Research Article

Drug Testing and Analysis

Received: 14 August 2009 Revised: 20 November 2009 Accepted: 20 November 2009 Published online in Wiley Interscience: 9 February 2010

(www.drugtestinganalysis.com) DOI 10.1002/dta.102

Simultaneous separation and confirmation of amphetamine and related drugs in equine plasma by non-aqueous capillary-electrophoresis-tandem mass spectrometry

X. Q. Li,[a] C.E. Uboh,[a,b]* L. R. Soma,[a] F. Y. Guan,[a] Y. W. You,[a] M. C. Kahler,[b] J. A. Judy,[b] Y. Liu[a] and J. W. Chen[a]

A non-aqueous capillary electrophoresis-mass spectrometry (NACE-MS) method was developed for simultaneous separation and identification of 12 amphetamine and related compounds in equine plasma. Analytes were recovered from plasma by liquid-liquid extraction using methyl tertiary butyl ether (MTBE). A bare fused-silica capillary was used for separation of the analytes. Addition of sheath liquid to the capillary effluent allowed the detection of the analytes by positive electrospray ionization mass spectrometry using full scan data acquisition. The limit of detection (LOD) for the target analytes was 10–200 ng/mL and that of confirmation (LOC) was 50–1000 ng/mL in equine plasma. Capillary electrophoresis (CE) and mass spectrometry (MS) parameters were optimized for full CE separation and MS detection of the analytes. Separation buffer comprised 25 mM ammonium formate in acetonitrile/methanol (20: 80, v/v) plus 1 M formic acid. Sheath liquid was isopropanol-water-formic acid (50 : 50:0.5, v/v/v). Samples were hydrodynamically injected and separated at 25 kV. Analytes were electrokinectically separated and mass spectrometrically identified and confirmed. This simple, fast, inexpensive and reproducible method was successfully applied to post race equine plasma and research samples in screening for amphetamine and related drugs.

Keywords: amphetamine; non-aqueous capillary electrophoresis; mass spectrometry

Introduction

Amphetamine and related drugs have been used and abused for a long time in sports, in the work place, and by recreational users because of their stimulatory effect on the central nervous system. Their popularity is attributed to the psychotropic and entactogenic properties.[1] The methylenedioxy-derivatives of amphetamine are the largest group of drugs that are most widely abused. These agents have gained notoriety because of their ability to induce strong feelings of empathy and comfort out of euphoria. They are used in human or equine athletes to alter performance, and thus, are included in the World Anti-Doping Agency (WADA) and the Association of Racing Commissioners International (ARCI) list of banned substances.[2–3] Commonly used methods in the analysis of these drugs include enzyme-linked immunosorbent assays (ELISA), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). ELISA has been the simplest, relatively inexpensive and most widely used method in target drug screening for these agents.[4–7] The low specificity of antibodies in recognizing the drugs, however, has led to a number of false positive test results, and above all, ELISA does not differentiate between amphetamine analogs. To overcome these drawbacks, GC-MS was the first technique used in the analysis of a large number of samples with high specificity and sensitivity for these drugs.[8–13] The major disadvantage with GC-MS is that it requires extremely tedious derivatization procedures, making this technique time-consuming and inefficient in the analysis of samples on a large scale. The use of LC-MS has grown rapidly in popularity in forensic and clinical applications; and thus, rapid and accurate detection of these potential drugs of abuse in athletes have been made possible.[14–20] The major advantage of LC-MS over GC-MS, besides sensitivity and selectivity, is that it offers high-throughput capability and less sample manipulations. Potential problems, however, such as leakage from high pressure and clogging of column from small particles make sample implementation on LC-MS a little more challenging than that in CE-MS. CE-MS is a good orthogonal technique to LC-MS. CE is driven by high voltage not high pressure as in LC-MS, and it is an open channel, which make it less prone to leakage

* *Correspondence to: Dr C.E. Uboh, University of Pennsylvania, School of Veterinary Medicine, New Bolton Center Campus, Department of Clinical Studies, 382 West Street Road, Kennett Square, PA, 19348, USA. E-mail: ubohcorn@vet.upenn.edu*

a *University of Pennsylvania, School of Veterinary Medicine, New Bolton Center Campus, Department of Clinical Studies, 382 West Street Road, Kennett Square, PA, 19348, USA*

b *Pennsylvania Equine Toxicology & Research Center, Department of Chemistry, West Chester University, 220 East Rosedale Avenue, West Chester, PA 19382, USA*

and offer better reproducibility. CE-MS combines high resolving power and separating speed of CE and the sensitivity and analyte information (e.g., molecular mass and structure elucidation) provided by MS. Separation by CE covers a broader scope of compounds including small molecules, peptides, biomarkers, and proteins, especially for polar compounds. In addition, CE is free of stationary phase, so it is much easier to change background electrolyte (BGE) and the column is less expensive than that of high performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC). CE consumes very small sample and buffer solution, especially organic solvents, thus making it a greener technique as opposed to HPLC or UPLC.

The limitation in CE-MS is that a number of buffers such as borate and phosphate commonly used in CE[21] are incompatible with the electrospray ionization source in MS. In an attempt to avoid the problem of ion suppression and the clogging of the ion inlet tube due to salt deposition and solidification from non-volatile buffer, non-aqueous capillary electrophoresis (NACE), involving volatile electrolytes and organic solvents has gained popularity in CE-MS.[22]

In the present study, we took advantage of the compatibility of NACE with mass spectrometer for simultaneous separation and identification of amphetamine and its structurally and pharmacologically related chemical compounds. There are many elegant publications in the literature on the analysis of amphetamine and related drugs by CE-MS.[23–27] Among these, CE-MS in aqueous media with volatile solvents has been largely used, but the problem of 'caking' out of solutes at high capillary temperature greatly affects widespread application of this technique. In NACE, the physical and chemical properties of organic solvents used are different from those employed in aqueous capillary electrophoresis (ACE), and these make NACE the separation buffer of choice which is very compatible with MS for enhancing resolution, selectivity, and sensitivity in the analysis of compounds with similar chemical structures. To our knowledge, we are unaware of any publication on the simultaneous separation of these 12 amphetamine-related drugs in equine plasma by NACE-MS. It is the desire of the Pennsylvania (PA) Racing Commissions to provide a level playing field to all participants in the sport of horse racing. Thus, every effort is made to protect the interests of the betting public and the welfare of the horse by ensuring that all horses competing in PA are free of amphetamine, amphetamine-related drugs, or any other performance-enhancing substances during competition. Thus, zero is the only acceptable plasma concentration of these compounds in the horse during competition. Confirmation of the presence of any of these compounds alone would be sufficient evidence to report a positive drug finding to the Racing Commission. Based on this premise, quantification was not necessary in the present study. This study was therefore undertaken to develop a method for simultaneous detection and confirmation of amphetamine and related compounds (Figure 1) of high abuse potential in equine sport using NACE-MS.

Experimental

Chemicals and reagents

Stock solutions (each1.0 mg/mL in methanol) of amphetamine (A), methamphetamine (MA), ephedrine (EPH), pseudoephedrine (PSE), phentermine (PHE), N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB), 3,4-methylenedioxyamphetamine (MDA), p-methoxymethamphetamine (PMMA), 3, 4-methylenedioxyethylamphetamine (MDE), 3,4-methylenedioxymethylamphetamine (MDMA) and 4-bromo-dimethoxyphenethylamine (BDM)) were purchased from Cerilliant (Round Rock, TX, USA). The powder form of 3, 4-methylenedioxypropylamphetamine (MDPA) was obtained from Sigma (St Louis, MO, USA). HPLC grade acetonitrile, methanol and isopropanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water (HPLC grade) was obtained from Chata (Fort Collins, CO, USA). Ammonium acetate and ammonium formate (HPLC grade) were purchased from Spectrum Laboratory Products (Garden, CA, USA). Methyl tertiary-butyl ether (MTBE) and formic acid (98%) were obtained from EM Science (Gibbstown, NJ, USA).

Preparation of saturated sodium borate solution (pH10)

Sufficient sodium borate was added to a beaker containing 1L water heated at 40 °C while stirring on a magnetic stirrer and adding more sodium borate until the solute could no longer dissolve in the solution. The mixture was then allowed to cool to room temperature; pH was adjusted to 10 with 50% sodium hydroxide, and it was labelled and stored at room temperature.

Standard solutions

Stock solution of MDPA was prepared in methanol. MDPA was weighed (5.0 mg) into a 10 mL brown glass bottle and 5.0 mL methanol was added to prepare a stock solution of 1.0 mg/mL. All other stock solutions were purchased from Cerilliant (1.0 mg/mL methanol). Serial dilutions of the standard solution of each analyte in the separation buffer were prepared from the stock solutions. A mixture of the standard solution of the 12 analytes was prepared by adding 20 µL stock solution of each analyte into a 5 mL brown glass bottle, and then 1760 µL separation buffer was added to obtain 10 ng/µL of each analyte. All standard solutions were stored at 4 °C and discarded after 90 days.

Preparation of non-aqueous buffer

Ammonium formate (25 mM) and formic acid (1 M) in acetonitrile/methanol (20: 80, v/v) mixture was used as the separation buffer. For preparation of the buffer, 1.6 g ammonium formate was dissolved in 200 mL methanol. This solution was transferred into a volumetric flask (1 L), formic acid (39 mL) and acetonitrile (200 mL) were added and mixed. Sufficient methanol was added to bring it to1L. The buffer was filtered through 0.45 µm microfilter (Whatmann, Clifton, NJ, USA) and degassed for 10 min in an ultrasonic bath, containing 300 mL distilled water at room temperature prior to use.

For optimization of CE-MS parameters, different concentrations (5 mM, 10 mM, 25 mM, 50 mM and 75 mM) of ammonium formate were used to compare the resolution and stability of current supply. Various ratios of acetonitrile to methanol in the separation buffer (v/v) at 5 : 95, 10 : 90, 20 : 80, 30 : 70, 50 : 50, 70 : 30, 80 : 20 and 0 : 100 were also compared. Different concentrations of formic acid (0.5M, 1.0M and 1.5M) were evaluated for peak shape, resolution and sensitivity by enhancing ionization.

Sample collection and preparation

Post-race blood samples were collected from the first three winners on a race card and any specials at the discretion of the judges, and delivered to our laboratory for forensic analysis. Samples

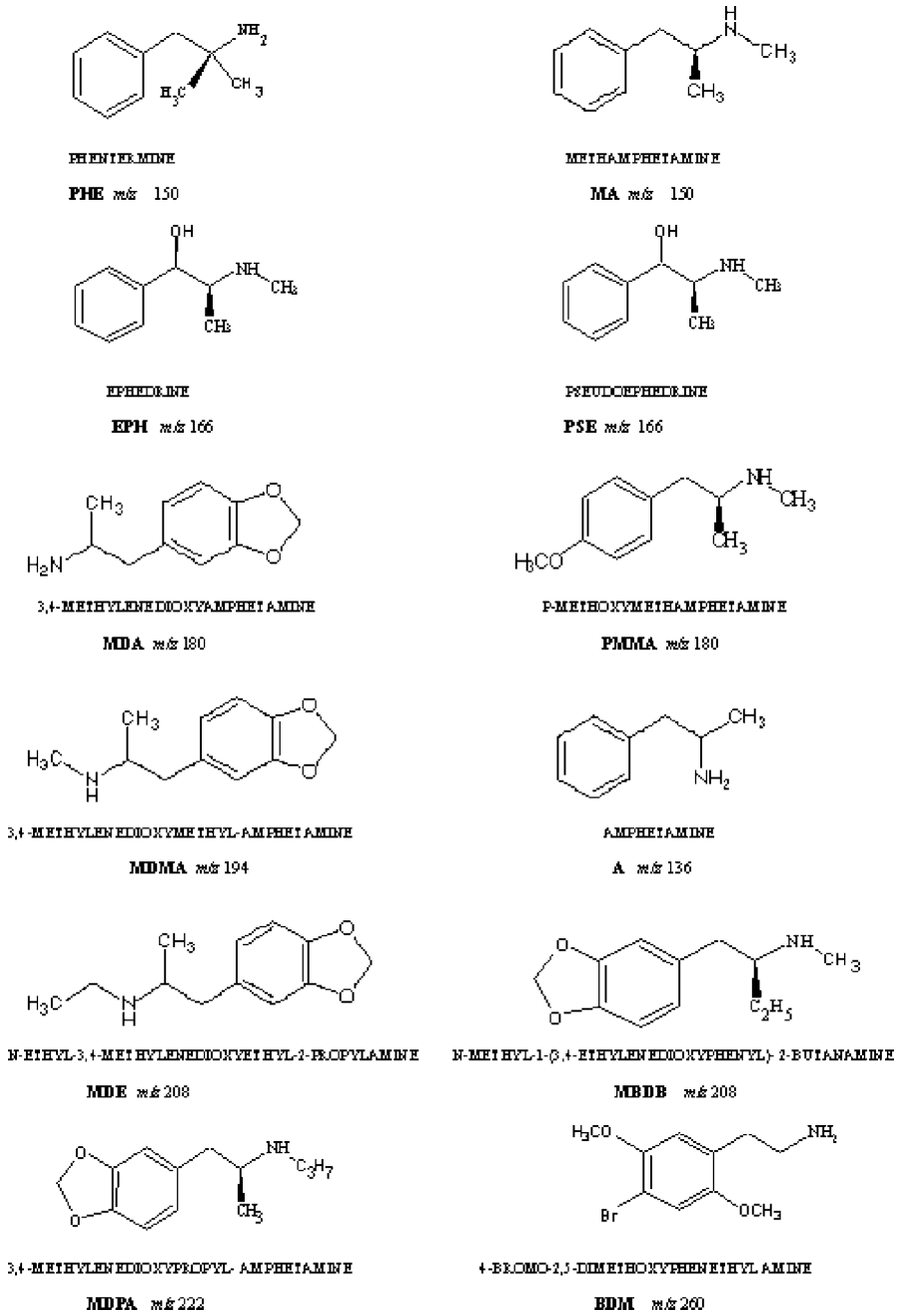

Figure 1. Chemical structures of the analytes.

were centrifuged at 3000 rpm (1610 × g) to obtain plasma. The harvested plasma was stored at 4 °C pending analysis. To each plasma sample (1 mL) in a 16 × 150 mm test tube, saturated sodium borate buffer (1 mL; pH 10) was added and thoroughly mixed by vortex prior to adding 5 mL MTBE and mixing. The tube was capped and mixed on a rotorack for 10 min, and centrifuged at 3000 rpm (1610 × g) for 10 min. The organic layer (top) was transferred into a pre-labeled culture tube (12 × 75 mm) and the content was dried at 50 °C (Techni-Dri-Block DB-3, Duxford, Cambridge, UK) under a steady flow of air or nitrogen until approximately 1 mL was left to which 10 µL freshly prepared HCl in methanol (10%, v/v) was added to induce salt formation and prevent splashing of the analytes along the sides of the tube during drying; drying was continued to completion. The dried residue was reconstituted in 60 µL methanol-buffer (90 : 10, v/v) and mixed by vortex for 10 sec. The sample was then transferred into a 0.2 mL PCR tube (Axygen, Union City, CA, USA), capped and centrifuged at full speed for 15 min in a microcentirfuge (Fisher Scientific, San Jose, CA, USA) to settle any particulates. The sample was now ready for NACE-MS analyses.

Preparation of plasma calibrator

An aliquot (100 µL) of the standard solution mixture (10 ng/µL of each analyte) was added into 0.9 mL blank equine plasma and mixed by vortex for 10 s. The plasma sample was extracted, using the method described above for sample preparation. The reconstituted extract was used as calibrator in the analysis of test samples.

Matrix effects

Matrix effect on ionization of amphetamine and related drugs was evaluated. Blank plasma (1.0 mL) was extracted as described under sample preparation; 100 µL of the standards mixture (10.0 ng/µL) was added to the drying extract of blank plasma when nearly 1.0 mL was remaining and drying was continued to dryness. The dried residue was reconstituted in 60 µL methanol-buffer (90 : 10, v/v). Water (1.0 mL) instead of plasma was used to compare matrix effect contributed by plasma with that of water. The same volume of standard solution was added in the drying extract of water as described above. The dried residue was reconstituted in 60 µL methanol-buffer (90 : 10, v/v). Matrix effect was calculated by comparing the peak intensity of each analyte in the extracted plasma and that in water according to the Eqn (1).

$$\text{Matrix effect (\%)} = (I_{\text{water extract}} - I_{\text{plasma extract}})/I_{\text{water extract}} \times 100 \quad (1)$$

where $I_{\text{plasma extract}}$ was the intensity of an analyte in blank plasma extract; and $I_{\text{water extract}}$ was that of an anlayte in the extracted water. Negative and positive values indicated plasma-induced ion suppression or enhancement effect on the analyte, respectively.

Capillary electrophoresis

Capillary electrophoresis experiments were performed on a PACE/MDQ (Beckman Coulter, Fullerton, CA, USA) equipped with a diode-array detector, autosampler and power supply for delivering high voltage (30 kV). The experiments were carried out using cationic mode (anode (+) at the inlet and cathode (−) at the outlet). Separation was performed in a bare fused-silica capillary column (80 cm × 50 µm i.d.) (Polymicro Technologies, Phoenix, AZ, USA). The capillary ends were trimmed by capillary column cutter with diamond blade (Agilent Technologies, Santa Clara, CA, USA) to obtain an even cut which was 90° to the axis of the capillary column. At the outlet end of the capillary, a 20 mm portion of the polyimide coating was removed by direct heating for 4–5 sec on a capillary window maker (MicroSolv Technology Corporation, Eatontown, NJ, USA). The burned polyimide coating was wiped with alcohol-wet Kimwipe to expose the glass frame of the capillary and thus, prevent interaction of polyimide with NACE buffer, and to ensure effective streamline spraying and uninterrupted mixing of sheath liquid with capillary column effluent at the tip of the capillary. To obtain the best peak shape, better sensitivity, steady and stable supply of current for analyte separation, different capillary tip positions at 2 mm, 1 mm inside, 0 mm flushed, 1 mm or 2 mm protruding from the stainless tube were evaluated. Separation voltage was 25 kV with initial ramping for 17 sec. Capillary cartridge was maintained at 25 °C. Sample was hydrodynamically injected at 10 psi for 10 sec. During sample injection, spray voltage and sheath gas flow were off to eliminate the disturbing effect on stacking phenomenon.[28]

Capillary conditioning

Before initial use, the capillary was sequentially conditioned with 0.1 M HCl, methanol and water each for 10 min, followed by 0.1 M NaOH in reverse mode for 10 min, forward mode for 20 min, then water for 10 min to ensure that sodium hydroxide was completely washed off; separation buffer flush was introduced for 10 min before electro-conditioning at 25 kV for 60 min. Electro-conditioning of the capillary with separation buffer for 45 min was performed every day prior to analysis of samples.[20] To prevent changes in the migration times of the analytes, autosampler was programmed to use the next vial containing fresh separation buffer for separation after every 6 samples had been analyzed. When not in use, the capillary was flushed with 0.1 M NaOH followed with water for 5 min, and stored dry.

Mass spectrometry

Mass spectrometric analyses were performed on a Finnigan LCQ Duo ion trap (Thermo Fisher Scientific, San Jose, CA, USA) equipped with electrospray ionization (ESI) interface operated in positive mode. CE-MS ion source adapter kit (Thermo Fisher Scientific, San Jose, CA, USA) was used to couple the Beckman-Coulter PACE/MDQ to the mass spectrometer. The coupling interface provided coaxial sheath liquid for make-up flow (5 µL/min) delivered by Harvard syringe pump 11(Harvard Apparatus, Hollston, MA, USA) and sheath gas (6 arbitrary units) to enhance nebulization in ESI. Different ratios of isopropanol and water in the sheath liquid composition (75 : 25, 50 : 50, and 40 : 60) were used to optimize the sensitivity and resolution. Different percentages (0.1%, 0.5%, 1.0% and 5.0% (v/v)) of formic acid were also evaluated for achieving better separation of the analytes. Sheath gas was nitrogen. Different sheath gas flow rates from 3 to 20 arbitrary units were evaluated to obtain best resolution, sensitivity and peak shape.

ESI source and MS parameters were as follows: scan mode; full scan; mass range: 100–300; ESI voltage, 4500 V; ion transfer temperature, 170 °C, capillary voltage, 3.0 V, source current, 80 µA. Automatic gain control (AGC) was employed for three microscans and a maximum injection time of 200 ms. Xcalibur software (version 2.0.1, Thermo Fisher Scientific, San Jose, CA, USA) was used for CE-MS instrument control, data acquisition and processing.

Results and Discussion

CE-MS data

During analyses, CE current was stable at 8.9 µA. Figure 2 shows total ion current (TIC) electropherograms of the 12 amphetamine and related drug standards (100 ng/uL) in full scan mode (*m/z* range: 100–300). Although some of the electropherograms overlapped in TIC, they were resolved in the extracted ion current (EIC) electropherogram except those for MBDB and MDE, which had the same migration time (t_m) of 23.55 min (Figure 3). EPH and PSE are diastereomers and thus, have the same protonated molecule, *m/z* 166, and product ions, *m/z* 148 in MS^2, *m/z* 117 and *m/z* 133 in MS^3 (Table 1). Despite the isobaric characteristics of EPH and PSE in protonated molecule and product ions, both analytes had distinctly different t_m (22.66 min *vs* 23.52 min) by which they were identified and differentiated from one another. MA and PHE; MDE and MBDB are two pairs of constitutional isomers[29] with the same protonated molecule, $[M + H]^+$, at *m/z* 150 and *m/z* 208, respectively. MA and PHE were separated by their respective t_m (21.25 min vs. 22.76 min) (Figure 3). Different strategies, such as, separation voltage, buffer pH and sheath gas flow rate were evaluated to resolve MDE from MBDB by t_m, but were not successful using the present method.

CE-MS/MS data

Full scan product ion spectrum (MS/MS) provided unique distinguishing information for the detection, separation and

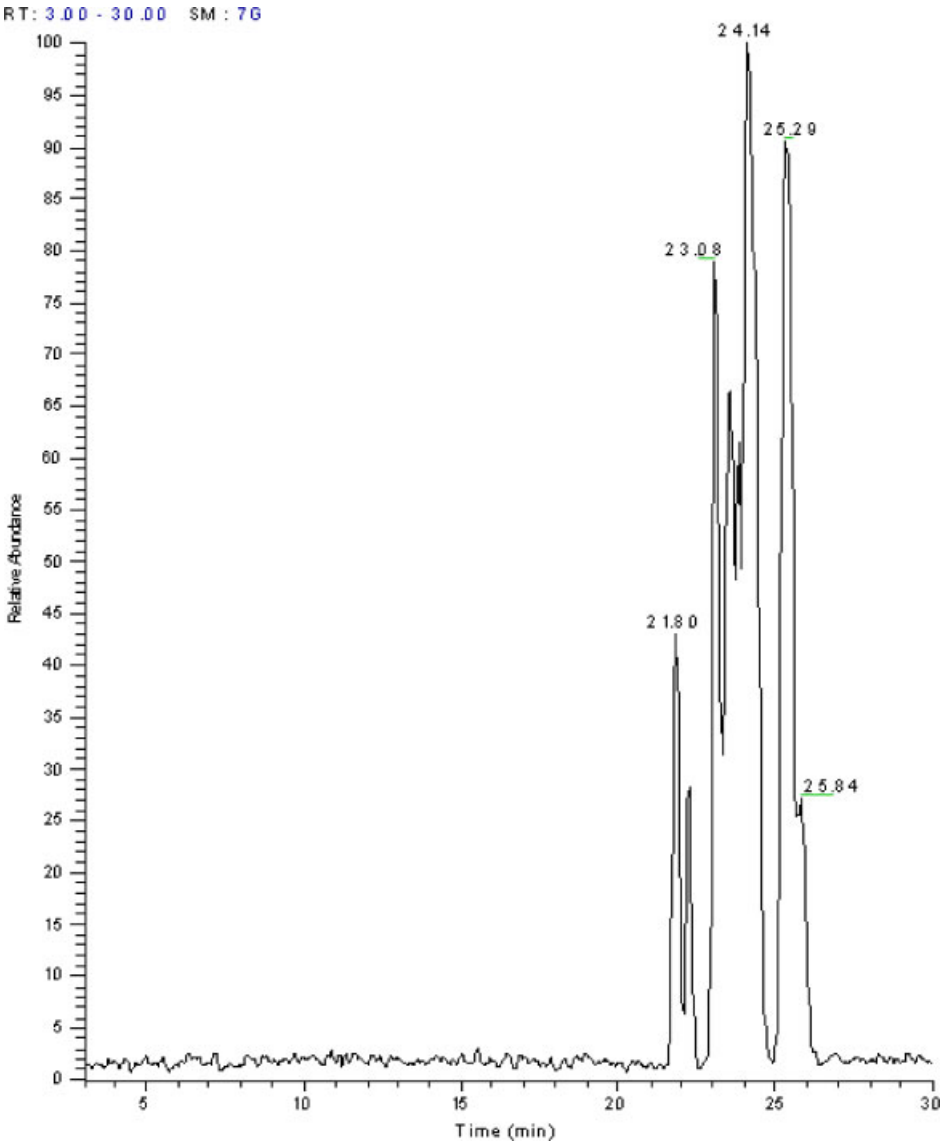

Figure 2. TIC of the 12 analytes (100 ng/μL each). Experimental conditions: separation on bare-fused silica capillary of 80 cm × 50 μm i.d.dimensions; separation voltage, 25 kV; hydrodynamic injection at 10 psi for 10 sec; separation buffer, 25 mM ammonium formate in acetonitrile/methanol (20: 80, v/v), plus 1 M formic acid; Mass spectrometric analysis was in positive electrospray ionization mode; sheath gas, 6 arbitrary units,; sheath liquid was isopropanol-water (50 : 50, v/v) with 0.5% formic acid at 5 μL/min.

identification of these analytes. In CE-MS/MS full scan mode, specificity and signal-to-noise ratios were enhanced compared with CE-MS. For example, MDE and MBDB were not differentiated from each other by CE-MS; however, their MS^2 product ions were distinctly different. The most intense product ion for MDE was *m/z* 163 and that for MBDB was *m/z* 177. Both analytes were easily separated by full scan product ion spectra (CE-MS/MS). Fragmentation parameters of amphetamine and related drugs are shown in Table 1. EIC electropherogram of CE-MS/MS of the analytes studied are shown in Figure 4. All analytes were successfully separated and identified.

Advantages of NACE buffer

In CE-MS, the separation buffer is more than just a carrier of the analytes. It determines the charge state of the analytes and carries the current that drives the separation. Thus, the type of salt in the separation buffer with concentration of the buffer plays an important role in ionization and separation. NACE, as a separation buffer, has been shown to be a powerful medium for improving separation and selectivity of compounds with similar chemical structure, especially ionizable molecules.[30] All analytes in this study were basic drugs by chemical classification (Figure 1), and for that reason an acidic buffer was chosen for better separation and ionization. In addition, at low pH, electro-osmotic flow (EOF) is very low, and thus, plays a minor role, which favours reproducibility of results. Ammonium formate and ammonium acetate are often used as electrolytes in NACE-MS because they are volatile salts. The use of ammonium formate resulted in sharper and narrower peak shape than ammonium acetate, probably because of its lower ionic strength. Results obtained showed that resolution, and sensitivity improved with high ratio of methanol and high buffer concentration. Thus, optimum separation was obtained using 25 mM ammonium

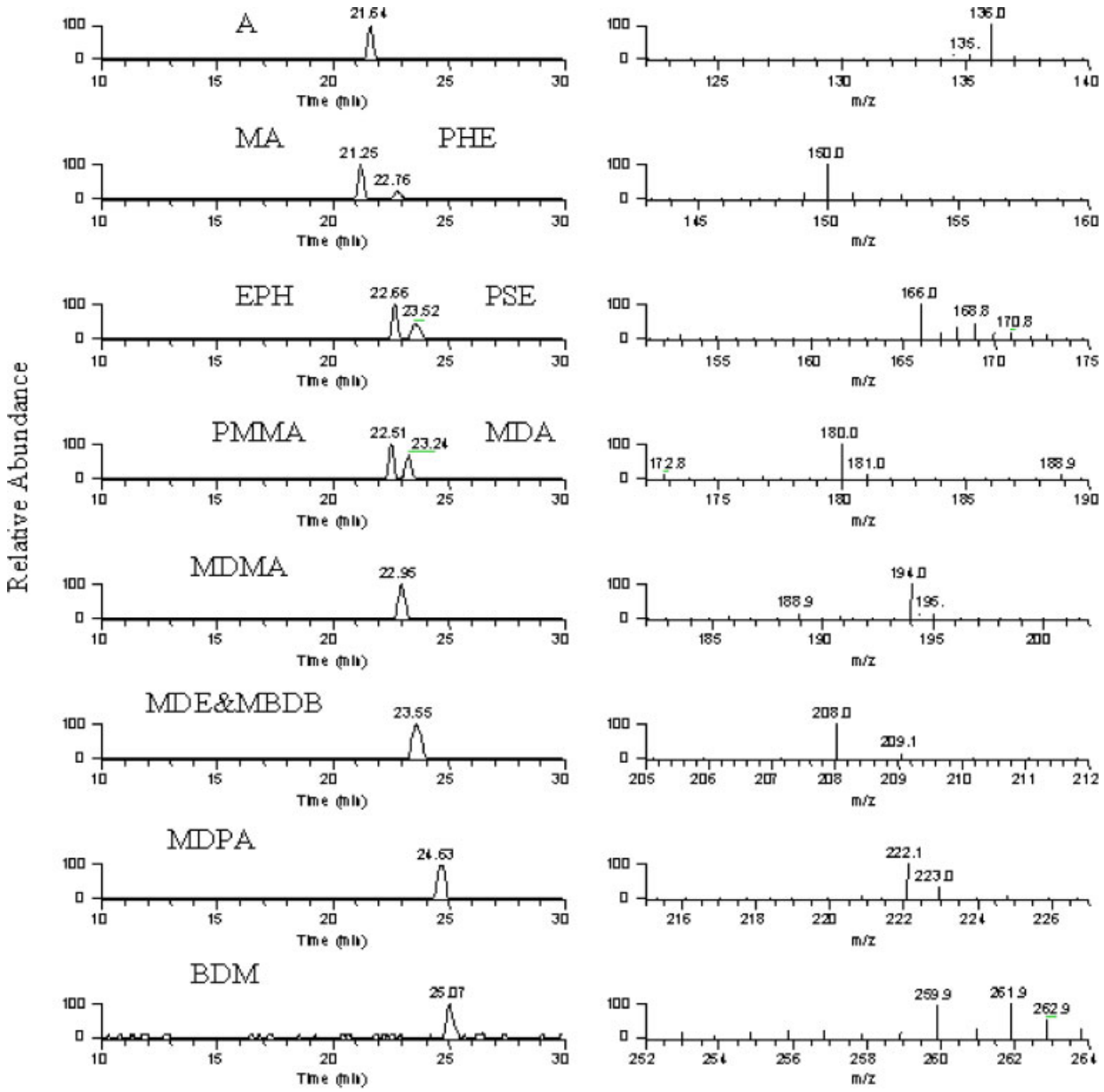

Figure 3. NACE-MS of the 12 analytes (100 ng/μL each). Left panel shows electropherograms, while right panel represents extracted mass spectra showing $[M + H]^+$ ions. Diagnostic ions in the spectra (from top to bottom) were *m/z* 136 for amphetamine (A); *m/z* 150 for methamphetamine (MA) and phentermine (PHE); *m/z* 166 for ephedrine (EPH) and pseudoephedrine (PSE); *m/z* 180 for p-methoxymethamphetamine (PMMA) and 3,4-methylenedioxyamphetamine (MDA); *m/z* 194 for 3,4-methylenedioxymethylamphetamine (MDMA); *m/z* 208 for N-ethyl-3,4-methylenedioxy-2-propylamine (MDE) and N-methyl-1-(3,4-ethylenedioxyphenyl)-2-butanamine (MBDB); *m/z* 222 for 3,4-methylenedioxypropylamphetamine (MDPA) and *m/z* 260 for 4-bromo-2,5-dimethoxyphenethylamine (BDM). Experimental conditions were the same as in Figure 2.

formate in acetonitrile/methanol (20: 80, v/v), plus 1M formic acid to enhance ionization.

Mass spectrometry optimization

CE provides very fast and efficient separation, thus, its peak width is very narrow. The mass spectrometer which is coupled to CE requires sufficient resolution and scan speed to capture the CE peak. In the quadrupole ion trap, the rate at which ions are sequentially scanned out of the trap according to their m/z value is very high, maybe 5000–10 000 m/z units per second. In order to obtain the best MS performance, the position of the capillary outlet, sheath liquid composition, and sheath gas flow rate were optimized. The optimal capillary outlet position was to place the outlet end of the capillary in sheath liquid maintaining electrical continuity to obtain maximum separation of the analytes. Results obtained showed that capillary tip at a flushed position (0 mm) maintained stability of the supply of current, spray and repeatability.

It has been reported that an appropriate sheath liquid and its flow rate are very critical for achieving good performance.[31] Results obtained in this study showed that isopropanol resulted in a more stable current delivery than methanol; however, with higher percentage (>70%) of isopropanol, supply of current was less stable leading to frequent current failure. The composition of sheath liquid for separation of these analytes was

Table 1. Fragmentation parameters of 12 amphetamine and related drugs

Compounds	Precursor ion $[M+H]^+$ (*m/z*)	Most intense productions (*m/z*)	Isolation width	Collision energy (%)	t_m (min)
A	136	119(MS^2)	3	27	21.64
		91(MS^3)	3	25	
MA	150	119, (MS^2)	3	33	21.25
		91(MS^3)	3	37	
MDA	180	163(MS^2)	3	22	23.24
		135,133,105(MS^3)	3	35	
MDMA	194	163(MS^2)	3	28	22.96
		135,133, 105(MS^3)	3	35	
MDE	208	163(MS^2)	3	28	23.55
		135,133,105(MS^3)	3	35	
EPH	166	148(MS^2)	3	27	22.66
		133,117(MS^3)	3	45	
PSE	166	148(MS^2)	3	25	23.52
		133,117(MS^3)	3	45	
MDPA	222	163(MS^2)	3	35	24.53
		135,133,105(MS^3)	3	38	
PMMA	180	149(MS^2)	3	27	22.51
		121(MS^3)	3	35	
MBDB	208	177(MS^2)	3	27	23.55
		147,135(MS^3)	3	25	
PHE	150	133(MS^2)	3	35	22.76
		91,105(MS^3)	3	26	
BDM	260	243(MS^2)	3	22	25.07
		228,164(MS^3)	3	35	

t_m = migration time in minutes.

isopropanol-water (50 : 50, v/v) with 0.5% formic acid delivered at 5 μL/min.

Low pressure was used for delivering sheath gas with sheath liquid in ESI interface to assist with droplets formation that could affect sensitivity and resolution.[32] Results obtained from this study showed that at 4 to 8 arbitrary units, good resolution of the analytes was achieved. Sensitivity was drastically reduced when sheath gas flow was below 3 arbitrary units, suggesting the formation of large charged droplets. When sheath gas flow was higher than 10 arbitrary units, resolution of the analytes was completely lost. Thus, sheath gas flow at 6 arbitrary units was chosen for good and reproducible resolution and sensitivity for separation of all the analytes.

Matrix effect

Endogenous compounds extracted from a sample matrix might suppress or enhance ionization of analytes recovered from that matrix resulting in changes in signal intensities of the analytes, especially when ESI mode was applied. Matrix effects may occur in any biological samples, including plasma, urine, postmortem tissue samples, saliva and other test samples. As shown in Table 2, the average ion suppression or enhancement for all these analytes was less than 15% except for phentermine, which means that the contribution by matrix effect on these ananlytes in plasma was not significant under the current experimental conditions.

Criteria for detection and confirmation of analytes in test sample

When 'real-world' samples from racetracks in PA were screened for these target drugs (Figure 1), the criteria for detection were: (1) diagnostic ions (Table 1) for these drugs should be present in full scan MS spectrum or MS/MS spectrum; (2) t_m of a drug in the suspect sample and that of the calibrator should agree to within ±0.2 min. Thus, once there was any suspect electropherogram that met the above criteria following initial screening result, confirmation method was subsequently applied to confirm the presence or absence of the suspect analyte in the test sample.

Typically, the identity of an analyte in a test sample can be confirmed by comparing the MS^n spectra with those of the reference standard. Thus, the confirmation method of each analyte was developed according to its unique MS/MS and MS^3 spectra. For example, a method for the confirmation of MDE was developed using the product ions *m/z* 163 for MS^2 and *m/z* 135, 133, 105 for MS^3. Confirmation criteria were: (1) diagnostic product ions (MS^2 and MS^3) of each analyte (Table 1) should be present; they should be within ±20% when compared with those of the calibrator, and interfering ions should not be more than 20%; and (2) t_m of the analyte in the suspect sample and that of the authentic reference standard should agree to within ±0.2 min.

LOD for detection and confirmation

Limit of detection (LOD) for the analytes in equine plasma was measured using spiked samples to determine the presence of the analytes at a signal-to-noise ratio greater than 3 : 1 for the qualifying ions ($[M + H]^+$). LOD of all analytes in equine plasma was 10–200 ng/mL (Table 3). Limit of confirmation (LOC) for the analytes in plasma was defined as that concentration of the analyte that represents electrophoregraphic signal-to-noise ratio level greater than 3 : 1 for the qualifying ion (MS^3). LOC in plasma was 50–1000 ng/mL depending on the analytes (Table 3).

Analysis of real-world sample

The method was successfully used in screening post-race equine plasma samples collected from racehorses following competition and in screening for drugs in postmortem tissue samples from racehorses that suddenly collapsed and died during training or racing in PA. Screening result for plasma sample (#97982) from one of the racetracks in PA is shown in Figure 5. A presumptive positive for the presence of EPH was indicated in the sample because the sample and EPH reference standard shared identical t_m and diagnostic ion of *m/z* 166. Ephedrine and PSE had the same protonated molecule, *m/z* 166, and the same product ion, *m/z* 148 in MS^2, and *m/z* 117, *m/z* 133 in MS^3. Both analytes were differentiated only by the unique individual t_m. Thus, a mixture of the two standards (EPH and PSE) was used as the reference standard. The presence of EPH in sample #97982 was confirmed by t_m, MS^2 (*m/z* 148) and MS^3 (*m/z* 133,117) spectra (Figure 6), indicating the reliability of the method for screening and confirming the presence of EPH in equine plasma. The presence of EPH in the same sample was also confirmed at our GC-TOF/MS workstation. This CE-MS method was also successfully used to confirm the presence of MDMA in equine postmortem sample.

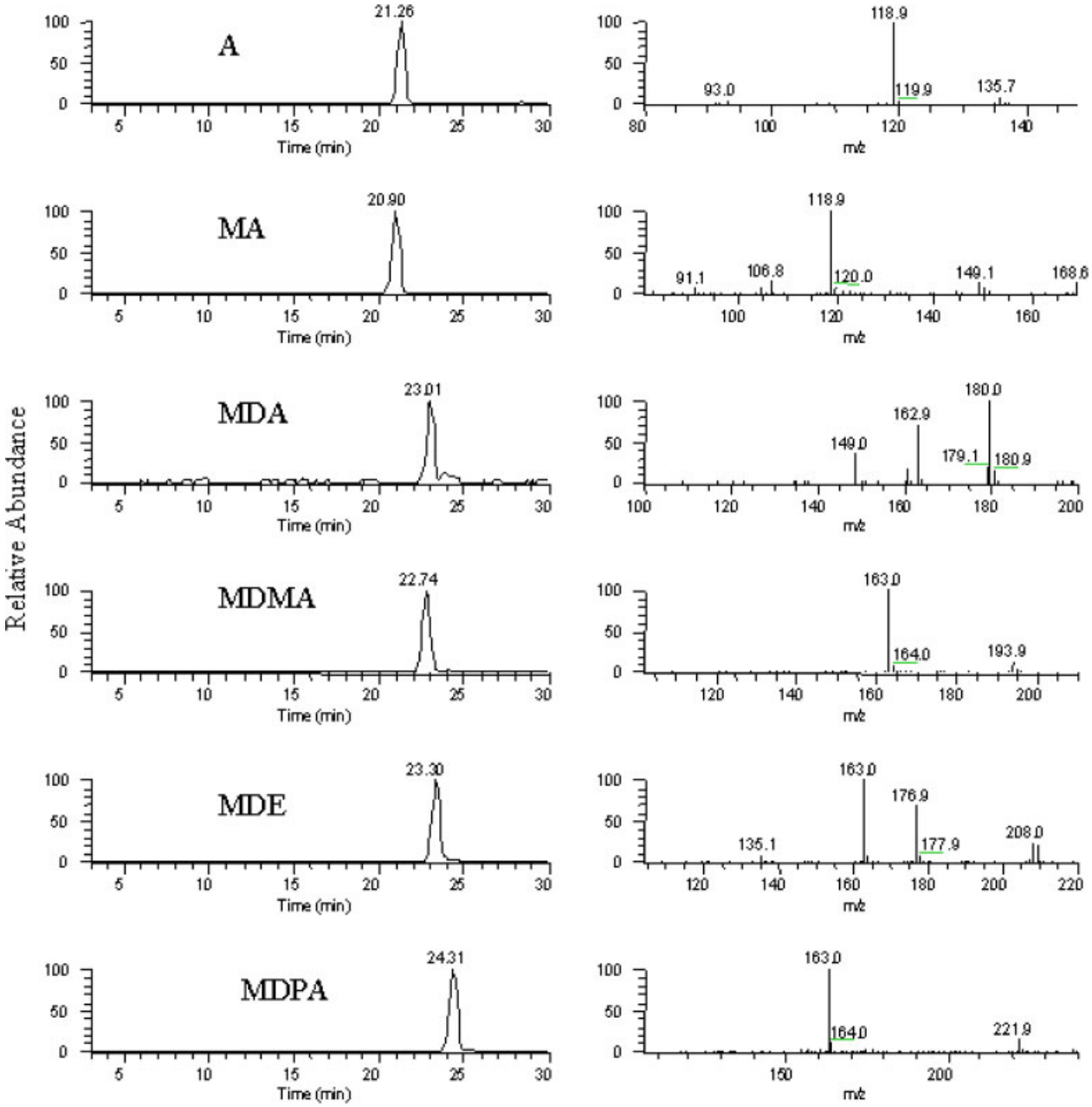

Figure 4. NACE-MS/MS of the12 analytes (100 ng/μL each). Left panel represents electropherograms while right panel shows MS/MS spectra. Diagnostic ions were the most intense product ions (from top to bottom) at *m/z* 119 for amphetamine (A), *m/z* 119 for methamphetamine (MA), *m/z* 163 for 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethylamphetamine (MDMA), N-ethyl-3,4-methylenedioxy-2-propylamine (MDE), and 3,4-methylenedioxypropylamphetamine (MDPA), *m/z* 148 for ephedrine (EPH) and pseudoephedrine (PSE), *m/z* 149 for p-methoxymethamphetamine (PMMA), *m/z* 177 for N-methyl-1-(3,4-ethylenedioxyphenyl)-2-butanamine (MBDB), *m/z* 133 for phentermine (PHE), and *m/z* 243 for 4-bromo-2,5-dimethoxyphenethylamine (BDM). Experimental conditions were the same as in Figure 2.

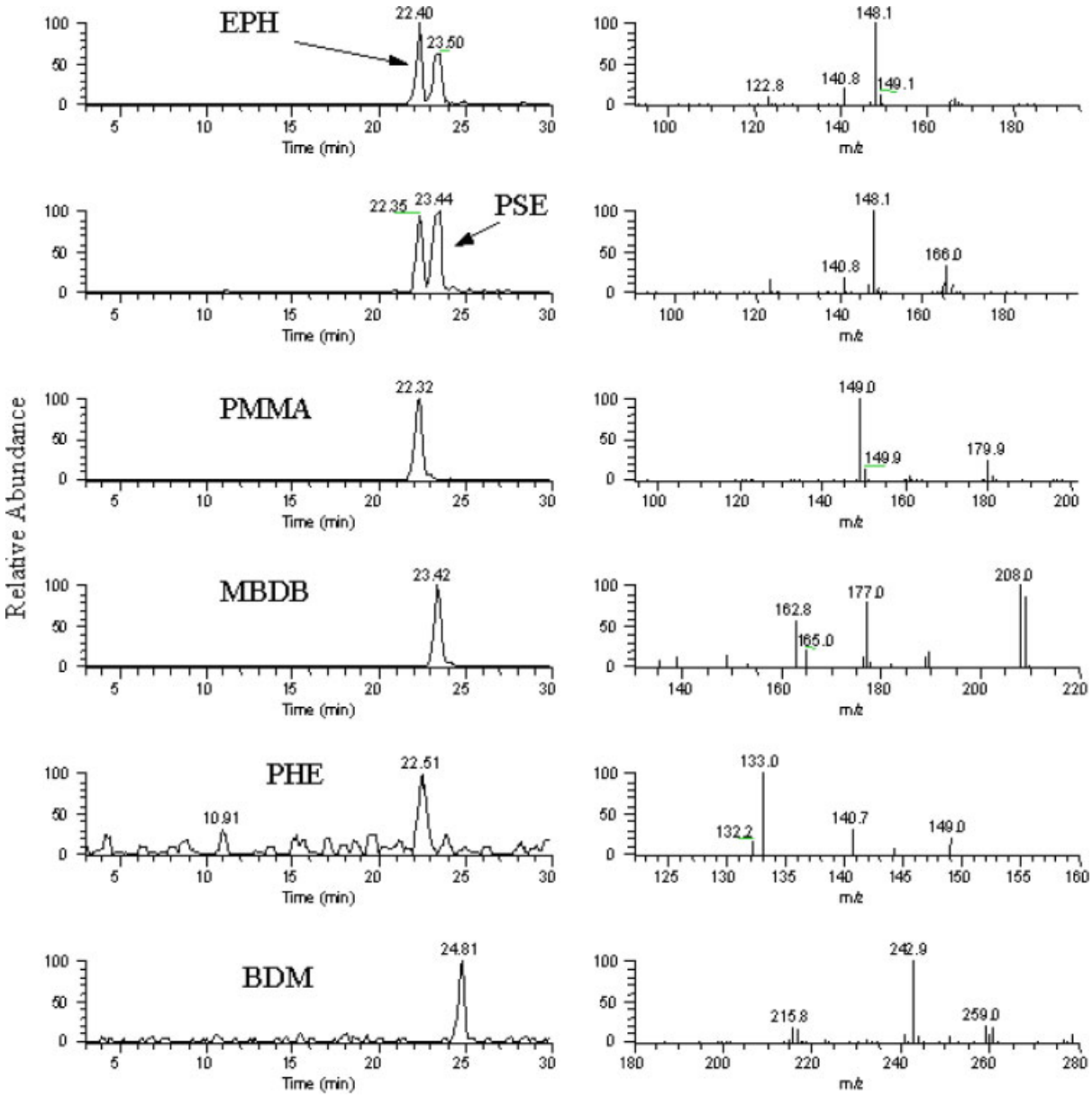

Figure 4. (*Continued*).

Table 2. Matrix effect on 12 amphetamine and related drugs

Compounds	Ion suppression/ enhancement(%)*	Compounds	Ion suppression/ enhancement(%)*
A	+0.38	PSE	−2.36
MA	+2.40	MDPA	−3.85
MDA	−5.25	PMMA	−5.87
MDMA	−6.00	MBDB	−7.89
MDE	−4.22	PHE	−15.78
EPH	+1.22	BDM	−10.33

* "+" represention enhancement; while "−" represention suppression.

Table 3. Limit of detection and confirmation

Compounds	LOD (ng/mL)	LOC (ng/mL)
A	10	50
MA	10	50
MDA	200	1000
MDMA	200	1000
MDE	200	1000
EPH	100	500
PSE	100	500
MDPA	200	1000
PMMA	200	1000
MBDB	200	1000
PHE	200	1000
BDM	200	1000

Conclusion

CE-MS combines high efficiency and resolution of CE with high selectivity and sensitivity of MS in the analysis of all 12 analytes

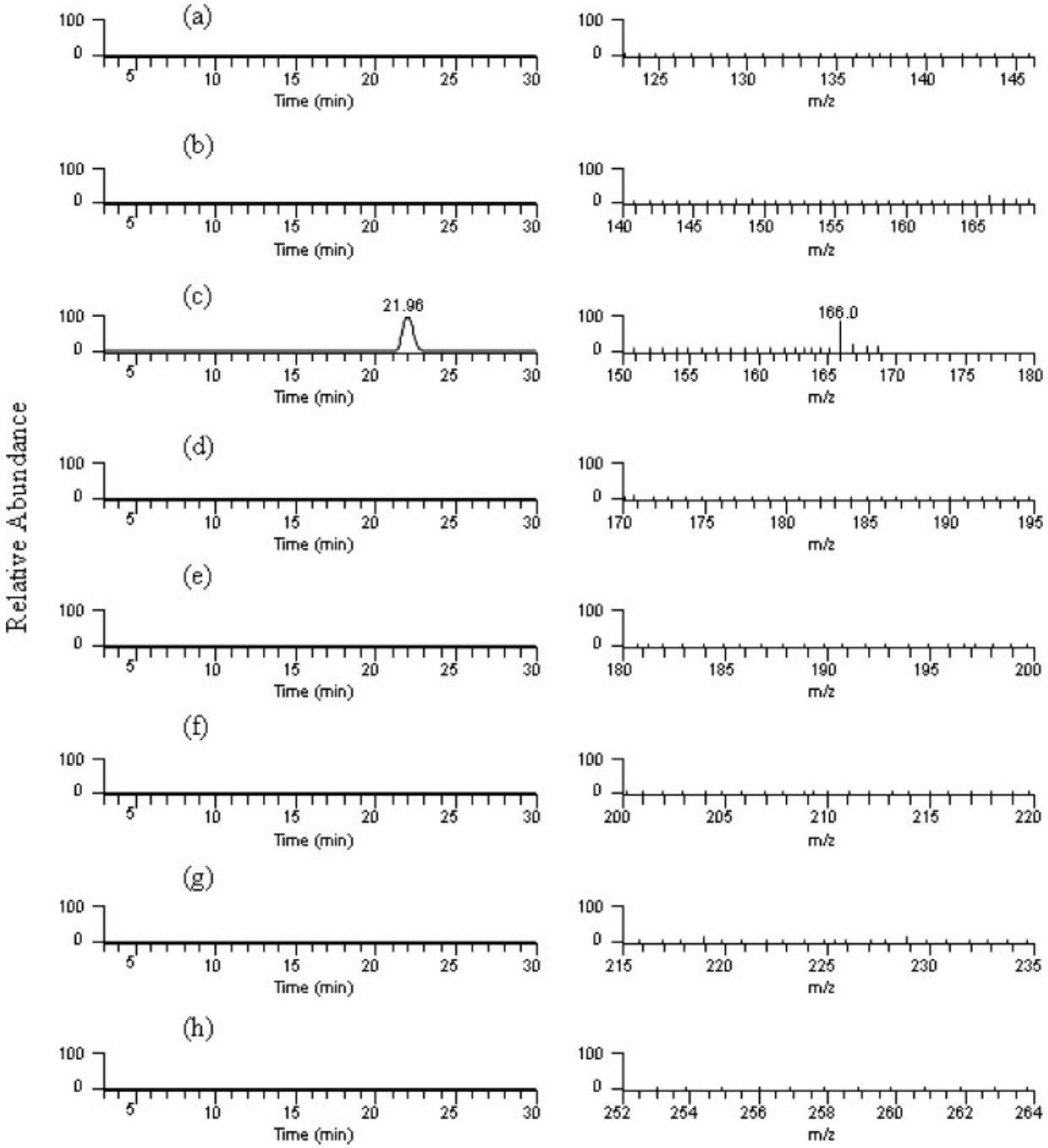

Figure 5. CE-MS screening result of a 'real world' plasma sample # 97982 obtained from a horse post competition in PA. Left panel shows electropherograms, while right panel represents extracted mass spectra showing $[M+H]^+$ ions. Panel (a) represents screening for A; (b) for MA and PHE; (c) for EPH and PSE; (d) for PMMA and MDA; (e) for MDMA; (f) for MDE and MBDB; (g) for MDPA and (h) for BDM. Sample #97982 in panel (c) contained a suspect electropherogram for either EPH or PSE (*m/z* 166). All other target analytes were not detected.

in equine plasma. In this study, simultaneous screening and confirmation method for amphetamine and related drugs was developed and successfully applied to screening of equine plasma samples collected from racehorses post competition in PA. Key parameters, such as sheath liquid composition, sheath gas flow rate and buffer composition were carefully optimized for reproducible results. The method offers an opportunity for multidrug target screening and confirmation. The only shortcoming of the method was the length of time (26 min) for complete resolution of all 12 analytes. However, the method provided the first simultaneous separation of 12 amphetamine and related drugs in equine plasma by NACE-MS. This method is efficient, selective, and sensitive to the detection and confirmation of the presence of 12 amphetamine and related drugs in equine plasma.

Acknowledgements

The authors thank the PA Racing Commissions for providing financial support for this study. The PA Harness Horsemen Association at Pocono and Chester Downs, Meadows Standardbred Owners

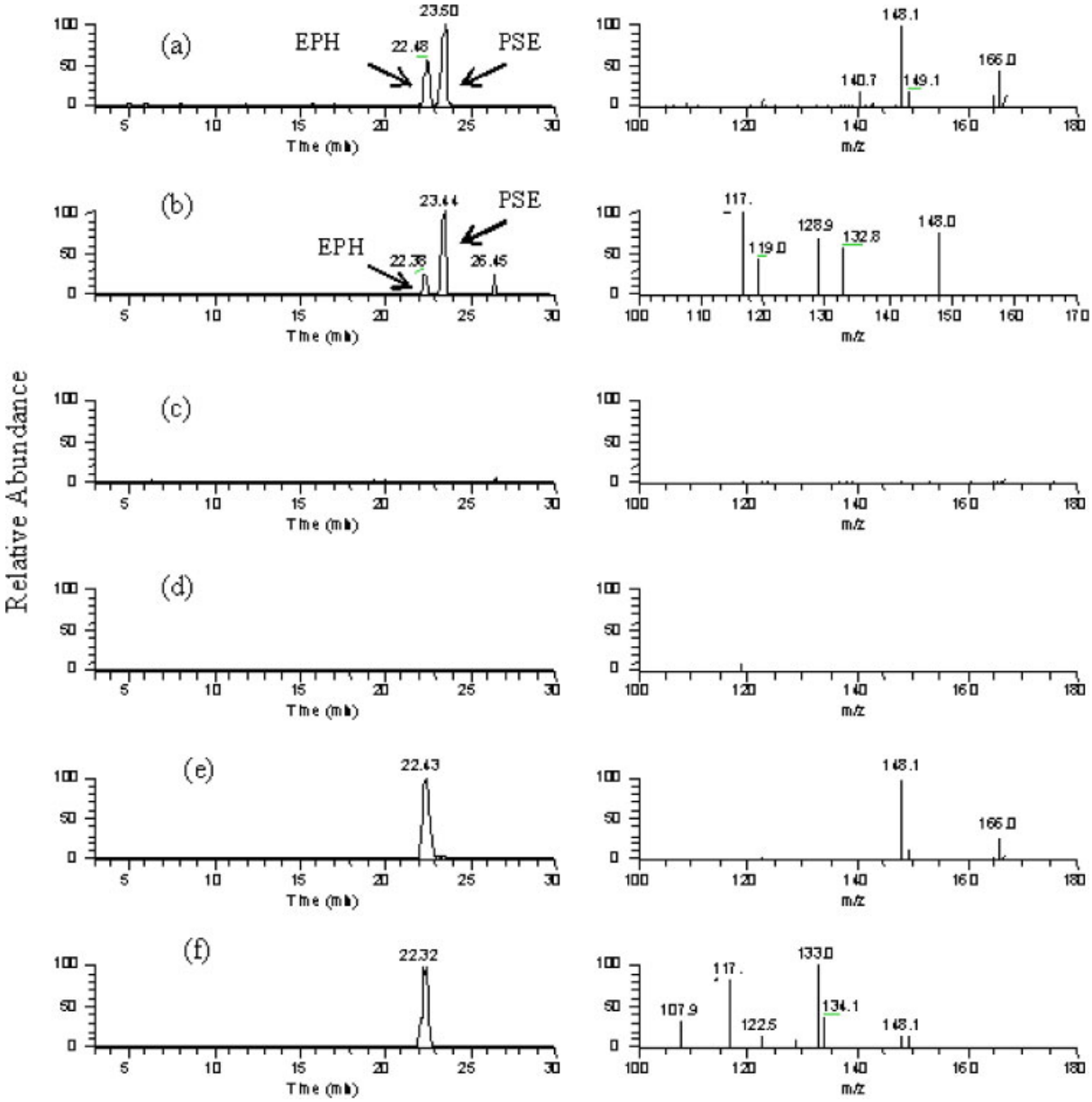

Figure 6. Confirmation of the presence of EPH in a 'real world' plasma sample # 97982. Left panel shows electropherograms, while right panel represents extracted mass spectra. From top to bottom, (a) electropherograms and MS^2 spectra of EPH and PSE standard, (b) electropherograms and MS^3 spectra of EPH and PSE standard, (c) absence of identifiable electropherogram and MS^2 spectra of the analytes in blank equine plasma (d) absence of identifiable electropherogram and MS^3 spectra of the analytes in blank equine plasma (e) electropherogram and MS^2 spectra of sample #97982 (f) electropherogram and MS^3 spectra of sample #97982. The presence of EPH in sample #97982 was confirmed by both MS^2 and MS^3 spectra, and by t_m.

Association and Horsemen Benevolent and Protection Association at Penn National and Presque Isles Downs also made financial contributions and to them the authors are grateful.

References

[1] D. E. Nichols, *J. Psychoactive Drugs* **1986**, *18*, 305.
[2] The world Anti-Doping Code: The 2008 Prohibited List International Standard. Available at: http://www.wada-ama.org/rtecontent/document/2008_List_Format_en.pdf.
[3] Uniform classification guidelines for foreign substances and recommended penalties and model rule. Available at: http://www.arci.com/druglisting.pdf.
[4] K. A. Moore, C. Werner, R. M. Zannelli, B. Levine, M. L. Smith, *Forensic Sci. Int.* **1999**, *106*, 93.
[5] T. Kupiec, L. DeCicco, V. Spiehler, G. Sneed, P. Kemp, *J. Anal. Toxicol.* **2002**, *6*, 513.
[6] M. Laloup, G. Tilman, V. Maes, B. G. De, P. Wallemacq, J. Ramaekers, N. Samyn, *Forensic Sci. Int.* **2005**, *153*, 29.
[7] L. G. Apollonio, I. R. Whittall, D. J. Pianca, J. M. Kyd, W. A. Maher, *J. Anal. Toxicol.* **2007**, *31*, 208.
[8] R. O. Hughes, W. E. Bronner, M. L. Smith, *J. Anal. Toxicol.* **1991**, *15*, 256.
[9] B. K. Gan, D. Baugh, R. H. Liu, A. S. Walia, *J. Forensic Sci.* **1991**, *36*, 1331.
[10] C. Jurado, M. P. Gimenez, T. Soriano, M. Menendez, M. Repetto, *J. Anal. Toxicol.* **2000**, *24*, 11.
[11] A. Kankaanpaa, T. Gunnar, K. Ariniemi, P. Lillsunde, S. Mykkanen, T. Seppala, *J. Chromatogr. B.* **2004**, *810*, 57.
[12] L. W. Chung, G. J. Liu, Z. G. Li, Y. Z. Chang, M. R. Lee, *J.Chromatogr. B.* **2008**, *874*, 115.

[13] A. A. Marais, J. B. Laurens, *Forensic Sci. Int.* **2009**, *183*, 78.
[14] M. J. Bogusz, K. D. Kruger, R. D. Maier, *J. Anal. Toxicol.* **2000**, *24*, 77.
[15] H. Kataoka, H. L. Lord, J. Pawliszyn, *J. Anal. Toxicol.* **2000**, *24*, 257.
[16] H. K. Nordgren, O. Beck, *J. Anal. Toxicol.* **2003**, *27*, 15.
[17] H. P. Hendrickson, A. Milesi-Halle, E. M. Laurenzana, S. M. Owens, *J. Chromatogr. B.* **2004**, *806*, 81.
[18] M. Concheiro, S. M. Simoes, O. Quintela, C. A. de, M. J. Dias, A. Cruz, M. Lopez-Rivadulla, *Forensic Sci. Int.* **2007**, *171*, 44.
[19] K. Zaitsu, M. Katagi, T. Kamata, H. Kamata, N. Shima, H. Tsuchihashi, T. Hayashi, H. Kuroki, R. Matoba, *Forensic Sci. Int.* **2008**, *177*, 77.
[20] M. Mueller, F. T. Peters, G. A. Ricaurte, H. H. Maurer. *J. Chromatogr. B.* **2008**, *874*, 119.
[21] J. C. Hudson, M. Golin, M. Malcolm, C.F. Whiting, *J. Can. Soc. Forens. Sci.* **1998**, *31*, 1.
[22] G. N. W. Leung, H. P. O. Tang, T. S. C. Tso, T. S. M. Wan, *J. Chromatogr. A.* **1996**, *738*, 141.
[23] L. Geiser, S. Cherkaoui, J. L. Veuthey, *J. Chromatogr. A.* **2000**, *895*, 111.
[24] Y. T. Iwata, T. Kanamori, Y. Ohmae, K. Tsujikawa, H. Inoue, T. Kishi, *Electrophoresis* **2003**, *24*, 1770.
[25] M. Nieddu, G. Boatto, G. Dessi, *J. Chromatogr. B.* **2007**, *852*, 578.
[26] R. Gottardo, F. Bortolotti, P. G. De, J. P. Pascali, I. Miksik, F. Tagliaro, *J. Chromatogr. A.* **2007**, *1159*, 185.
[27] J. Schappler, D. Guillarme, J. Prat, J. L. Veuthey, S. Rudaz, *Electrophoresis* **2008**, *29*, 2193.
[28] A. B. Wey, W. Thormann, *J. Chromatogr. A* **2001**, *924*, 507.
[29] O. D. Sparkman, *Mass Spectrometry Desk Reference*, Global View Publisher: Pittsburgh, **2006**.
[30] I. Bjornsdottir, S. H. Hansen *J. Chromatogr.* **1995**, *711*, 313.
[31] M. Hashimoto, Y. Ishihama, M. Tomita, T. Soga. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3579.
[32] K. Huikko, T. Kotiaho, R. Kostiainen, *Rapid Commun. Mass Spec.* **2002**, *16*, 1562.