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Direct-injection screening for acidic drugs in plasma and neutral drugs in equine urine by differential-gradient LC–LC coupled MS/MS

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Abstract

Direct-injection LC–LC hybrid tandem MS methods have been developed for undertaking broad-based screening for acidic drugs in protein-precipitated plasma and neutral doping agents in equine urine. In both analyses, analytes present in the matrix were trapped using a HLB® extraction column before being refocused and separated on a Chromolith® RP-18e monolithic analytical column using a controlled differential gradient generated by proportional dilution of the first column's eluent with water. Each method has been optimised by the adoption of a mobile phase and gradient that was tailored to enhance ionisation in the MS source while maintaining good chromatographic behaviour for the majority of the target drugs. The analytical column eluent was fed into the heated nebulizer (HN) part of the Duospray® interface attached to a 4000 QTRAP® mass spectrometer. Information dependent acquisition (IDA) with dynamic background subtraction (DBS) was configured to trigger a sensitive enhanced product ion (EPI) scan when a multiple reaction monitoring (MRM) survey scan signal exceeded the defined criteria. Ninety-one percent of acidic drugs in protein-precipitated plasma and 80% of the neutral compounds in equine urine were detected when spiked at 10 ng/ml.

Keywords: Direct injection; Hybrid MS/MS; Equine drug testing; Differential LC/LC gradient

1. Introduction

Laboratories conducting doping surveillance analysis on equine racing samples are under pressure to expand their scope of testing to cope with an ever-increasing number of readily available drugs, both legal and illegal, which are potential doping agents. Consequently, there has been a discernable shift from their historical reliance upon pharmacological class-based analyses, which typically involve a relatively small (usually <20) number of target analytes, towards the adoption of broader techniques (e.g. refs. [1,2]) able to screen many more compounds within each run. This movement has been assisted by the increase in the affordability of LC/MS instruments, which are much better suited than GC/MS instruments to the task of analysing for a diverse range of compounds. For example, when the chemical space covered by different analytical techniques is plotted graphically using relative molecular mass and analyte polarity for the axes, electrospray and atmospheric pressure chemical ionisation, collectively called atmospheric pressure ionisation (API), cover an area about four times greater than GC/MS [3]. Also, unlike GC, samples analysed by LC do not generally require a time-consuming derivatization step using hazardous chemicals to make the analytes amenable to chromatography. The latest API-LC/MS instruments also offer superior performance to older models and methods for screening hundreds of drugs simultaneously can now be developed (e.g. refs. [4,5]) without compromising sensitivity [6] to the point where the false negative rate becomes unacceptably high. In line with this trend away from class-based analyses, we have recently reported [7] on the use of a direct-injection LC–LC hybrid MS/MS analysis for the screening of equine urine for 250 positive TurboIonSpray® responsive drugs and now present two complementary analytical methods that can be used in conjunction with this analysis. The methods we report on here use API by means of heated nebulization and were developed for broad-based screening for negatively ionisable drugs in protein-precipitated plasma and for detecting the presence, in equine urine, of doping agents that produce positive ions. In horserace sample testing, these groups of doping agents are often referred to as "acidic" and "neutral" drugs, respectively.

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Many acidic drugs are commonly used during the course of legitimate equine therapeutic treatment and we wanted to limit the inter-horse detection time variability as much as possible when designing our method. It has been stated that post-administration blood concentrations of compounds that ionise in aqueous solution, as the acidic drugs do, are more predictable than the corresponding urinary levels [8] and data from several recent equine pharmacokinetic studies [9–13] seems to support that contention. Consequently, we opted to develop a broad-based screen of negative heated nebulizer (−HN) responsive substances using protein-precipitated plasma as our analytical matrix. We believe that this approach allows veterinarians to treat racehorses with greater confidence that a particular dose, given outside of the detection period, will not give rise to an inadvertent positive result. An additional benefit of selecting this matrix is that our current pre-race sample collection operation is set up to exclusively undertake venipuncture capture of blood and, therefore, urine is not available for testing until after the race has been run.

Corticosteroids are potent neutral drugs that have a noted ability to mask painful conditions in racehorses and, for this reason, we considered them as the most important pharmacological sub-group within the positive heated nebulizer (+HN) ionisable target group. However, the basis for our prohibited substance rules is the *International Agreement on Breeding, Racing and Wagering* (IABRW) [14] that is published by the International Federation of Horseracing Authorities and this provides a threshold for the endogenous corticosteroid hydrocortisone that only applies to urine samples. Therefore, the use of the protein-precipitated plasma for neutral drug screening was not a viable option and we are currently restricted to using post-race urine as the sample type for conducting this screening analysis.

We have evaluated both of these methods and have found that the speed of analysis, limits of detection and robustness are satisfactory for use in our doping surveillance screening of racehorses competing at the Singapore Racecourse. In particular, we found that these direct-injection analyses were both faster and less labour intensive than the liquid/liquid extraction plus derivatization GC/MS and off-line solid phase extraction LC/MS methods that they replaced and this has benefited the overall efficiency of the laboratory's operations.

2. Experimental

2.1. Materials and reagents

The reference standards used were obtained from various suppliers shown by number in column 3 of Tables 3 and 4. The number correspond with 1, Sigma/Aldrich (Singapore); 2, United States Pharmacopeia (Rockville, MD, USA); 3, G.D. Searle & Co. (Chicago, IL, USA); 4, Boehringer Ingelheim (Singapore); 5, gift from the Australian Racing Forensic Services Limited (Sydney, Australia); 6, Cambridge Isotope Laboratories (Andover, MA, USA); 7, Cerilliant (Round Rock, TX, USA). Phenobarbitone-D_5 was obtained from Cerilliant (Round Rock). Hydrocortisone (≥98%) was obtained from Sigma (Singapore) and the internal standard (≥98%) hydrocortisone-D_4 (9, 11, 12, 12) was from the Cambridge Isotope Laboratories (Andover). The hydrocortisone (98.9 ± 0.2%) standard reference material (SRM) was from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). Water used to prepare the aqueous mobile phase was purified using a Millipore Elix® pre-treatment system to feed a MilliQ® reverse osmosis water purification unit. The total organic carbon in the purified water was less than 10 ppb and resistance was greater than 18 MΩ. Acetonitrile and methanol were HPLC grade from Fisher Scientific (Pittsburgh, PA, USA) and formic acid (>98%) was Cica® grade from Kanto Kaguka (Singapore). The ammonium acetate GR was obtained from Merck Pty. Ltd. (Singapore). Organic solvents and aqueous buffers were passed through a 0.2 μm Whatman (Maidstone, England) filter made of either cellulose nitrate (aqueous) or PTFE (organic) material before being used as LC mobile phases.

2.2. Instrumentation configuration

An Agilent (Singapore) HP1100 series LC consisting of a G1316A column oven with six-port valve, G1311A quaternary, G1310A isocratic pump plus a G1367A autosampler retrofitted with a multi-draw option kit that extends the maximum injection volume to 500 μl, was connected to an Applied Biosystems (Singapore) MDS Sciex 4000 QTRAP® hybrid tandem mass spectrometer operating under Analyst 1.4.1 software. The Shimadzu (Singapore) LC6A was used as the second isocratic pump. Two (Valco Instruments Company, Houston, TX, USA) 10-port valves (040-0811V) with microelectric valve actuators, controlled by Analyst 1.4.1 software, were mounted next to the DuoSpray® TIS and HN dual spray source. The Oasis HLB® (2.1 mm × 20 mm, 25 μm) (Waters Asia, Singapore) extraction column and Chromolith® Performance RP-18e (4.6 mm × 100 mm plus guard column) analytical column, were installed into the flow path as described in ref. [7]. The autosampler, plumbed so that needle could be back-flushed by the quaternary pump, was used in a programmed mode to load the extended loop three times with the volume entered (100 μl) into the Analyst sample batch list. After an additional volume (100 μl) was drawn up and the needle returned to the seat, the valve switched to mainpass position and the run was triggered.

2.3. Sample preparation and instrumental settings—analysis of equine plasma using HN in the negative ion mode

2.3.1. Sample preparation

Equine plasma (500 μl) in an Axygen (Union City, CA, USA) 1.5 ml plastic microsample tube was mixed with an equal volume of acetonitrile containing 2000 ng/ml of the internal standard phenobarbitone-D_5. After the mixture was briefly vortex mixed and then left at 4 °C for 60 min, the precipitated proteins were removed by centrifugation in a Heraeus Biofuge® Pico at a RCF of 16,000 × *g* for 10 min. The supernatant (600 μl) was transferred into an Axygen (Union City) 96-well plate.

2.3.2. HPLC settings

Shown in Table 1A.

2.3.3. Switching valves

The six-port valve in the heated column compartment started with position 1 → 2 and then switched at 5.00 min to 1 → 6. At the end of the run, the valve was switched back to the starting position.

The first 10-port valve was started in the position that bypassed the analytical column and was switched at 1.5 min. The valve was switched back to the bypass position at the end of the run. The second 10-port valve was switched to channel the flow from the first 10-port valve to the Chromolith RP-18e column and HN at the start of the batch analysis and remained there for the duration in which the method was used.

2.3.4. Settings used for the 4000 QTRAP®

The heated nebulizer at 600 °C was used with a curtain gas 35 psi, nitrogen collision gas (CAD) set to high, GS1 80 psi and GS2 0 psi. The nebulizer current was −3 μA with the interface heater on. The entrance potential was set to 10 V for all transitions. An intensity threshold of 0 counts per second (cps) was

Table 1A
LC program used for the analysis of equine plasma using HN in the negative mode

Step	Time (min)	Flow rate (μl/min)	A (%), 0.15% formic acid water	B (%), 10 mM ammonium acetate, ~pH 7	C (%), acetonitrile	D (%), 10 mM ammonium acetate, pH 10
(i) Quaternary pump (Agilent HP1100)						
0	0.00	4000	Not used	100	0	Not used
1	0.40	4000		100	0	
2	0.50	500		100	0	
3	1.50	500		0	100	
4	3.00	4000		0	100	
5	5.00	4000		0	100	
6	5.10	4000		100	0	
7	7.00	4000		100	0	

Step	Pump 1, Agilent HP1100		Pump 2, Shimadzu LC6A	
	Time (min)	Flow rate (μl/min) (water)	Time (min)	Flow rate (μl/min) (95:5, water:methanol)
(ii) Isocratic pumps				
0	0.00	0	0.00	300
1	0.50	3500		
2	1.50	3500		
3	3.00	0		
4	5.00	0		
5	5.10	4000		
6	7.00	4000	7.00	300

Table 1B
LC program used for the analysis of equine urine using HN in the positive mode

Step	Time (min)	Flow rate (μl/min)	A (%), 0.15% formic acid water	B (%), 10 mM ammonium acetate, ~pH 7	C (%), acetonitrile	D (%), 10 mM ammonium acetate, pH 10
(i) Quaternary pump (Agilent HP1100)						
0	0.00	2000	100	Not used	0	Not used
1	0.50	1000	70		30	
2	4.00	2000	0		100	
3	6.00	2000	0		100	
4	6.10	4000	100		0	
5	7.00	4000	100		0	

Step	Pump 1, Agilent HP1100		Pump 2, Shimadzu LC6A	
	Time (min)	Flow rate (μl/min) (water)	Time (min)	Flow rate (μl/min) (95:5, water:methanol)
(ii) Isocratic pumps				
0	0.00	0	0.00	300
1	0.50	1500		
2	4.00	0		
3	6.00	0		
4	6.10	3000		
5	7.00	3000	7.00	300

set for both MRM experiment 1 ($Q1$ and $Q3$ unit resolution) experiment 2 ($Q1$ and $Q3$ high and unit resolution, respectively), declustering potential (DP), collision energy (CE) plus other instrumental parameters used for the MRMs are shown in Table 3. The dwell time for all MRM transitions was 20 ms.

2.4. Sample preparation and instrumental settings—analysis of equine urine using HN in the positive ion mode

2.4.1. Sample preparation

Equine urine (900 μl) in an Axygen (Union City) 1.5 ml plastic microsample tube was combined with 100 μl of methanol containing 900 ng of the internal standard d_4-hydrocortisone. Particulate matter was removed by centrifugation in a Heraeus Biofuge® Pico at a RCF of 16,000 × g for 10 min. The supernatant was transferred into an Axygen (Union City) 96-well plate.

2.4.2. HPLC settings

Shown in Table 1B.

2.4.3. Switching valves

The six-port valve in the heated column compartment started with position 1 → 2 connected and then switched at 6.00 min to 1 → 6. At the end of the run, the valve was switched back to the starting position.

The first 10-port valve was started in the position that bypassed the analytical column and was switched to the column at 1.3 min. The valve was switched back to the bypass position at the end of the run.

2.4.4. Settings used for the 4000 QTRAP®

The heated nebulizer at 600 °C was used with a curtain gas 25 psi, nitrogen collision gas (CAD) set to high, GS1 80 psi and GS2 50 psi. The current was 4 μA with the interface heater on. The entrance potential was set to 10 V for all transitions. An intensity threshold of 0 cps was set for MRM experiment 1 ($Q1$ and $Q3$ unit resolution) and 100 cps for experiment 2 ($Q1$ and $Q3$ high resolution), declustering potential, collision energy plus other instrumental parameters used for the MRMs are shown in Table 4. The dwell time for all MRM transitions was 20 ms.

2.4.5. Quantitation of hydrocortisone

For the assessment of the suitability of using the +HN method for undertaking hydrocortisone quantitation, two sets of calibration samples (0, 750, 1250, 1500, 2000, 3000 and 5000 ng/ml) and spiked samples were prepared as described below. A primary stock solution (PSS), at approximately 1 mg/ml hydrocortisone, was prepared by weighing approximately 10 mg of Sigma (Singapore) hydrocortisone and dissolving this into 10 ml of methanol in a volumetric flask. The actual concentration was recorded for the following step. The PSS was used to prepare a working stock solution (WSS) by pipetting the appropriate volume of the PSS into a 10 ml volumetric flask to give a final concentration of 100 μg/ml in methanol. WSS (between 0 and 500 μl) was added to 10 ml of equine urine, which had previously been analysed and shown to contain a low level of hydrocortisone (<50 ng/ml), to produce the seven calibration levels (up to 5.0 μg/ml). Control samples at a concentration of 750 ng/ml (negative) and 1250 ng/ml (positive) were prepared in a similar manner using the SRM material. A 9.0 μg/ml solution of hydrocortisone-D_4 in methanol was prepared in a volumetric flask. Two batches of calibration samples were prepared independently. The samples and calibrators were processed as described in Section 2.4.1 above. These were analysed (in duplicate injections) in the following sequence: (a) first batch of calibrators, (b) equine urine and reagent blanks, (c) six negative control, (d) six positive control samples and lastly (e) the second calibration batch. Measurement uncertainty for the method was calculated using the 95% confidence level.

2.5. Information dependent acquisition parameters and dynamic background subtraction settings

The IDA criteria were to acquire an EPI spectrum (maximum fill time 20 ms) when the signal was greater than 1E+4 cps (acidic) and 2E+4 cps (neutral) for the most intense peak on the inclusion list within the corresponding time window. An exclusion list was used to avoid the triggering of an EPI in the time range segments before and after the one in which the analyte was expected to elute. Former target ions were always excluded after two occurrences and an exclusion list was used to avoid EPI being prematurely triggered before the time window in which the drug was expected to appear. The dynamic background subtraction settings used were an average of one background subtraction and a five data points smooth.

2.5.1. Method validation

Primary stock solutions of all the reference standards used were prepared at a concentration of either 1 or 0.1 mg/ml of methanol. The reference standard were combined into a working stock solution that gave a concentration of 1000 ng/ml for each of the targeted drugs in the equine matrix. The spiked urine and plasma samples were serially diluted 1:10 with the corresponding blank matrix to achieve a concentration of 100, 10 and 1 ng/ml. Three aliquots of each group spiked samples, along with three urine or plasma samples (urine and plasma blanks), taken from the stock used to prepare the spiking were processed along with the spiked samples. To assess the intra-day variability, six spiked urine samples were analysed using the relevant method and to assess the inter-day variability, this was done on 3 separate days. A drug was considered as detected if its MRM peak area was >1E+3 and the S/N (calculated using the standard deviation of noise) was >10 at the corresponding retention time for the authentic reference standard. The specificity of the method was evaluated by examining the data obtained from the analysis of the matrix blank samples and, where necessary, instrumental settings (e.g. increasing $Q1$ and/or $Q3$ resolution) were adjusted until the blank samples generated no significant peaks in the relevant time window.

2.6. Thoroughbred racehorse drug administration

2.6.1. Phenylbutazone administration

Blood samples from 27 race conditioned horses (approximately 500 kg body mass) were collected into 8 ml Vacuette® (Greiner Bio-one, Kremsmuenster, Austria) lithium heparin gel separator tubes before administration and then at 6 and at 24 h after intravenous administration of 2 g phenylbutazone per racehorse. Samples were centrifuged to separate the plasma from the blood cells and then stored at 4 °C. When required for analysis, the samples were left to reach ambient temperature (approximately 22 °C) before being processed as a composite of two (with a blank plasma) using the methods described in Section 2.3.1.

2.6.2. Betamethasone intra-articular injection

A race conditioned thoroughbred racehorse was administered of Celestone Chronodose® (Schering-Plough, Singapore) by intra-articular injection of 1 ml (betamethasone 5.7 mg in total, as betamethasone sodium phosphate 3.9 mg plus betamethasone acetate 3 mg in an aqueous vehicle) into both the left and right front leg joints. A naturally voided urine sample was collected 36 h after administration and stored at 4 °C for 1 week before analysis.

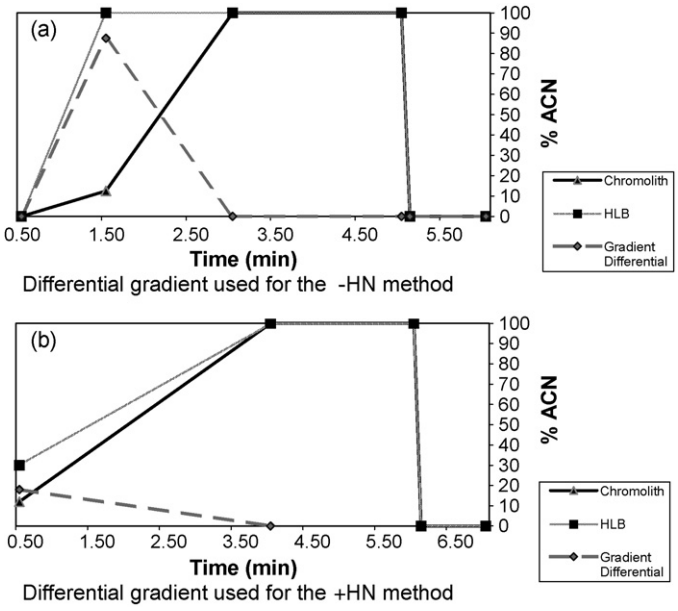

Fig. 1. Differential gradient for isolation, refocusing and separation of: (a) acidic drugs in protein-precipitated plasma samples and (b) neutral drugs in equine urine samples.

3. Results and discussion

Apart from a minor modification, which involved the addition of second analytical column connected to the HN interface of the DuoSpray® source, the instrumental configuration used for both of these methods was the same as described previously [7] for screening 250 basic drugs in equine urine. This use of common flow paths and an identical solid phase extraction platform (HLB®) for extracting both the TurboIonSpray® (TIS) and HN responsive drugs, allows batches to be screened without any re-plumbing of the LC or other operator intervention being necessary when switching between the methods. There are obvious time and manpower savings generated by using the instrument in this fashion.

We undertook pretreatment of the plasma samples using protein precipitation, as this has been reported to assist in releasing protein bound drugs [15] and enhance method robustness [16]. After evaluating the outcome of using different ratios of the precipitant, we elected to use an equal volume of acetonitrile, as enough of the protein was precipitated from the plasma to allow for more than 100 injections of the supernatant without causing a noticeable increase in the LC system backpressure. Using a 1:1 ratio has an added advantage because the target drugs, when dis-

Table 2
Peak area and retention time reproducibility data for +HN and −HN methods (100 ng/ml level)

	Intra-assay ($n=6$)			Inter-assay ($n=3$)
	Day 1	Day 2	Day 3	
(A) Analyte peak area				
(a) Hydrochlorothiazide				
Average cps[a]	5.6E+05	4.5E+05	4.9E+05	5.0E+05
%R.S.D.	16.2	21.0	19.2	11.8
(b) Dexamethasone				
Average cps	3.6E+04	3.4E+04	2.6E+04	3.2E+04
%R.S.D.	17.9	21.7	22.9	17.0
(B) Retention time				
(a) Hydrochlorothiazide				
Average RT[b] (min)	3.358	3.357	3.358	3.358
%R.S.D.	0.22	0.30	0.51	0.02
(b) Dexamethasone				
Average RT (min)	2.518	2.528	2.525	2.524
%R.S.D.	0.30	0.30	0.42	0.20

[a] cps, counts per second.
[b] RT, retention time.

solved in (50:50) acetonitrile:water, were retained on the HLB® solid phase and, hence, dilution of the supernatant with water prior to injection was not required. Furthermore, signal suppression did not appear to be an issue when using this procedure, as both plasma and water sample spikes treated in this manner gave approximately the same peak height for all of the target drugs.

Preparation of the urine samples used for the neutral drug analysis was by dilution of the sample 9:1 with methanol, as described previously [7], followed by centrifugation to eliminate the precipitate that formed. In our experience, this procedure assists in reducing the material that is found in many equine samples, that precipitates, coagulates or, by some other mechanism, blocks the flow path after injection.

Monolithic columns have become increasingly popular over the past few years and we selected this type for separating both the acid and neutral drugs that had been trapped on the extraction cartridge. Our decision was made upon the basis that monoliths produce a lower backpressure than conventional packed LC columns [17] and this made them better suited to our objective of developing a rapid separation of the compounds of interest by using the highest mobile phase flow rate that was compatible with the API source. While it was evident that some chromatographic peak resolution was sacrificed using this approach, we decided that this was not a critical issue, because we intended to use the mass spectrometer to resolve any co-eluting drugs.

The 4000 QTRAP® was set up to undertake rapid MRM survey scanning for the analytes using the triple quadrupole (QqQ) mode. This enabled us to continuously monitor the analytical column eluent to establish whether any of the target analytes were present and the more time consuming EPI scan mode was

Table 3
MRM acquisition parameters and the limit of detection achieved for negative HN target analytes

Number	Drug name	From sup.	MRM transition parameters				RT[a] (min)	Detected at spiked level?[b] (ng/ml)			
			*Q*1	*Q*3	DP	CE		1	10	100	1000
(A) MRM experiment 1 (*Q*1 + *Q*3 unit resolution)											
1	Butalbital	1	223.4	180.2	−52	−16	2.61		Y	Y	Y
2	Carboxy celecoxib	5	410.1	366.2	−85	−30	2.52		Y	Y	Y
3	Cromolyn	1	467.0	379.0	−55	−22	2.40			Y	Y
4	Ethoxzolamide	2	257.3	178.2	−40	−26	2.69		Y	Y	Y
5	Fenbufen	1	253.4	209.3	−40	−14	2.57	Y	Y	Y	Y
6	Hydroxy celecoxib	5	396.1	302.2	−78	−37	2.71		Y	Y	Y
7	Meclofenamic acid	1	294.2	258.2	−68	−18	2.66		Y	Y	Y
8	Oxaprozin	1	292.3	220.3	−69	−32	2.61			Y	Y
9	Secobarbital	2	238.3	195.3	−57	−17	2.69		Y	Y	Y
	Phenobarbitone-D_5[c]	7	236.0	193.1	−40	−17	2.60	NT[d]	NT	NT	Y
(B) MRM experiment 2 (*Q*1 high + *Q*3 unit resolution)											
10	Althiazide	1	382.2	341.2	−62	−21	2.65		Y	Y	Y
11	Amobarbital	2	225.3	182.3	−69	−17	2.65		Y	Y	Y
12	Aprobarbital	2	209.3	166.2	−41	−16	2.61		Y	Y	Y
13	Bendroflumethiazide	1	420.2	289.3	−109	−36	2.73	Y	Y	Y	Y
14	Benzthiazide	1	430.2	308.2	−93	−35	2.65	Y	Y	Y	Y
15	Brinzolamide	2	382.2	217.2	−114	−30	2.61		Y	Y	Y
16	Butabarbital	2	211.0	168.2	−47	−18	2.61		Y	Y	Y
17	Celecoxib	3	380.3	316.3	−116	−33	2.90	Y	Y	Y	Y
18	Chlorthalidone	1	337.0	190.0	−87	−28	2.56		Y	Y	Y
19	Diclofenac	1	294.2	250.2	−37	−12	2.61	Y	Y	Y	Y
20	Flufenamic acid	1	280.3	236.3	−62	−23	2.65	Y	Y	Y	Y
21	Flunixin	2	295.4	251.4	−82	−24	2.61		Y	Y	Y
22	Hydrochlorthiazide	1	295.9	205.0	−77	−36	2.52	Y	Y	Y	Y
23	Ketoprofen	1	253.0	209.3	−68	−12	2.57		Y	Y	Y
24	Mefenamic acid	1	240.2	196.3	−52	−25	2.69	Y	Y	Y	Y
25	Meloxicam	4	350.0	286.2	−47	−21	2.56		Y	Y	Y
26	Methyclothiazide	2	357.9	322.0	−26	−19	2.61	Y	Y	Y	Y
27	Meticrane	1	274.0	210.1	−85	−30	2.56			Y	Y
28	Nimesulide	1	307.0	229.1	−61	−27	2.76	Y	Y	Y	Y
29	Phenylbutazone	1	307.2	279.2	−54	−26	2.65	Y	Y	Y	Y
30	Picrotoxin	1	309.1	193.1	−58	−25	2.56		Y	Y	Y
31	Tolfenamic acid	1	260.0	216.0	−53	−21	2.69	Y	Y	Y	Y
32	Trichlormethiazide	1	284.0	248.1	−81	−26	2.51		Y	Y	Y
			Detected/total tested					12/32	29/32	32/32	32/32
			Percentage					38	91	100	100

[a] RT, averaged retention time.
[b] Y, detected.
[c] Denotes the internal standard.
[d] NT, not tested.

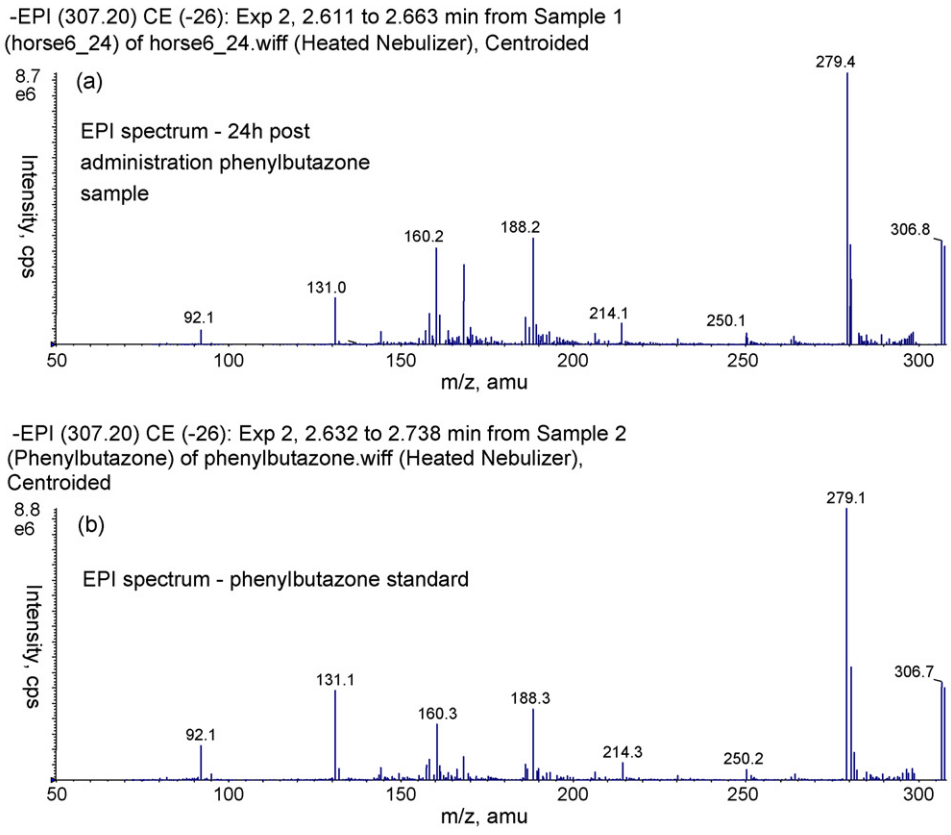

Fig. 2. EPI spectrum obtained from the phenylbutazone 24 h post-administration sample (a) and phenylbutazone reference standard (b).

only triggered if a target signal exceeded the defined IDA criteria. Inclusion plus exclusion lists were used in combination with DBS to enhance the effectiveness of the data dependent acquisition process. The Analyst® quantitation and query wizard was also used to flag all the MRM peaks that fell within the expected retention time window and exceeded the intensity threshold. This list was manually cross-checked against the IDA generated data and, if the software had failed to trigger the

Table 4
MRM acquisition parameters and the limit of detection achieved for positive HN target analytes

Number	Drug name	From sup.	MRM transition parameters				Average RRT[a] (min)	Detected at spiked level?[b] (ng/ml)			
			*Q*1	*Q*3	DP	CE		1	10	100	1000
(A) MRM experiment 1 (*Q*1 + *Q*3 unit resolution)											
1	Amcinonide	2	503.3	399.3	66	12	1.38			Y	Y
2	Betamethasone	1	393.4	373.4	25	13	1.08	Y	Y	Y	Y
3	Dexamethasone	1	393.4	373.4	25	13	1.10	Y	Y	Y	Y
4	Flunisolide	2	435.4	321.3	65	22	1.12	Y	Y	Y	Y
5	Triamcinolone acetonide	1	435.4	321.3	65	22	1.11	Y	Y	Y	Y
6	Fluorometholone	1	377.2	339.1	27	23	1.16	Y	Y	Y	Y
7	Methylprednisolone	1	375.2	253.2	61	28	1.05		Y	Y	Y
8	Prednisolone	1	361.2	279.4	40	22	0.99			Y	Y
9	Prednisone	1	359.2	295.2	59	20	1.00		Y	Y	Y
(B) MRM experiment 2 (*Q*1 + *Q*3 high resolution)											
10	Capsaicin	1	306.2	137.0	57	23	1.30	Y	Y	Y	Y
11	Hydrocortisone	1	363.2	327.0	66	23	1.00	NT[c]	NT	Y	Y
	Hydrocortisone-D_4[d]	6	367.2	331.2	109	23	1.00	NT	NT	Y	Y
			Detected/total tested					6/10	8/10	11/11	11/11
			Percentage					60	80	100	100

[a] RRT, relative retention time (relative to IS at 2.99 min).
[b] Y, detected.
[c] NT, not tested.
[d] Denotes the internal standard.

required EPI scan, the sample was reanalysed using a non-IDA method to obtain the desired data.

When analysing the acidic drugs, we selected a differential gradient (Fig. 1a) with a large percentage difference in the content of organic solvent between the extraction and analytical column during the first 1.5 min and, thereafter, the differential was reduced in a linear manner to zero at 3.1 min. This program permitted the drugs to be trapped and then eluted off the analytical column into the HN with an average peak width (FWHH) of 0.09 min and the asymmetry value ranged between 1.0 and 1.3 (10% peak height). The reproducibility of the analyte peak area and retention time was evaluated and Table 2 provides the intra- and inter-assay %R.S.D. values obtained for hydrochlorothiazide at the 100 ng/ml spiking level. These data are broadly representative of the variation observed for all the acidic analytes where, on average, the inter-assay R.S.D. for the retention time of the standards was 0.47% and the inter-assay R.S.D. for the individual peak areas was <25%. These results compare favourably with other methods [18–21] that have been proposed for the direct-injection LC analysis of acidic drugs in plasma samples.

The multiple reaction monitoring settings used to monitor the 32 analytes that we targeted using −HN conditions are listed in Table 3. Achieving maximum sensitivity was not our foremost priority, as high concentrations of these drugs were anticipated in samples from doped horses. Hence, during the method development stage we started out using the high-resolution setting for $Q1$ to take advantage of the additional selectivity that was obtained when using this configuration. However, it became apparent that this increased the lower LOD for some targets beyond a level that we considered satisfactory for our purpose and these transitions were shifted into a separate experiment using the more sensitive, but less specific, unit resolution setting for both $Q1$ and $Q3$. The results, shown in Table 3, demonstrate that the combination of two MRM experiments provided an acceptable LOD for this group of drugs. We believe that our results demonstrate that our long-standing practice of pooling samples into composites of two to enhance the speed and efficiency of the sample batch processing can continue without unreasonably compromising the post-administration detection time window for the target drugs.

The applicability of this method for detecting acid drugs in post-administration samples during the interval when the drug is likely to be having maximal effect was evaluated by analysing blood samples collected from 27 horses at 6 and at 24 h after intravenous administration of phenylbutazone. The unchanged drug was detected in all samples and negative (i.e. below LOD) results were obtained from the analysis of more than 300 blood samples taken from horses between 110 and 158 h after administration. These data gave us confidence that the method would be able to detect administrations that were probably having an effect on the horse and, at the same time, gave us assurance that this can be done without inadvertently producing positive results from samples collected from horses which were treated several days beforehand. Positive screening results can also be confirmed using this method. For example, Fig. 2 shows that the EPI spectrum of the 24 h sample (a) and the phenylbutazone standard (b) are a very acceptable match and, consequently, we anticipate that undertaking a confirmatory analysis of a positive sample would provide similarly definitive data on which to base the decision.

The differential gradient (Fig. 1b) adopted for the neutral drugs was markedly different from the one described above for acidic drugs. The differential at the start of the run was the relatively small value of 20% and was reduced in a linear fashion to 0% at 4 min. When using this gradient, the peaks had a FWHH of 0.07 min and an asymmetry values that ranged from 1.1 to 1.6. Table 2 gives the inter- and intra-assay peak area and retention time reproducibility for dexamethasone at the 100 ng/ml level. These results are very similar to those obtained from the analysis of the other neutral analytes spiked at this level, where the %R.S.D. of the analyte peak areas and retention times were less than 25 and <0.5%, respectively. In our preliminary work, analytes were screened using unit setting for $Q1$ and $Q3$ to get the maximal sensitivity. However, after evaluating the first set of results, we became aware that we needed to modify the method to eliminate an interfering peak observed, within capsaicin's expected retention time window, in a few urine blank samples. This issue was resolved by relocating this MRM transition to the high resolution setting for both $Q1$ and $Q3$. To enhance the accuracy of the quantitation analysis, hydrocortisone and the internal standard were also included in the experiment with higher resolution settings.

Table 4 shows the LOD achieved for the neutral drugs spiked into blank urine samples. In all cases, these were deemed as adequate for our purposes, as fortuitously, the lowest sensitiv-

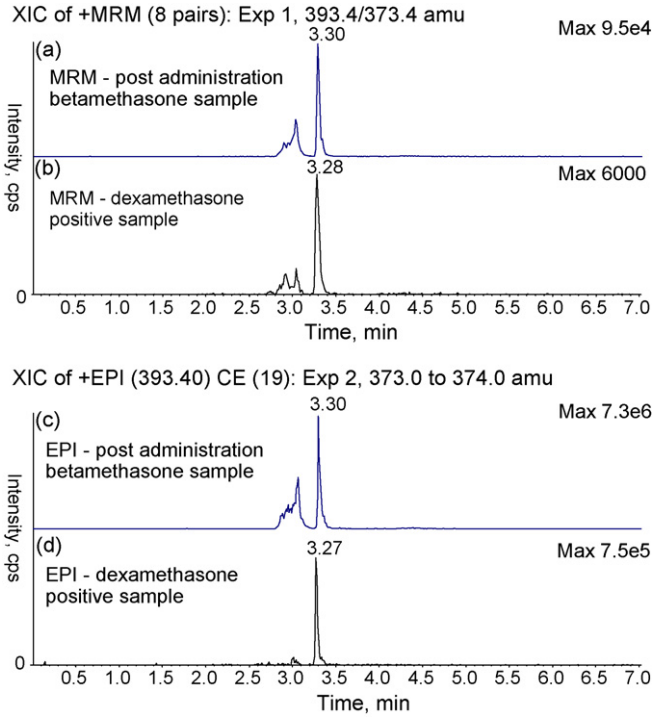

Fig. 3. Extracted ion chromatograms from MRM (393.4 → 373.4) and EPI (range *m/z* 373.0–374.0). (a and c) From the Celestone® post-administration and (b and d) from the dexamethasone positive sample.

ity was observed for those corticosteroids that are generally given to horses in larger doses. Therefore, correspondingly higher concentrations would be anticipated post-administration of these particular drugs. The more potent synthetic corticosteroids were detected at the 1 ng/ml level and this is compatible with the 2 ng/ml dexamethasone Minimum performance criteria that was established by the International Federation of Horseracing Authorities in Article 6 of the IABRW [14]. This LOD is also effective from a practical viewpoint as, for example, the urine level for an intra-muscular administration of 26 mg of dexamethasone sodium phosphate was reported as 1.2 ng/ml in a 48 h urine sample [22]. Therefore, this direct-injection screening method would give a detection period for dexamethasone IM administration that would cover the period over which this drug is expected to exert a marked effect on the racehorse. As discussed above, undertaking this screening analysis on urine samples has the disadvantage of producing greater uncertainty with respect to the actual detection window for therapeutic drugs. However, if so desired, a corticosteroid (other than hydrocortisone) detected on direct-injection screening of the urine sample could be confirmed by using one of the published extraction methods for the determination of these compounds in equine plasma (e.g. refs. [23,24]) and this would help to address this issue of detection time variability.

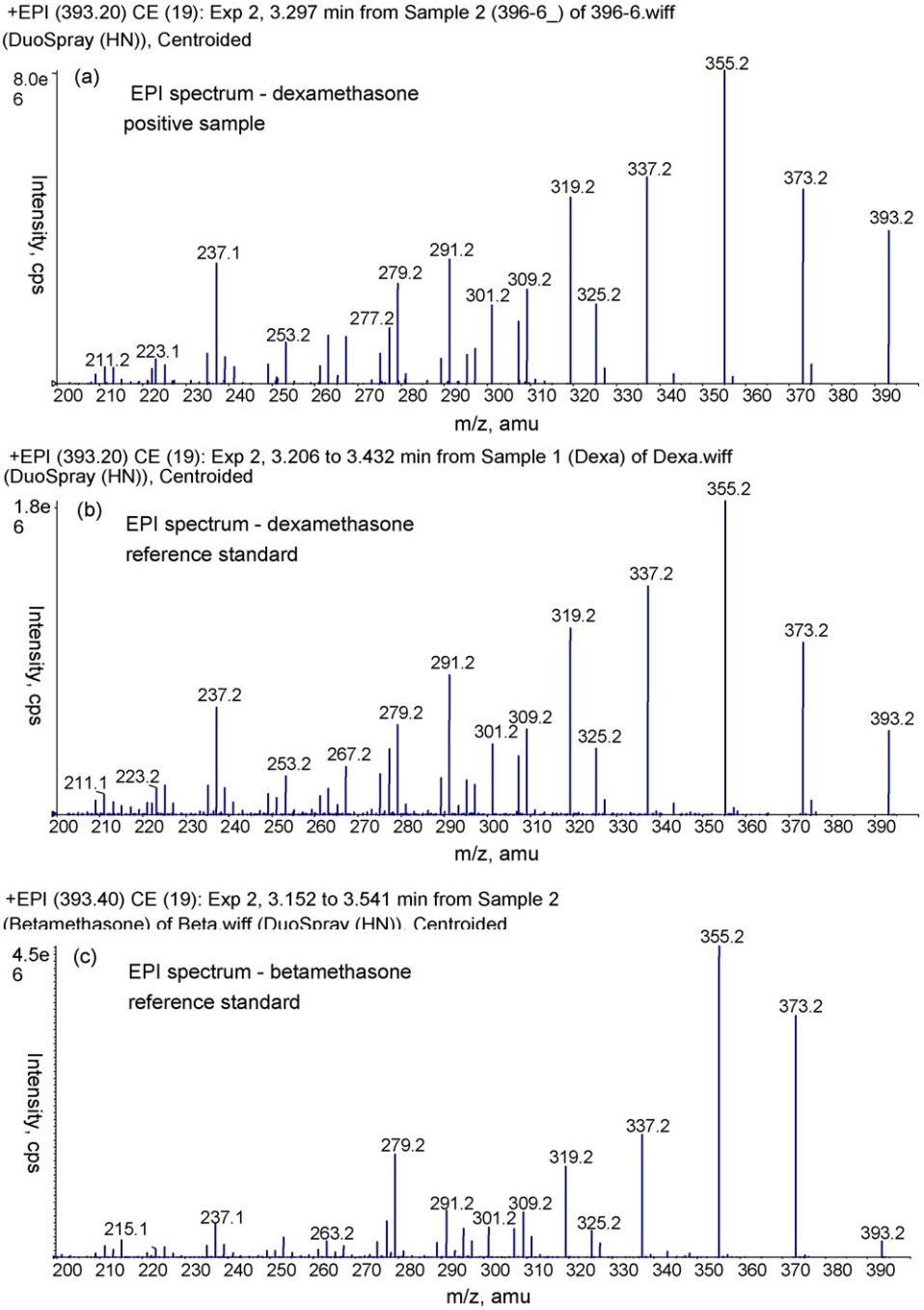

Fig. 4. EPI spectra obtained from the positive sample (a), dexamethasone reference standard (b) and (c) betamethasone reference standard.

Under the IABRW, the endogenous corticosteroid hydrocortisone's regulatory threshold is set at 1 μg/ml of equine urine. Consequently, we considered that it was important that any screening method that targeted corticosteroids should be able to flag samples that potentially exceeded this limit. To assess this capability we used calibrators to produce a calibration curve and then quantitated "unknown" samples spiked with the NIST hydrocortisone SRM (98.9 ± 0.2%) to assess the suitability of this approach as a way of screening out any samples that would require confirmatory quantitative analysis. The linearity of the averaged (two batches in triplicate) calibration curve was $r^2 = 0.9994$ and we were able to correctly identify all six of the *positive* control (+25%) spiked samples analysed as exceeding the threshold. All of the *negative* control (−25%) samples were classified as falling below the threshold. These results are consistent with our calculated measurement uncertainty value of ±0.2 μg/ml at the 95% confidence level.

Anabolic steroids are an important pharmacological subgroup that fall within the neutral drugs commonly screened for in equine doping surveillance. For this reason, we included the MRM transitions for monitoring these drugs in our initial +HN method set up. However, limited separation between these reference standards was observed when using the LC–LC gradient described here and, more importantly, highly similar EPI spectra were produced from fragmentation of endogenous and exogenous steroids of the same chemical formula. Therefore, the method was found by us to be generally unsuited for detection of the majority of the anabolic steroids we analysed and the entire sub-group was dropped from the screening method. We intend to revisit this issue after our library of anabolic drugs has been expanded by the acquisition of greater number of reference standards that have a chemical composition that is sufficiently different from the commonly encountered equine endogenous anabolic steroids.

The effectiveness of this neutral drug screen was evaluated by analysing urine from a racehorse given betamethasone by intra-articular administration and a post-race sample that had previously been confirmed to be positive for dexamethasone by using solid phase extraction coupled to API-LC/MS/MS using a chiral separation column. Fig. 3a and b shows the extracted ion chromatogram for the 393 > 373 MRM transition used for the analysis of the dexamethasone positive and betamethasone administration sample, respectively. In both cases, the target drug was clearly detected. Fig. 3c and d shows a similar pattern for the extracted ion range (*m*/*z* 373–374) from EPI scans of the precursor ion (*m*/*z* 393) of these samples and it can be seen that the dexamethasone positive sample's spectrum (Fig. 4a) is a good match to the one obtained from the dexamethasone standard (Fig. 4b). The match between the sample and the betamethasone standard's spectrum (Fig. 4c) is not as good and this would provide useful collateral information that will assist in the differentiation of these isomers.

4. Conclusion

We have demonstrated that it is possible to use differential gradient LC–LC coupled with a hybrid MS/MS to undertake a broad-based direct-injection screen for 32 acidic and 11 neutral drugs in plasma and urine, respectively. While the total number of targeted compounds pales in comparison with our previously published method screens for 250 analytes, this is simply a reflection of the fact that the vast majority of standards in our possession produced their optimal response under TIS rather than the HN conditions used. Consequently, from the beginning, only a small number of our available drugs were allocated for analysis by the −HN and +HN methods. Ultimately we anticipate that the total number of targets will increase in tandem with our continuing expansion of our library of reference drug standards and there is reason to think that eventually we will be able to screen for hundreds of drugs per analytical run. The cost effective approach of using a single instrument to undertake several complementary broad-spectrum screens was of great benefit in helping us to keep with the rapid expansion in the number of potential doping agents. Moreover, the simplicity of the sample preparation steps, coupled with the speed of analyses, permits a higher sample throughput and this has aided the overall efficiency of our laboratory's operations.

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