloride, prednisolone, prednisone, and ranitidine hydrochloride were obtained from USP (Rockville, MD, USA). Beclomethasone, desoximetasone, 21-deoxydexamethasone, dichlorisone and fludrocortisone were from Steraloids (Newport, RI, USA). Clenbuterol, 21-desoxycortisone, flumethasone and heptaminol hydrochloride were acquired from Sigma (St. Louis, MO, USA). Benzoylecgonine, methadone hydrochloride and n-norpropoxyphene maleate were obtained from Alltech (Deerfield, IL, USA). Atenolol, fluclorolone acetonide, fluocinolone acetonide, haloperidol, hydrocortisone glucuronide, nortriptyline hydrochloride, perphenazine, sotalol hydrochloride and triamcinolone acetonide were obtained from BP (Middlesex, UK). Bisoprolol fumarate and potassium losartan were purchased from Merck (Darmstadt, Germany). Trifluperidol was from Janssen Pharmacetica (NJ, USA), benperidol from Janssen–Cilag (Buckinghamshire, UK), and d_4-hydrocortisone from ESR (New Zealand). Romifidine and telmisartan were from Boehringer Ingelheim (Ingelheim, Germany), buspirone hydrochloride from Bristol–Myers Squibb (NY, USA), budesonide from Douglas Pharmaceutical (Auckland, New Zealand), salmeterol xinafoate from Glaxo Wellcome (Middlesex, UK), bromocriptine mesylate from Hind Wing (Hong Kong), practolol from ICI (Now Zeneca Plc, UK), etafedrine from Merrel Dow Research (Ohio, USA), carteolol hydrochloride from Otsuka (Tianjin, China), sildenafil citrate from Pfizer (NY, USA), hydroxyalprazolam from Radian (Texas, USA), nalbuphine hydrochloride from Research Biochemicals Incorporated (MA, USA), buprenorphine hydrochloride from Reckitt & Colman Products Ltd. (Hull, UK), carvedilol from Roche (Mannheim, Germany), irbesartan from Sanofi (Paris, France), and candesartan cilexetil from Takeda Chemical Industries (Osaka, Japan). Esmolol hydrochloride was from The Boots (Isando, South Africa). β-Glucuronidase (from Patella vulgata, lyophilized powder), protease (from bovine pancreases, type I, 6.9 U/mg solid) and sodium hydroxide (pellets, analytical grade) were purchased from Sigma (St. Louis, MO, USA). Acetic acid (96%), hydrochloric acid (30%), potassium hydroxide (pellets), potassium phosphate and sodium chloride (GR grade) were obtained from Merck (Darmstadt, Germany). Sodium sulphate was purchased from Farco Chemical Supplies (Beijing, China). Dichloromethane (GR grade), ethyl acetate (GR grade), isopropanol (LC gradient grade; LiChrosolv®) and methanol (LiChrosolv®; LC grade) were obtained from Merck (Darmstadt, Germany). Bond Elut Certify® cartridges (130 mg, 3 mL) were purchased from Varian (Harbor City, CA, USA). HPLC grade deionised water was obtained from an in-house water purification system (Milli-Q, Molsheim, France). Ammonia solution (33%; extra pure grade) was from Riedel–deHaen (Seelze, Germany).

2.2. Sample preparation and extraction procedures

Urine (3 mL) was spiked with d_4-hydrocortisone (300 ng) and nadolol (300 ng) as the internal standards (I.S.) for the screening of corticosteroids and basic drugs, respectively, and diluted with potassium phosphate buffer (pH 6.0, 0.1 M, 1 mL). The pH was adjusted, if necessary, to 6.0 using either potassium hydroxide (0.1 M) or hydrochloric acid (0.1 M). Protease (5 mg/mL, 60 μL) and β-glucuronidase (18,000 U/mL, 360 μL) were added and the urine sample was incubated at 65 °C for 3.5 h. The enzyme treated urine was then diluted with potassium phosphate buffer (pH 6.0, 0.1 M, 1.6 mL) before loading onto a Bond Elut Certify® cartridge, which had been pre-conditioned with methanol (2 mL), deionised water (2 mL), and potassium phosphate buffer (pH

6.0, 0.1 M, 2 mL). The cartridge was then washed with phosphate buffer (pH 6.0, 0.1 M, 2 mL) followed by acetic acid (1.0 M, 2 mL), and then eluted with dichloromethane/ethyl acetate (4:1, v/v, 3 mL) to collect Fraction 1 for the analyses of corticosteroids. Fraction 1 was further washed with NaOH/NaCl (1 M/0.15 M, 2 mL). The organic extract was filtered through an anhydrous sodium sulphate drying tube and evaporated to dryness under nitrogen at 60 °C. The dried residue was then reconstituted with methanol (50 μL) and transferred to a conical insert in a Chrompack autosampler vial for LC-MS-MS analysis. The SPE cartridge was further washed with methanol (2 mL), dried for 5 min with nitrogen at 20 p.s.i., and eluted with 2 mL of ethyl acetate/dichloromethane/isopropanol (5:4:1, v/v) containing 2% of concentrated aqueous ammonia to collect Fraction 2 for the analyses of basic drugs. Fraction 2 was evaporated to dryness under nitrogen and the residue reconstituted in methanol (50 μL). The content was transferred to a conical insert in a Chrompack autosampler vial for LC-MS-MS analysis.

2.3. Instrumentation

LC-MS-MS analysis was performed on a Thermo Finnigan TSQ Quantum mass spectrometer equipped with a Surveyor Autosampler and a MS Pump system (Thermo Finnigan, San Jose, CA, USA). Solid-phase extraction (SPE) was carried out using a RapidTrace® SPE workstation (Zymark Corporation, Hopkinton, MA, USA).

2.4. LC conditions for the screening of corticosteroids

A reversed-phase Supelcosil™ LC-8-DB column (3.3 cm × 2.1 mm i.d., 3 μm; Supelco, Bellefonte, PA, USA) was used for the analyses. The mobile phase was composed of 10 mM ammonium acetate (pH 6.8) as solvent A and methanol as solvent B. A linear gradient was run at 0.2 mL/min, with 20% solvent B at the start ($t = 0$ min), increasing to 80% solvent B at $t = 1$ min, then to 100% B at $t = 2.5$ min, and finally hold at 100% solvent B for 1 min (until $t = 3.5$ min). The gradient was then returned to 20% solvent B at $t = 3.6$ min, and stabilised until $t = 5$ min before starting the next injection. Injection volume was 5 μL each.

2.5. LC conditions for the screening of basic drugs

A reversed-phase Supelcosil™ LC-8-DB column (3.3 cm × 2.1 mm i.d., 3 μm; Supelco, Bellefonte, PA, USA) was used for the analyses. The mobile phase was composed of 5 mM acetic acid as solvent A, ammonium formate (pH 3.8) as solvent B and acetonitrile as solvent C. A linear gradient was run at 0.2 mL/min, with 40% solvent A and 40% solvent B at the start ($t = 0$ min), decreasing to respectively 5% solvent A and 5% solvent B at $t = 3$ min, and hold for 0.5 min (until $t = 3.5$ min). The gradient was then returned to 40% solvent A and 40% solvent B at $t = 3.6$ min, and stabilised until $t = 8$ min before starting the next injection. Injection volume was 5 μL each.

2.6. MS conditions for the screening of corticosteroids and basic drugs

The atmospheric pressure ionisation (API) source was operated in negative and positive ESI modes for the screening of corticosteroids and basic drugs respectively. A capillary temperature at 270 °C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 40 and 20 arbitrary TSQ quantum units respectively. Detection of the drugs was performed in the MRM mode with a single time segment. The peak widths for the selection of the precursor and the corresponding product ions in Q1 and Q3 were both at 0.7 amu (FWHM). The scan width for the selected product ions was set at 1 amu and the scan time at 20 or 50 ms per scan. Argon was used in the collision cell and was set at 1.2 mTorr for all experiments. The collision-induced-dissociation (CID) energies ranged from 10 to 22 eV for the corticosteroids and 11 to 50 eV for the basic drugs. The actual CID energies used for individual drugs are shown in Figs. 1 and 2. Data processing was performed using the Finnigan Xcalibur Version 1.3 software.

2.7. Drug administration studies

Post-treatment urine samples were used to evaluate the performance of the LC-MS-MS method. For triamicinolone acetonide, a thoroughbred gelding (castrated horse) was treated intra-articularly with 18 mg of triamicinolone acetonide. Urine samples were collected naturally before administration and then up to 7 days post-administration. For the Ulcerguard™ (ranitidine HCl) administration, another thoroughbred gelding was administered orally with 3.3 g of ranitidine HCl three times daily for 2 weeks. Urine samples were collected naturally before administration and then up to 2 weeks post-administration.

2.8. Quantification of triamcinolone acetonide and ranitidine in drug administration urine samples

The sample processing and LC-MS procedures used for the quantification of triamcinolone acetonide and ranitidine in the post-treatment urine samples were identical to that described for the screening methods. For each batch of samples, calibrators were prepared by spiking the target analyte at 5 levels (0, 5, 10, 20 and 50 ng/mL) in different 3-mL aliquots of a negative urine and processed in duplicate. A five-level calibration curve was established by plotting the drug-to-I.S. peak area ratios versus the drug concentrations in the calibrators using linear regression. The concentrations of the target analyte in the post-administration urine were determined from the calibration curve. Samples with concentrations falling outside the calibration range were diluted with deionised water before analysis. A QC sample (a negative urine spiked at 10 ng/mL) was analysed in duplicate

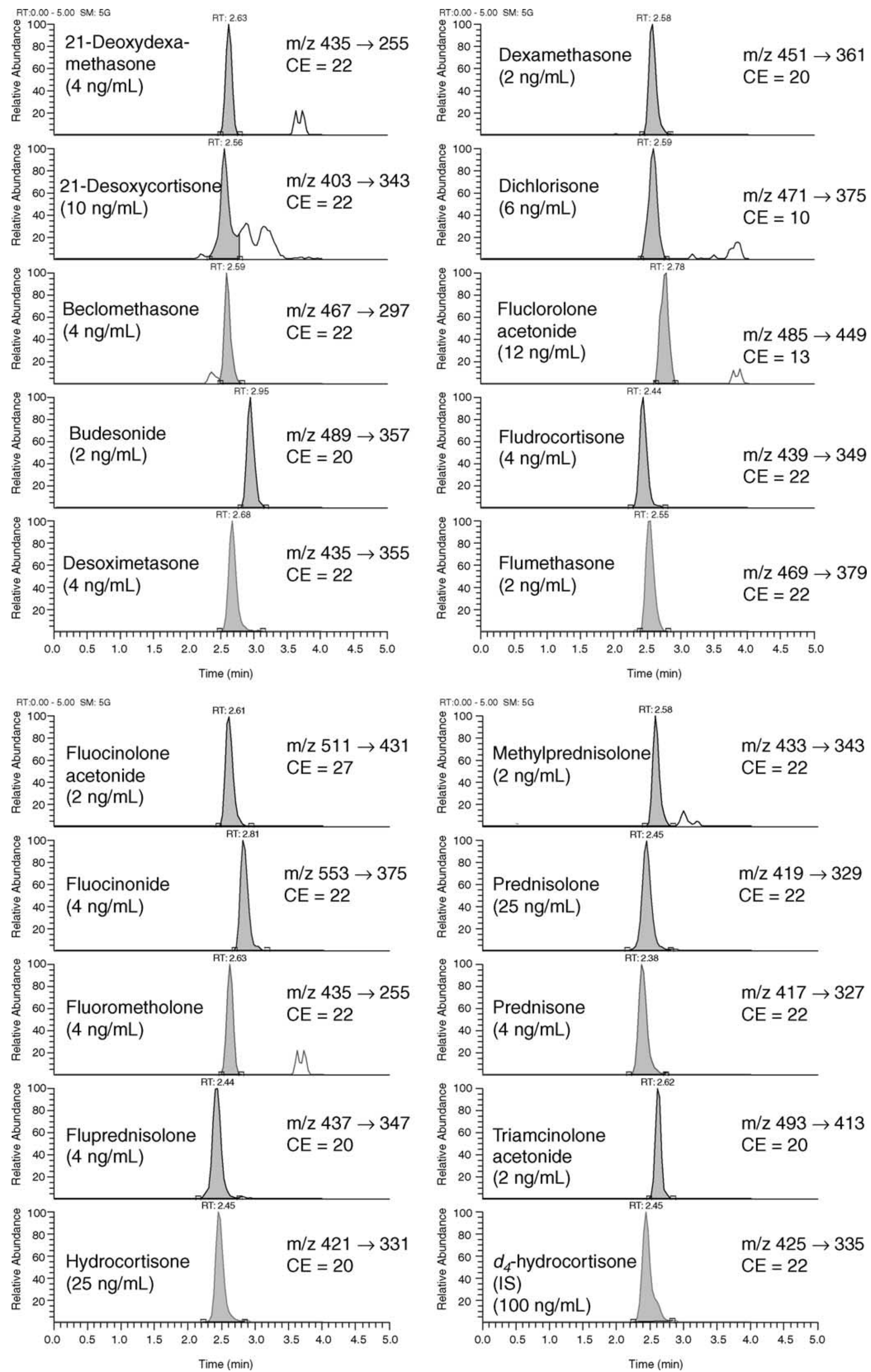

Fig. 1. Typical selected product-ion chromatograms of the 19 targeted corticosteroids obtained from the analysis of a spiked urine sample.

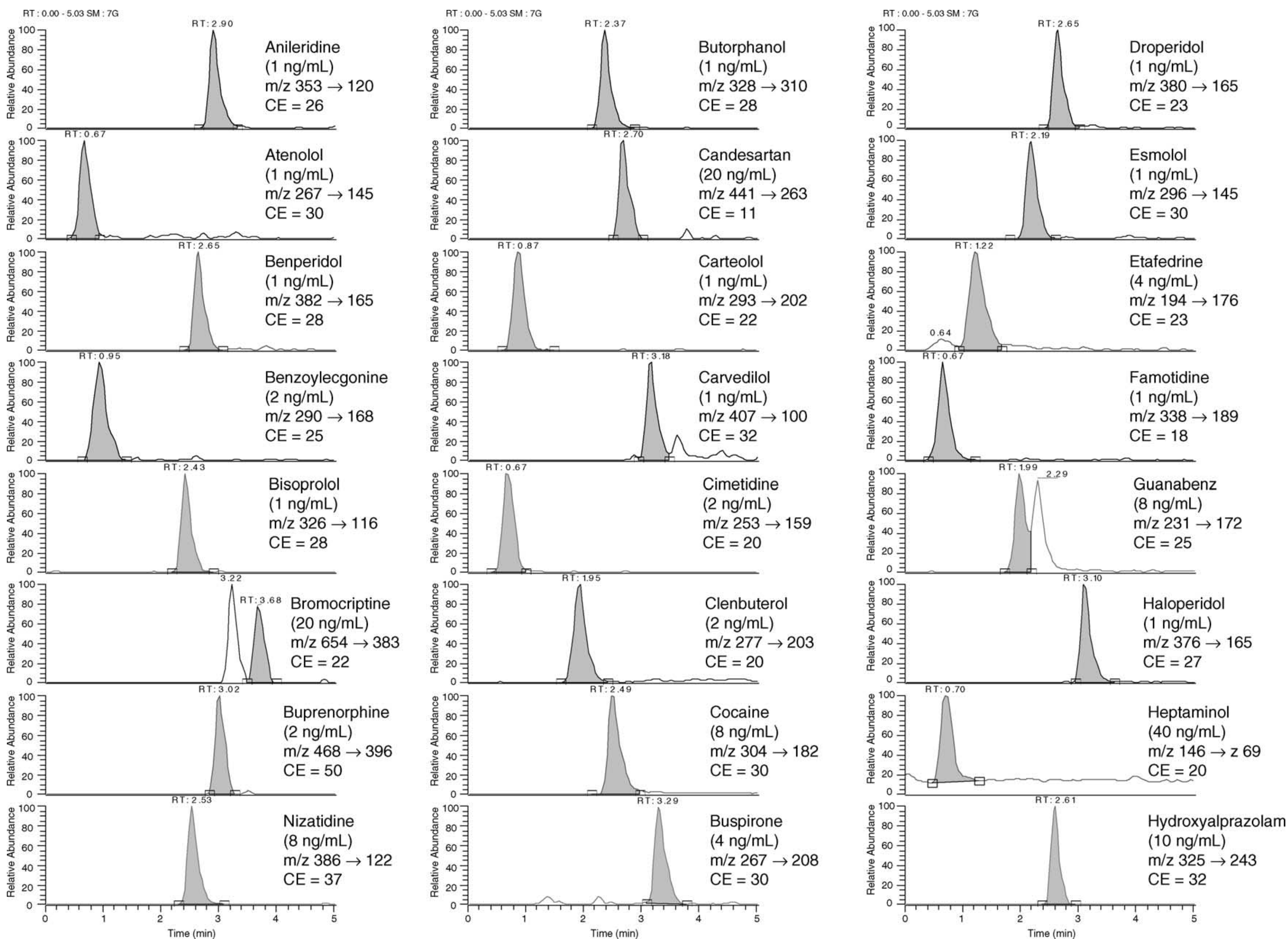

Fig. 2. Typical selected product-ion chromatograms of the 48 targeted basic drugs obtained from the analysis of a spiked urine sample.

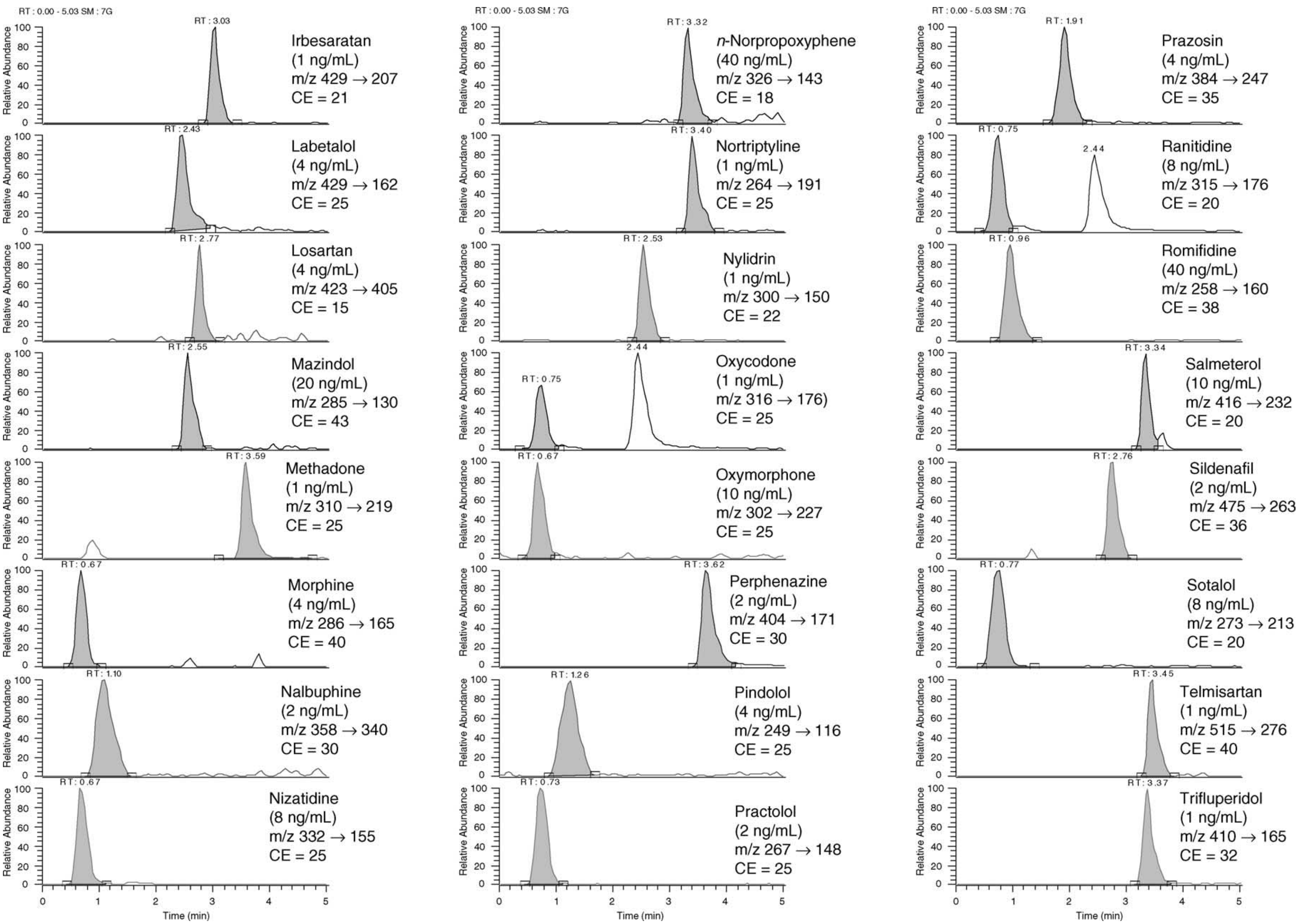

Fig. 2. (*Continued*.)

for each batch of samples to verify that the analysis was in control.

3. Results and discussion

3.1. Method sensitivity and specificity

Fig. 1 shows the selected product-ion chromatograms for 19 corticosteroids from a quality control sample (a spiked horse urine sample), which is routinely run with each batch of samples. The levels of corticosteroids spiked in the quality control sample represent those that can be consistently detected by the LC-MRM method. All target analytes were detected within 3 min and the overall LC-MS turnaround time was 5 min inclusive of post-run and solvent equilibration times. Similarly, selected product-ion chromatograms for 48 basic drugs from a quality control sample are shown in Fig. 2. The overall LC-MS turnaround time for the basic drug screen was 8 min, and all target analytes could be detected within

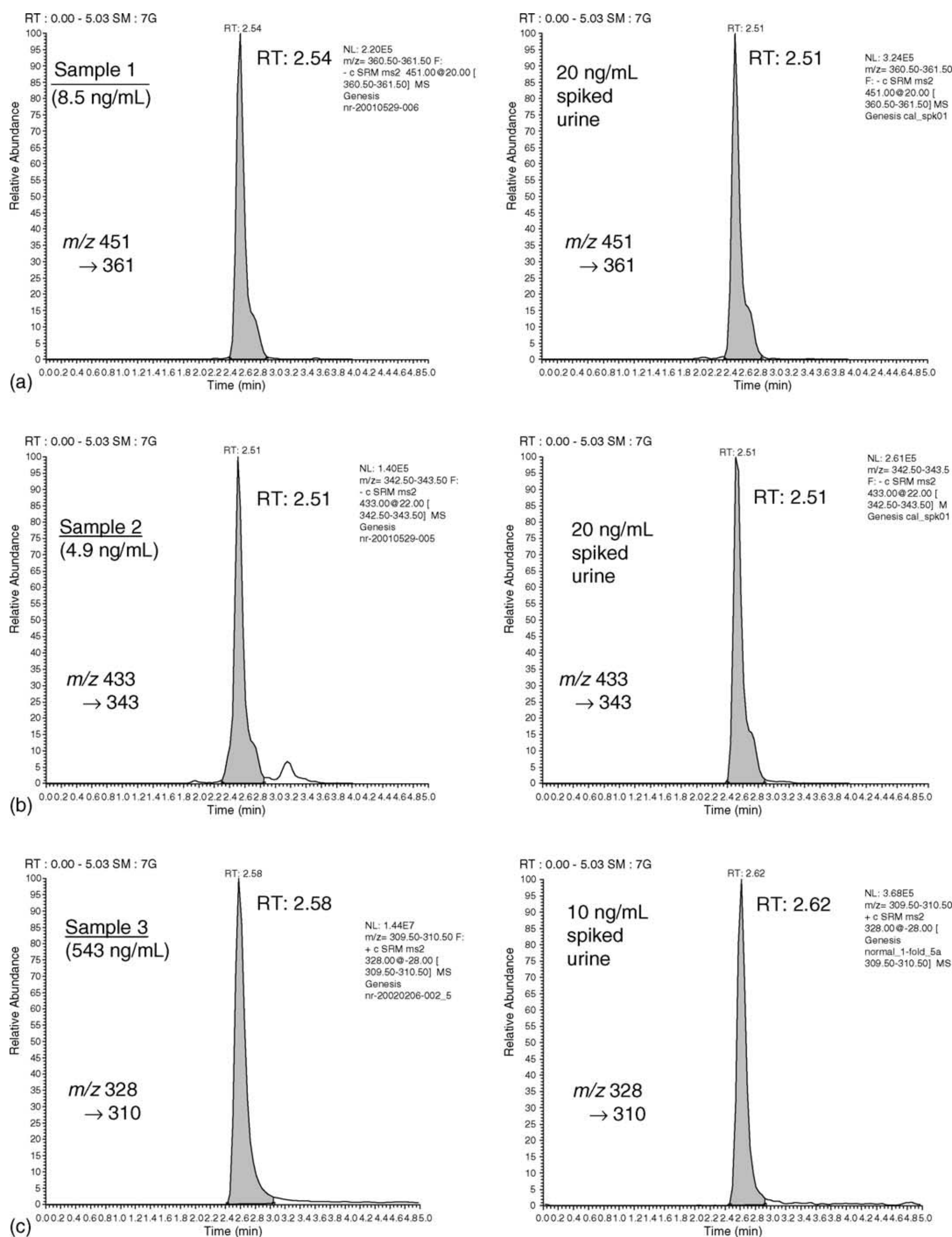

Fig. 3. Selected product-ion chromatograms of: (a) dexamethasone; (b) methylprednisolone; (c) butorphanol; and (d) clenbuterol obtained from the referee samples and spiked urine samples.

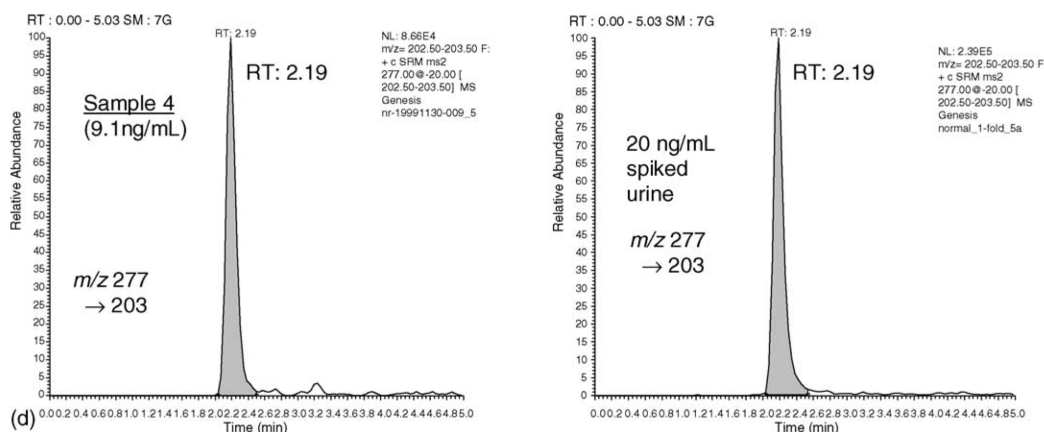

Fig. 3. (*Continued*.)

4 min. The signal-to-noise ratios for all analytes were well in excess of 3:1 (the general definition for limit of detection), indicating that both methods have ample sensitivity for the target analytes at the spiked levels.

Method specificities of the two LC-MS screening methods were assessed by testing different negative post-race horse urine samples ($n = 35$). Interferences from the matrices at the expected retention times of the target ions were not observed.

3.2. Inter-day precision

The reproducibility of the analyte-to-I.S. peak area ratios and the relative retention times for the 19 corticosteroids and 48 basic drugs were examined by replicate analyses ($n = 10$) of a spiked urine sample on 4 different days. The internal standards were respectively d_4-hydrocortisone (100 ng/mL) for the corticosteroids and nadolol (100 ng/mL) for the basic drugs. The spiked drug concentrations and results of the precision study are summarised in Tables 1 and 2. For corticosteroids, the inter-day precision for the peak area ratios ranged from 2 to 11%, and that for the relative retention times from 0 to 0.49%. For basic drugs, the inter-day precision for the peak area ratios ranged from 5 to 17%, and that for the relative retention times from 0.65 to 1.83%. These results indicated that the method has acceptable precision to be used on a routine basis.

3.3. Screening of corticosteroids and basic drugs in positive samples

In order to demonstrate that the above screening methods are applicable to real positive samples, four referee horse urine samples (or generally referred to as 'B' samples) from different overseas racing authorities reported to contain dexamethasone, methylprednisolone, butorphanol and clenbuterol were analysed. Fig. 3a and b show that both dexamethasone and methylprednisolone were clearly detected using the new method with good signal-to-noise ratios. The retention times matched well with those obtained from the spiked urine standards. The concentrations were determined to be 8.5 ng/mL for dexamethasone and 4.9 ng/mL for methylprednisolone using one-point calibration at 20 ng/mL. Similar results are shown in Fig. 3c and d for butorphanol and clenbuterol. Samples flagged as suspicious by the screening method are normally confirmed by analysing another aliquot of the sample in question using the same extraction method and analysed using an ion-trap LC-MS in full scan product-ion scanning mode. Alternatively, confirmation can be performed using a triple-quadrupole LC-MS in MRM mode. However, at least three significant transitions will be monitored in order to provide a higher degree of proof. For the confirmation of dexamethasone, a slower solvent gradient was used to distinguish it from its 16-epimer betamethasone.

Table 1
Inter-day precision data (% RSD) on peak area ratios and relative retention times for the 19 corticosteroids obtained from the analysis of a spiked urine sample using d_4-hydrocortisone as the internal standard

Drug	Spike concentration (ng/mL)	Peak area ratio % RSD	Relative retention time % RSD
21-Deoxydexamethasone	40	2	0.42
21-Desoxycortisone	100	4	0.03
Beclomethasone	40	11	0.42
Budesonide	20	7	0.25
Desoximetasone	40	3	0.49
Dexamethasone	20	5	0.39
Dichlorisone	60	10	0.27
Fluclorolone acetonide	120	9	0.37
Fludrocortisone	40	4	0.43
Flumethasone	20	4	0.21
Fluocinolone acetonide	20	5	0.39
Fluocinonide	40	5	0.08
Fluorometholone	40	9	0.45
Fluprednisolone	40	3	0.30
Hydrocortisone	250	3	0.00
Methylprednisolone	20	4	0.27
Prednisolone	250	3	0.00
Prednisone	40	4	0.22
Triamcinolone acetonide	20	11	0.43

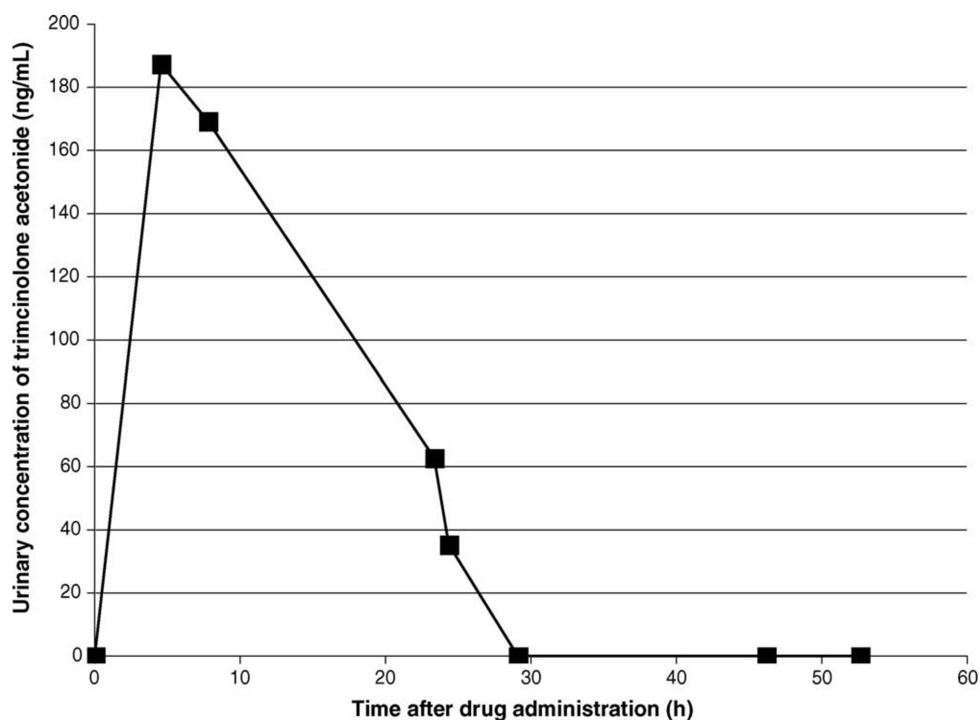

Fig. 4. Urinary elimination of triamcinolone acetonide after an intra-articular administration of 18 mg of triamcinolone acetonide to a thoroughbred gelding.

3.4. Urinary excretion studies

Triple-quadrupole mass spectrometer operating in MRM mode has been shown to be a reliable technique for quantitative analysis of drugs in human biological samples [12–16]. The technique also worked well in the quantification of post-administration urine samples collected in this study. The correlation coefficients of the calibration curves in all cases were greater than 0.99 and the measured concentrations of the QC samples were within ±10%. Fig. 4 shows the urinary elimination profile for triamcinolone acetonide. Using the LC-MS methods developed in study, the administration of triamcinolone acetonide could be detected for about 30 h, and that of ranitidine for about 2 weeks. (Fig. 5).

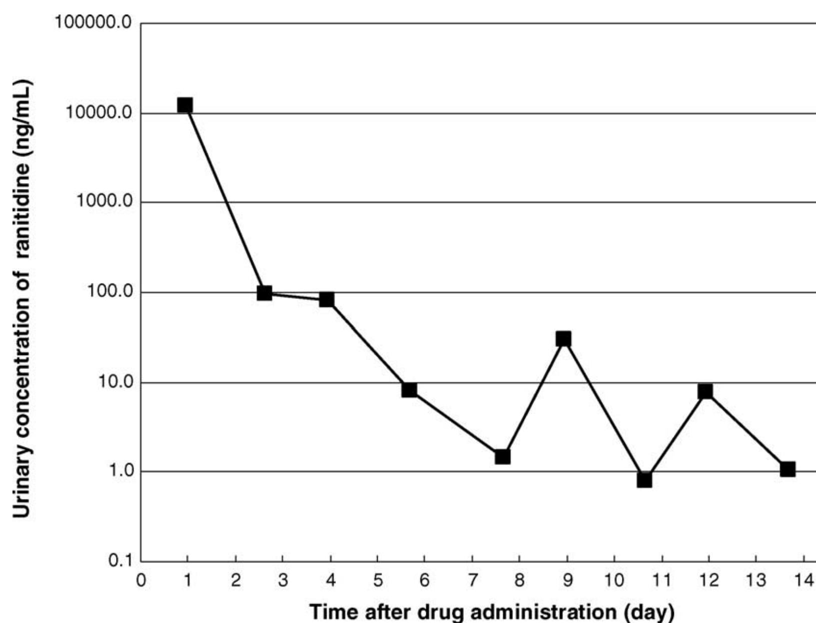

Fig. 5. Urinary elimination of ranitidine after an oral administration of Ulcerguard^{TM} (ranitidine HCl), given 3.3 g of ranitidine HCl 3 times per day for 2 weeks, to a thoroughbred gelding.

Table 2
Inter-day precision data (% RSD) on peak area ratios and relative retention times for the 48 basic drugs obtained from the analysis of a spiked urine sample using nadolol as the internal standard

Basic drugs	Spike concentration (ng/mL)	Peak area ratio % RSD	Relative retention time % RSD
Anileridine	20	7	0.71
Atenolol	20	11	1.13
Benperidol	20	6	0.79
Benzoylecgonine	40	7	1.01
Bisoprolol	20	7	0.72
Bromocriptine	400	7	0.70
Buprenorphine	20	15	0.90
Buspirone	80	6	0.74
Butorphanol	20	5	0.77
Candesartan	400	10	0.90
Carteolol	20	7	1.11
Carvedilol	20	9	0.88
Cimetidine	40	6	0.72
Clenbuterol	40	5	0.91
Cocaine	160	6	0.70
Desipramine	40	6	0.73
Droperidol	20	6	0.89
Esmolol	20	7	0.71
Etafedrine	80	5	1.11
Famotidine	20	11	1.45
Guanabenz	160	6	0.75
Haloperidol	20	5	0.74
Heptaminol	800	6	0.72
Hydroxyalprazolam	200	9	0.76
Irbesartan	20	7	0.77
Labetalol	80	6	0.65
Losartan	80	8	0.76
Mazindol	400	11	0.77
Methadone	20	6	0.72
Morphine	80	17	1.83
Nalbuphine	40	5	0.70
Nizatidine	160	6	0.70
n-Norpropoxyphene	800	8	0.74
Nortriptyline	20	8	0.73
Nylidrin	20	7	0.74
Oxycodone	20	6	1.33
Oxymorphone	200	8	1.03
Perphenazine	40	5	0.73
Pindolol	80	8	0.90
Practolol	40	7	1.40
Prazosin	80	5	0.82
Ranitidine	160	5	1.18
Romifidine	800	5	1.12
Salmeterol	200	6	0.84
Sildenafil	40	15	0.73
Sotalol	160	7	1.04
Telmisartan	20	12	0.85
Trifluperidol	20	6	0.68

4. Conclusion

Using a fast LC column coupled to a triple-quadrupole MS, two high-throughput LC-MS methods were developed for the screening of corticosteroids and basic drugs. The detection of 19 corticosteroids and 48 basic drugs could be achieved within a 5- and 8-min LC-MS-MS run respectively. Both methods could detect the targets at low ppb levels. Both methods showed acceptable precision to be used on a routine basis. Matrix interference was not observed at the expected retention times of the target ions. The applicability of the methods has been demonstrated by the analyses of drug-administration and positive samples.

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