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Fast and Sensitive Chiral Analysis of Amphetamines and Cathinones in Equine Urine and Plasma Using Liquid Chromatography Tandem Mass Spectrometry

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Abstract

A simple, rapid, sensitive and reproducible method for enantiomer analysis of methamphetamine, amphetamine, cathinone and methcathinone was developed and validated. The compounds were extracted from equine plasma and urine using a fast liquid-liquid extraction procedure. Only one milliliter plasma and one hundred microliter urine sample is needed for analysis. The extraction procedure had good recovery (>70%) and the matrix effect was negligible. Enantiomer differentiation and confirmation were achieved using liquid chromatography with chiral stationary phase and mass spectrometry detection. The method demonstrated excellent reproducibility with intra-day and inter-day precision of lower than 5%. The lower limits of detection for all of the compounds studied here were at low pg/mL level for both plasma and urine. This is the first report of the analysis of four chiral compounds in equine plasma and urine. Routine application was demonstrated for (S)- and (R)-enantiomer differentiation.

Keywords

Amphetamines and Cathinones, Equine Plasma and Urine, Chiral Analysis, Liquid Chromatography Tandem Mass Spectrometry

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1. Introduction

Methamphetamine, amphetamine, cathinone and methcathinone are powerful central nerve system (CNS) stimulants and are abused in many countries [1]-[8]. According to Uniform Classification Guidelines for Foreign Substances and Recommended Penalties and Model Rules from Association and Racing Commissioner International (RCI), methamphetamine, amphetamine, cathinone and methcathinone are all Class I drugs based on their ability to influence the outcome of a horse race. Class A Penalty will be imposed if any of these drugs are confirmed present at any level in post-race equine urine or plasma.

Amphetamine is a metabolite of methamphetamine [8] [9]. Cathinone and methcathinone both have a β-keto group and they are structurally close to amphetamine. These four compounds all contain a chiral center and have S and R enantiomers. The two enantiomers are non-superimposable mirror images of each other. In animal studies, the CNS stimulant activities increase in the order of (S)-amphetamine, (S)-cathinone, (S)-metham-phetamine and (S)-methcathinone [1]. Research results showed that the S-enantiomers of the four compounds demonstrated more CNS stimulant potency than the corresponding R-enantiomers due to the binding affinity differences to their receptor sites [9]-[13]. In the United States, (R)-methamphetamine is available in some over-the-counter nasal decongestant products, such as Vicks-Vapor inhaler and its generic equivalents. The illicitly manufactured methamphetamine consists mainly the S-enantiomer. Unambiguous identification of enantiomers can be extremely useful in clinical, forensic, and toxicological applications to distinguish illicit consumption, origin and the metabolism of the target compound [8]-[13].

Over the years, gas chromatography (GC), capillary electrophoresis (CE), and high performance liquid chromatography (HPLC) have been used for enantiomer analysis [5] [8] [9] [12]-[25]. Chiral separations can be classified into three categories [15]. The first one is pre-column derivatization with a chiral derivatization reagent to generate diastereomers; the second one involves adding a chiral agent to the mobile phase to form adducts with the enantiomers; and the third one is a direct method using chiral stationary phase[20] [25]. GC methods involve complicated and time consuming pre-column derivatization steps [3] [8] [12]-[14]. Liquid chromatography mass spectrometry (LC-MS) has gained more and more popularity for drug analysis because it is generally faster, easy to use and has better sensitivity and specificity [22] [23]. Chiral additive in mobile phase is usually not compatible for mass spectrometry detection [20] [25]. Direct chiral separation using chiral stationary phase without previous derivatization is a convenient choice for LC-MS analysis. The published methods with this approach had poor sensitivity and very long acquisition time [17] [18] [23]. We are presenting a fast and sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) chiral analysis method that can be used for the differentiation of the enantiomers of four compounds after an easy extraction from equine plasma and urine. To the author's knowledge, this is the first report of this kind in racing industry.

2. Experimental

2.1. Chemicals & Reagents

S(+)-Methamphetamine, R(−)-Methamphetamine, S(+)-Amphetamine, R(−)-Amphetamine, S(−)-Cathinone, R(+)-Cathinone, S(−)-Methcathinone, and R(+)-Methcathinone were purchased from Cerilliant (Round Rock, TX, USA). Formic acid and ammonium formate were from Sigma-Aldrich (St. Louis, MO, USA). Potassium hydroxide (KOH), Methyl-tert butyl ether (MTBE), and LC-MS grademethanol and acetonitrile were obtained from Spectrum Chemical (Gardena, CA, USA).

All of the stock solutions were prepared at 1 mg/mL in methanol. The working standard solutions (1 µg/mL, 10 ng/mL and 1 ng/mL) were prepared by serial dilution of the eight stock solutions with acetonitrile. All solutions were stored at −20˚C in tightly sealed glass bottles. All drug standard solutions were allowed to equilibrate to room temperature for at least 30 minutes before use. No internal standard was used since it is a confirmation analysis and quantitation is not necessary.

2.2. Sample Preparations

Liquid-liquid extraction was used to extract cathinones and amphetamines from equine plasma and urine. Urine and plasma samples were prepared using similar procedures. The extraction was conducted using 1 mL of plasma or 0.1 mL urine mixed with 1 mL water. The mixture was then mixed thoroughly with 50 µL 1 M KOH to ensure that the samples were basic. Five milliliter of MTBE was added for liquid-liquid extraction. The tubes

were gently shaken for 10 minutes on a rotorack and centrifuged at 3000 revolution per minute (RPM) for 10 minutes. The top organic layer was transferred to Pyrex disposable glass test tubes (16 × 100 mm), and the contents were dried at 40˚C under nitrogen in a turbovap evaporator. The extracts were reconstituted with 100 µL solvent (water and methanol, volume ratio of 1:1) and transferred to polypropylene autosampler vials. For each LC-MS/MS analysis, ten microliters of the dissolved extract was used.

2.3. LC-MS/MS Conditions

The LC-MS/MS system comprised a Shimadzu liquid chromatography system and an AB Sciex 4000 Qtrap mass spectrometer. The LC system consisted of LC-20ADXR pumps, a SIL-20ACXR auto-sampler, a DGU-20A5 degasser and a CTO-20A column oven (Shimadzu Scientific Instruments, Columbia, MD, USA). Analyte separation was performed on an Astec Chirobiotic V2 column (2.1 × 150 mm, 5 micron particle size) from Sigma (St. Louis, MO, USA). Column oven temperature was maintained at 25˚C. The mobile phase was a mixture of methanol and 50 mM ammonium formate/0.01% formic acid in LC-MS grade water (95/5, v/v). The isocratic total flow was 0.45 mL/min and the total analysis time was only6 minutes.

Qtrap 4000 (AB Sciex, Foster City, CA, USA) was used for mass spectrometry analysis. Positive ionspray voltage was set at 4000 volts. Ultra-pure nitrogen was used for ion source solvent evaporation and vacuum control. Gas 1, Gas 2, and curtain gas settings were 65, 45 and 40 psi, respectively. The ion source temperature was set at 400˚C. Multiple-reaction-monitoring (MRM) was used for analysis due to its high sensitivity and specificity. The neat standards were infused using a syringe pump for optimization of ion source parameters. The quantifying ion is the most intense ion among all of the MRM transitions for one compound. The chromatographic peak areas of the quantifying ions are used for calculations of recovery, precision and matrix effect in the validation process. The other ions are qualifying ions that are used for ion chromatographic peak area ratio calculations. The quantifying ions, qualifying ions and the corresponding settings for declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) for each compounds are listed in **Table 1**.

Table 1. MRM acquisition ions used and the corresponding electronic parameters for methamphetamine, amphetamine, cathinone and methcathinone.

		DP (volts)	EP (volts)	CE (volts)	CXP (volts)
Methamphetamine					
Quantifying ion	91	44	9	26	7.5
Qualifying ion	119	44	9	16	7.5
Qualifying ion	65	44	9	56	11
Amphetamine					
Quantifying ion	91	35	9	26	7.5
Qualifying ion	119	35	9	13	3.5
Qualifying ion	65	35	9	52	9
Cathinone					
Quantifying ion	117	39	10	34	9
Qualifying ion	105	39	10	26	11
Qualifying ion	89	39	10	59	7
Methcathinone					
Quantifying ion	131	49	14	29	10
Qualifying ion	130	49	14	44	10
Qualifying ion	105	49	14	33	8

2.4. Method Validation

Validation of this liquid-liquid extraction LC-MS/MS acquisition method was performed by checking specificity, extraction recovery, matrix effect, intra-day and inter-day precision, and limit of detection. Methamphetamine, amphetamine, cathinone and methcathinone are all RCI class 1 drugs, and positive confirmation of these drugs at any level in equine plasma or urine would be a violation. Since quantification is not necessary, the limit of quantification or linearity range was not assessed. Because each of the four compounds studied here has S and R enantiomers, eight analytes were evaluated.

Endogenous compounds and other matrix interferences could produce a false positive for analytes of interest. The method specificity was demonstrated by analysis of six different lots of blank equine plasma or urine samples. Each chromatogram was checked for peaks that might interfere with the detection of the eight analytes.

Extraction recovery was evaluated in three replicates. Plasma samples at concentration 0.1 ng/mL and urine samples at 1 ng/mL were extracted. Three blank plasma and urine samples were extracted in the same batch. After extraction, the blank plasma or urine extracts were spiked with the eight analytes to the corresponding concentration of 0.1 ng/mL (plasma) or 1 ng/mL (urine). Recovery was determined by comparing the average chromatographic peak areas between the samples spiked with the drug standards before and after extractions.

Ionization suppression or enhancement in electrospray ionization is usually from matrix effect. Triplicate samples at concentrations of 0.1 ng/mL (plasma) and 1 ng/mL (urine) were extracted and analyzed by this LC-MS/MS method. The same experiment was performed using deionized water. The plasma or urine matrix effect was calculated by comparing average chromatographic peak areas of the quantifying ions in plasma or urine with those in water. If the plasma or urine signal was stronger than that of water, it showed matrix enhancement, while a lower value indicated matrix suppression.

Intra-day precision was evaluated by analyzing six replicate plasma or urine samples at concentration of 0.1 ng/mL (plasma) or 1 ng/mL (urine) on the same day. For inter-day precision analysis, three sets of six replicate plasma or urine samples at the same concentrations as above were analyzed on three different days.

The lower limit of detection and lowest concentration at which the compounds can be confirmed were checked to find out the sensitivity of the method. Triplicate plasma samples at 2, 5, 10, 20, and 50 pg/mL were extracted and analyzed. Urine samples at concentrations of 10, 20, 50, 100, 200, and 500 pg/mL were also tested in triplicate.

3. Results and Discussion

3.1. Method Development

The aim of our research was to develop and validate a simple, fast, sensitive and selective LC-MS/MS method for confirmation and enantiomer separation of amphetamines and cathinones extracted from equine blood or urine. Since more than 50% of the pharmaceutical drugs are chiral, enantioselective chromatography has gained more and more attention [14] [15] [26]. There are many types of commercial chiral columns with different stationary phases [25] [27]. These stationary phases include polysaccharide derivatives, macrocyclic antibodies, proteins, cyclodextrins and pirke-type. Efficiency of enantioseparation is mainly determined by the chiral discriminative power of the chiral stationary phase employed. Macrocyclic antibody Vancomycin-based CHIROBIOTIC V2 column has demonstrated separation of amine compounds and worked well for our applications.

The separation of chiral drugs was performed under isocratic conditions. Several mobile phase modifiers were studied in order to obtain chiral separation in LC and to maintain satisfactory electrospray ionisation performance in positive ionization mode. Methanol, acetonitrile, formic acid, acetic acid, ammonium formate and ammonium acetate were tested for mobile phases or additives. Methanol, formic acid and ammonium formate were chosen because narrower chromatographic peaks with shorter retention times were achieved with this mobile phase. Also, ammonium formate and formic acid were used in this method due to their solubility in high content organic mobile phases and their volatility for fast solvent evaporation inelectrospray. High percentage of organic solvent methanol also makes solvent evaporation easier and promotes better sensitivity. **Figure 1** shows the separation of the four compounds and the eight isomers showed their unique retention times. All of the analytes were eluted in 5 minutes. Even though the (S)- and (R)-enantiomers are not baseline resolved, the resolution was sufficient to unambiguously differentiate the two isomers.

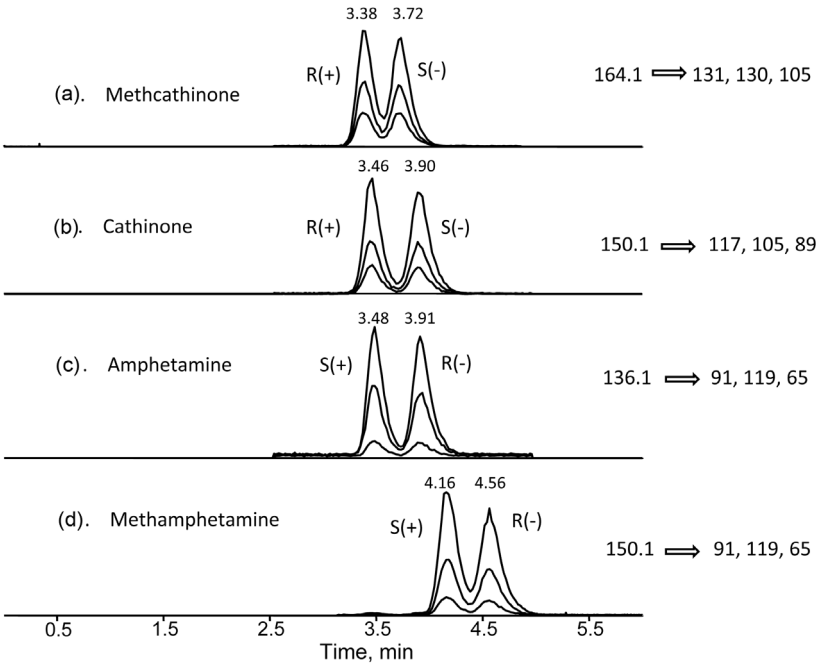

Figure 1. Chiral separation chromatograms of methcathinone, cathinone, amphetamine and methamphetamine extracted from a plasma sample spiked at 0.1 ng/mL.

3.2. Method Validation Results

Specificity testing was performed to make sure this method was free of endogenous compounds and other matrix interferences that could potentially produce a false positive signal and interfere with the analysis. Six different lots of blank equine plasma or urine samples were analyzed and the results indicated that the method is specific, because no interference peaks, capable of producing false positives, were observed in the MRM chromatograms.

A simple one step liquid-liquid extraction was used for sample processing. MTBE has a low boiling point and it evaporates quickly. Compared to the commonly used extraction solvent ethyl acetate, plasma and urine extracted with MTBE resulted in much cleaner extract, especially under basic conditions. The extraction recovery results for equine plasma and urine are listed in **Table 2**. Extraction recovery in plasma ranges from 84% to 100% and it is between 70% to 98% for urine. Urine samples had lower recovery compared to plasma. Even though cathinone has lowest extraction recovery among others (70% for urine and 84% for plasma), the results demonstrated that most of the drugs are extracted and recovered from both sample matrices.

Co-eluting compounds may cause ion suppressions or enhancements in the electrospray ionization process. These co-eluting compounds are extracted from the sample matrix together with the analytes, resulting in stronger or weaker signal. Triplicate plasma and urine extracts were compared with water extracts, which should be free of matrix effect (100%). In **Table 2**, matrix effect results showed slightly over 100% in general, which indicates both plasma and urine have some ion signal enhancements. This method is not a quantification method and the minor ion signal enhancement can be accepted.

Intra-day method precision was evaluated by analyzing six replicate plasma or urine samples on the same day. Inter-day method precision was evaluated by analyzing eighteen plasma or urine samples that were acquired on three different days. The precision of the method was determined by calculating the relative standard deviation (RSD) of peak areas of quantifying ions. The RSDs of allanalytes are below 5%, as listed in **Table 2**. The low RSDs indicated that the method had good precision for routine qualitative enantiomer analysis of metham-phetamine, amphetamine, cathinone and methcathinonein equine plasma and urine.

The lower limit of detection (LLOD) of this method in both equine plasma and urine is defined as the lowest concentration at which the compound of interest could be detected but not necessarily confirmed. It can be estimated as the lowest concentration at which all MRM products ions showed a signal-to-noise ratio ≥ 3. For medication violations, the racing industry commonly accepts confirmation criteria that include retention time and

Table 2. Urine and plasma validation results for methamphetamine, amphetamine, cathinone and methcathinone (S)- and (R)-enantiomers.

Urine validation results	Methamphetamine		Amphetamine		Cathinone		Methcathinone	
	S(+)	R(−)	S(+)	R(−)	R(+)	S(−)	R(+)	S(−)
Extraction recovery	94%	98%	86%	85%	70%	70%	85%	86%
Matrix effect	114%	106%	108%	109%	111%	113%	118%	117%
Precision RSD (intra-day) (6)	2.7%	3.7%	3.4%	2.3%	4.0%	4.4%	3.8%	3.2%
Precision RSD (inter-day) (6 + 6 + 6)	2.8%	4.0%	2.8%	3.2%	3.6%	3.7%	4.3%	3.3%
Lower limit of detection (pg/mL)	20	20	200	200	250	250	20	20
Lower limit of confirmation (pg/mL)	50	50	500	500	500	500	50	50
Plasma validation results	**Methamphetamine**		**Amphetamine**		**Cathinone**		**Methcathinone**	
	S(+)	R(−)	S(+)	R(−)	R(+)	S(−)	R(+)	S(−)
Extraction recovery	100%	97%	95%	98%	84%	84%	100%	94%
Matrix effect	109%	112%	118%	117%	98%	112%	113%	123%
Precision RSD (intra-day) (6)	3.7%	2.2%	4.3%	4.7%	3.5%	2.9%	3.8%	3.7%
Precision RSD (inter-day) (6 + 6 + 6)	4.5%	4.6%	4.8%	4.6%	3.9%	2.9%	4.3%	3.2%
Lower limit of detection (pg/mL)	2	2	20	20	10	10	2	2
Lower limit of confirmation (pg/mL)	5	5	50	50	25	25	5	5

spectral match, or comparison of the chromatographic peak area ratios of MRM signals for samples, calibrators and a neat standard. The lowest concentration at which the compounds can be confirmed was taken as the concentration at which the analyte responses were identifiable and discrete with a signal to noise ratio ≥ 10 for all MRM ionchromatogram peaks used. At this concentration, the product ion peak relative abundances should fall within the acceptable range when compared to the reference spectrum (Association of Official Racing Chemists (AORC) confirmation criteria: 10% absolute or 30% relative, whichever is greater) [28]. The results listed in **Table 2** demonstrated that methamphetamine and methcathinone are more sensitive and they both could be detected as low as 2 pg/mL in plasma and 20 pg/mL in urine. In this method, urine sample volume used is only 0.1 mL. If more urine sample is used, the LLOD could be lower even though more matrix effect may be present. Amphetamine has relatively higher LLOD because MRM transition *m/z* 136 - 65 is not sensitive. However, a LLOD of 20 pg/mL in plasma and 200 pg/mL in urine are still the lowest numbers reported for amphetamine chiral analysis from equine samples. Overall, this method presented very good sensitivity coupled with short analysis time.

3.3. Race-Track Sample Applications

In the past two years, 12 equine urine samples and 6 plasms samples were confirmed positive for metham-phetamine. Only one plasma and one urine sample from the same horse were R-methamphetamine and all of the other confirmations were (S)-enantiomer. **Figure 2** is an example showing an equine (S)-methamphetamine positive confirmation in both urine and plasma. It clearly demonstrated the retention time differences between the two isomers and resulted in an easy conclusion of which enantiomer is present in the samples.

4. Conclusion

A fast and easy method for enantiomer separation of amphetamines and cathinones was developed and validated.

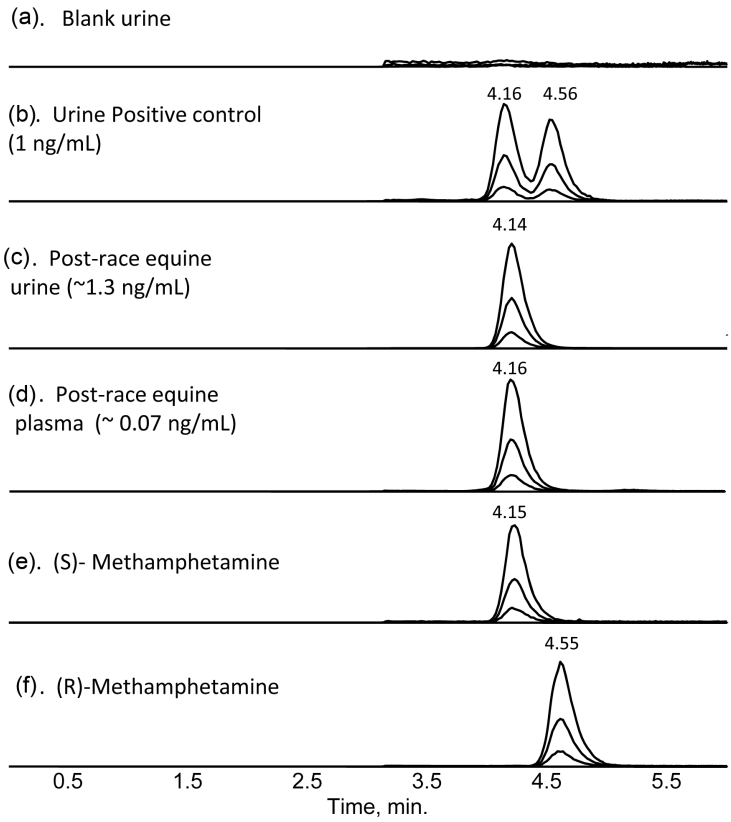

Figure 2. An example of (S)-methamphetamine identification in post-race horse urine and plasma samples. The three MRM transition traces are all shown. Product ion chromatograms of methamphetamine from analysis of (a) Blank urine; (b) Urine positive control; (c) Post-race equine urine sample; (d) The corresponding post-race equine plasma sample; (e) (S)-Methamphetamine standard; (f) (R)-Methamphetamine standard.

This method presented excellent reproducibility and sensitivity. Applicability was demonstrated by routine application of post-race equine urine and plasma samples.

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