Original Article

Method Development and Validation of Ephedrine and Pseudoephedrine in Horse Urine by Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

Background: A comprehensive, fast and precise method for the quantification of ephedrine and pseudoephedrine in horse urine was established and validated utilizing Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) followed by the necessities of Association of Official Racing Chemists (AORC), Federation of Equine International (FEI) and International Council for Harmonization (ICH) guidelines. **Methodology:** The processing of samples was performed by de conjugation accompanied by enzymatic hydrolysis, Solid Phase Extraction (SPE) procedure using HLB cartridges. **Results:** The total chromatographic run time of this method was 7.0 min and found linear over the concentration range of 4-40 ng/mL with a correlation of coefficient (r^2) of 0.99. The intraday and inter-day assay precision of ephedrine were extended from 1.64 to 2.86% and 1.61 to 3.12 %, respectively. In context of pseudoephedrine, the intraday and inter-day assay precision were ranged from 1.55 to 3.26% and 1.11 to 2.60 %, respectively. The absolute percent recovery (%) was found to be 85% for Ephedrine and 90% for Pseudoephedrine. The percent recovery was adequate to discriminate and quantitate the Ephedrine and Pseudoephedrine at or below the level prescribed by AORC Proficiency Testing (PT) list ie 20ng/ ml for equine urine sample. The LOD and LOQ were found 2 and 4 ng/ml respectively for Ephedrine and Pseudoephedrine. **Conclusion:** A comprehensive and precise method for quantitative estimation and diastereomeric differentiation of Ephedrine and Pseudoephedrine by LC-MS/MS was well established and validated according to AORC, FEI and ICH guidelines. This strategy may be additionally utilized for remedial medication checking purposes.

Key words: Ephedrine, Pseudoephedrine, ICH Guidelines, Doping control, Mass spectrometry, AORC, Validation.

Submission Date: 17-03-2020;
Revision Date: 29-06-2020;
Accepted Date: 13-08-2020

DOI: 10.5530/ijper.54.3s.167

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INTRODUCTION

Most equine doping samples are still urine, which offers some essential benefits in comparison to blood. Most analyses can be found in higher concentration resulting in a longer detection time. Furthermore, metabolites can be detected to fortify a positive result and to prove the passage of a drug through the horse urine.[1-3] Ephedrine is a drug belong to stimulant category. It is widely used to counteract the effects of anesthesia and also in therapies for the treatment of asthma and narcolepsy. It has been proven that Ephedrine has common side effects i.e heart attack; dizziness; high blood pressure; headache; nausea; nervousness; fast heart rate; tremor; loss of appetite; seizures; stroke; restlessness; trouble sleeping; stomach irritation, inability to urinate psychosis and abuse. Ephedrine has the high potential to boost horse and human athletic performance.[1]

Pseudoephedrine is a stimulant and sympathomimetic drug. Pseudoephedrine overdose can result in a mainly irregular heartbeat; sweating; hyperactivity; mydriasis; trouble sleeping; tachycardia; hallucinations;

hypertension; sinus arrhythmias; headache; anxiety; tremors; hyperthermia; head bobbing, hiding; psychiatric and symptoms like paranoia. Pseudoephedrine shares the pharmacologic properties of ephedrine but has less potent CNS-stimulating effects.[2,3]

Ephedrine and Pseudoephedrine are obtained from the plant ephedra silica and other members of the genus ephedra. Ephedrine has similar in molecular structure to phenylpropanolamine, methamphetamine and epinephrine (adrenaline). However, the effects of ephedrine are much less potent and longer acting than amphetamines. Ephedrine exhibits optical isomerism and has two chiral centers, giving rise to four stereoisomers, Pseudoephedrine is a stereoisomer of ephedrine that is used as a nasal decongestant. The decongestant effect of pseudoephedrine in equine was described in 1927.[4]

Ephedrine and Pseudoephedrine may be misused because of their performance enhancing potential and thus potential misuse should be controlled by racing laboratories. Ephedrine and Pseudoephedrine can be quantified in urine to screen the possible abuse in horses. The anti-doping rules for Federation of Equine International (FEI) ban the use of Ephedrine and Pseudoephedrine in horse sport. The concentration limit of Ephedrine and Pseudoephedrine in the proficiency testing program is defined 20ng/ml in equine urine by Association of Official Racing Chemists (AORC).[5]

In recent years, detection methods have been improved, resulting in the detection of prohibited substances including Ephedrine and Pseudoephedrine by gas chromatography nitrogen phosphorous detector (GC-NPD), gas chromatography-mass selective detector (GC-MSD), ion mobility MS (IM-MS) and liquid chromatography-tandem mass spectrometer (LC-MS/MS). While the GC-MSD has long been the suitable method in forensic testing and anti-doping field, LC-MS/MS in positive ESI has become a more common and sensitive technique for quantification of Ephedrine and Pseudoephedrine in horse urine in comparison with conventional GC-MSD.[6-13]

To our knowledge, information on separation methods for ephedrine and pseudoephedrine detectability and validation study while following AORC, FEI & ICH Guideline in horse urine matrix using LC–MS/MS was not reported. The structure of ephedrine and pseudoephedrine is shown in Figure 1(a) and Figure 1(b), respectively. A LC–MS/MS method was developed and validated to detect and quantify the Ephedrine and Pseudoephedrine in horse urine samples. Horse urine samples were analyzed using the developed method to demonstrate the applicability of the method as per ICH, FEI and AORC guidelines.[5,14,15]

MATERIALS AND METHODS

Material, Chemical and Standard Solution

Certified Reference Material (CRM) of Ephedrine, Pseudoephedrine and D3-Ephedrine were procured from National Measurement Institute (NMI), Australia and Toronto Research Chemicals (TCS), Canada. Sodium Bicarbonate ($NaHCO_3$), Potassium carbonate (K_2CO_3), Ammonium sulphate $(NH_4)_2SO_4$, Potassium Phosphate (K_3PO_4), Methanol (CH_3OH,), Tert-Butyl methyl ether ($(CH_3)_3COCH_3$), Formic acid (HCOOH), Hydrochloric acid (HCl), Sodium hydroxide (NaOH) were purchased from E Merck Ltd., India and β-Glucoronidase enzyme (*E. coli*) was supplied by Roche, Germany.

Instrumentation and chromatographic conditions

LC-MS/MS Analysis was performed using Thermo Scientific TSQ Triple Quadrupole Mass spectrometer Dionex Ultimate 3000 UHPLC Plus equipped with Automatic Liquid Sampler (ALS). Chromatographic separation was performed on a Inertsil ODS-3, C-8 column (4.6 mm × 50 mm, 3 µm) with a gradient elution of 0.1% formic acid in water (solvent A) and Methanol (solvent B).

Aliquots were prepared by taking 5ml of samples in test tube. Three gram of ammonium sulphate was added in each test tube and dissolved well on cyclo mixer. Then all the samples were centrifuged for 10 min and matrix was cleaned. Cleaned samples were taken in different test tubes. 6.0-6.5 pH was maintained by adding 1M HCl/NaOH in urine sample and any change was resisted in pH by adding 1 ml phosphate buffer. Ephedrine and pseudoephedrine mixture was spiked in different quantities into horse urine samples. Then 100µl Internal Standard (D3-Ephedrine) of 1µg/ml concentration was added into spiked urine samples. The 50µl enzyme β-glucoronidase from *E. coli* was added in all urine samples and mixed it properly, then samples were placed in incubator at 60 degree celsius temperature for 1 hr. Meanwhile, all the samples were placed in incubator & prequiliberate HLB cartridges with 2ml methanol, 2ml water and 2ml of 0.1 M phosphate buffer (pH 6.0). After incubation all the urine samples were again centrifuged for 5 min. All the urine samples were then loaded in HLB cartridges. Samples in HLB cartridge were washed with 3ml water, 1ml of 5% methanol and 0.1 M phosphate buffer. Then final elution was done by using 3ml methanol. After elution, all the eluted

methanol was dried using N_2 evaporator. All the dried residues were reconstituted with 100µl mobile phase (0.1% Formic acid + methanol, 50:50) and collected in vials and injected at LC-MS/MS.

Mass spectrometric analysis was performed on a triple quadrupole mass spectrometer operated in the multiple reaction monitoring (MRM) mode with the transitions of 166→77, 166→115,166 →117, 166→133 and 166→148 for Ephedrine/Pseudoephedrine with different retention times and 169→150.9 for D3-Ephedrine as shown in Table 1. LC-Quan-3.0 (Software) was used for calculation of concentration for Ephedrine and Pseudoephedrine.

The Ion transfer tube temperature was kept at 350°C, while vaporizer temperature was 400°C in Electron Impact (EI) mode, with a solvent delay of 2 min. Data were acquired in the Multiple Reaction Monitoring (MRM) mode, by using this diagnostic transition of the analyses (Ephedrine and Pseudoephedrine) as shown in Table 1.

Molecular Formula: $C_{10}H_{15}NO$

Molecular Weight: 165.23 g/mol

The method was established as per the requirements of ICH, FEI and AORC guidelines. To develop the linearity and range, a stock solution containing 1 mg/ml drug in ethanol was diluted to yield solutions in the concentration range 4-40ng/ml and keeping the injection volume constant (5 µL). To assess precision, five injections of five different QC concentrations (6, 8, 20 and 25 ng/ml) were made on the same day and intra-day precision was determined as relative standard deviation. These studies were also repeated on different days to determine inter-day precision. Accuracy was evaluated by fortifying a mixture of solution with three known concentrations of the drugs and recovery of the added drugs were evaluated. The specificity of the method for the drugs were established by analyzing horse urine samples collected from twelve different horse to investigate the potential interferences at the LC peak region for analysts and Internal Standard (IS) using the proposed extraction procedure and chromatographic-MS conditions. The Limits of Detection (LOD) and Limit of Quantification (LOQ) were determined experimentally, by analysis of samples spiked with decreasing concentrations of the analyses. LOD was defined as the concentration yielding a signal-to-noise ratio of 3. LOQ was calculated as the smallest concentration of analytic that could be measured with a signal-to-noise ratio of 10.

Recovery

Recoveries of the Ephedrine and Pseudoephedrine were determined for horse urine accompanied by the analysis of six different urine samples spiked at 20ng/ml.

RESULTS AND DISCUSSION

Method development and optimization

The optimization and quantification of the mass spectrometric condition were carried out in a multistep procedure. Initially, a full scan spectrum was acquired for each compound. Based on the full scan spectra followed by analysis of base peak, a suitable precursor ion was selected. Some injection was also run to collect the appropriate product ion succeeded byproduct scan mass spectra using different collision energies (CEs). An appropriate product ion and optimization of the collision energy was then carried out on both reference standard and extract from spiked horse urine samples. The Multiple Reaction Monitoring (MRM) diagnostics ions are shown in Table 1.

The experimental conditions for LC-MS/MS method ie., composition of mobile phase, flow rate and multiple reaction monitoring (MRM) for detection

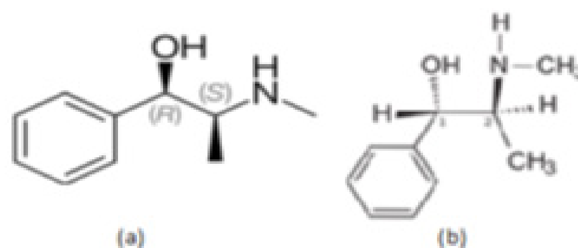

Figure 1: Structure of (a) (1R,2S)-(–)-Ephedrine and (b) (1S,2S)-(+)-Pseudoephedrine.

Table 1: Diagnostic MRM transition for Ephedrine, Pseudoephedrine and ISTD.

Name of Analyte	Precursor (*m/z*)	Product ion	RT (Minutes)	MRM	CE
Ephedrine/ Pseudoephedrine	166	77 115 117 133 148	4.86/5.79	166>77 166>115 166>117 166>133 166>148	35 29 21 22 20
D3-Ephedrine	169	150.9	4.90	169>150.9	20

were augmented to provide precise, accurate and reproducible results for the simultaneous determination of Ephedrine and Pseudoephedrine in horse urine.

The operational strategy for stability of the method was tried with spiked samples of 20 ng/ml in five different specific gravity (SG) urine specimens with 5 uL injection volume, comparing to 20 ng/mL of standard norms. The reason for the test was to assess the operational steadiness against the electrospray ionization (ESI) source contamination by urine samples after SPE clean-up. Figure 2 shows the representative chromatogram of ephedrine and pseudoephedrine with good resolution. Furthermore, Figure 3 shows the representative chromatogram D3-ephedrine, which was used as an internal standard. Figure 4 depicts the absence of any peak in drug free urine (DFU) at the retention time of standards, which represents the absence of co-elution and carry over during the injection of matrix. Based on these outcomes, it is possible to carry out analysis of horse urine samples after SPE clean-up for repeatable result by the high sensitivity LC-MS/MS technique with a low injection volume of 5 uL.

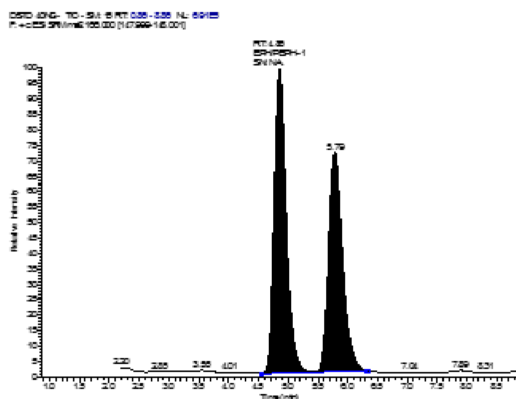

Figure 2: Representative MRM ion Chromatogram of Ephedrine and Pseudoephedrine in Horse Urine.

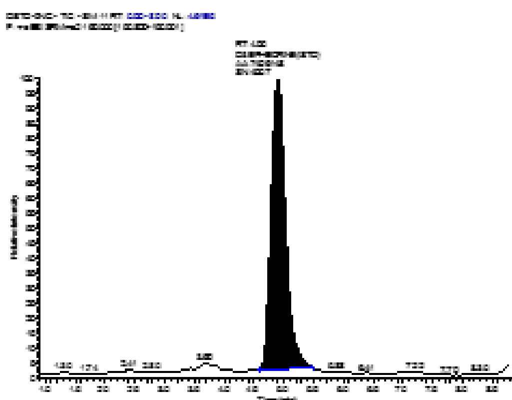

Figure 3: Representative MRM ion Chromatogram for D3-Ephedrine in Horse Urine

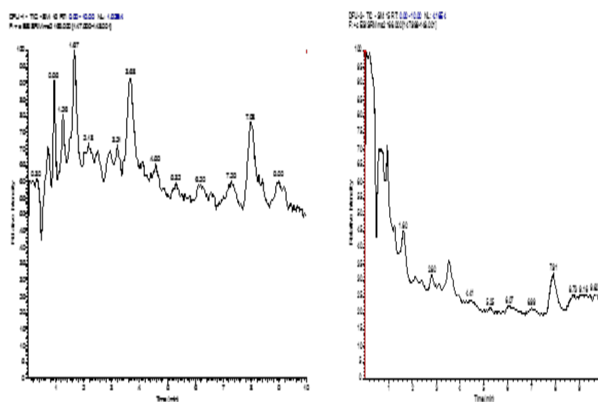

Figure 4: Representative MRM ion Chromatogram for Horse-Drug Free Urine (DFU).

The electrospray ionization was carried out in positive ionization mode for the analytical standards and its internal standard (IS). The accompanying instrument parameters for ionization were utilized: spray voltage: 4500 volt, Sweep gas: 2 Arb, Sheath gas: 50 Arb and Aux gas: 20 Arb.

Validation

The calibration curve was plotted utilizing five linear points viz., 4-40 ng/mL. The linearity standard curve represented a reliable reproducibility over the standard concentrations across the linear range. The response of calibration curve was organized by determining the best fit of peak-area ratios (peak area analyte/peak area IS) versus concentration and best fitted to the y= mx+c using weighing factor (1/X). The regression equation obtained were $y = 0.197x + 0.0002$ and the correlation coefficient, r^2, for the equation was 0.98. The precision and Accuracy were studied for intraday and inter-day samples ranged from 0.75- 1.7% and 87-111%, respectively, indicating the method was sufficiently precise. The measures values on intraday and inter-day were found to be within the expected limits as given in Table 2. The percentage recovery for Ephedrine and Pseudoephedrine was found to be 85% and 90% respectively at 20ng/ml spiking level. The percentage recovery was sufficient to reliable identify of Ephedrine and Pseudoephedrine in horse urine at or below the level prescribed by AORC proficiency testing list-2019 i.e20ng/ml. The LOD and LOQ was measured to be 2 and 4 ng/ml, respectively.

CONCLUSION

A comprehensive, fast and precise LC-MS/MS method for quantitative determination of Ephedrine and Pseudoephedrine was established and validated followed by ICH, FEI and AORC guidelines. This strategy

Table 2: Results from study of precision and accuracy.

Quality Controls (ng/ml)	Ephedrine			Pseudoephedrine		
	Mean±SD (*n*=5)	Accuracy (%)	Precision (%)	Mean±SD (*n*=5)	Accuracy (%)[a]	Precision (%)[b]
Intraday precision						
6	5.456±0.111	90.93	2.035	5.602±0.113	93.37	2.025
8	7.654±0.126	95.68	1.644	7.498±0.245	93.73	3.264
20	19.732±0.333	98.66	1.687	19.750±0.307	98.75	1.554
25	25.322±0.726	101.29	2.864	25.328±0.520	101.31	2.054
Inter day precision						
6	5.576±0.174	92.93	3.123	5.854±0.152	97.57	2.603
8	7.670±0.124	95.88	1.615	7.710±0.153	96.38	1.986
20	20.308±2.737	101.54	2.737	20.360±2.63	101.80	2.635
25	24.754±1.737	99.01	1.771	25.804±0.289	103.22	1.114

[a]Determined as (mean concentration/nominal concentration) x 100
[b]Calculated as % RSD (Standard deviation/mean).

was specific, accurate and reproducible for Ephedrine and Pseudoephedrine. The estimated run time of the developed method was 7 min at gradient program. This current method would get enhanced by combining the triple quadrupole with Liquid Chromatography, thereby can greatly improve the detection capabilities and minimizing false detection of target substance in complex horse urine matrix. This method was efficiently applied for screening and confirmation analysis for the testing of horse dope samples. Further, the extension of this work would be on the differentiation of results of Ephedrine and Pseudoephedrine analysis in horse urine due to presence of confounding/interfering factors. The higher sensitivity with shorter run time contains low sample volume, the better LOD/LOQ, the less tedious cleaning up and sample preparation procedure make this preferred analytical procedure using LC-MS/MS methodology.

ACKNOWLEDGEMENT

The author wishes to acknowledge the Ministry of Youth Affairs and Sports, Govt. of India for their continuous support.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ABBREVIATIONS

AORC: Association of Official Racing Chemists; **ICH:** International Council for Harmonization, **FEI:** Federation of Equine International; **HPLC:** High Performance Liquid Chromatography; **GC-MS:** Gas Chromatography- mass spectrometry; **SPE:** Solid Phase Extraction; **LC-MS:** Liquid chromatography mass spectrometry; **ESI:** Electrospray Ionization; **MRM:** Multiple Reaction Monitoring; **RT:** Retention time; **Arb:** Arbitrary unit; **CE:** Collision Energy; **HLB:** Hydrophilic Lipophilic Balance.

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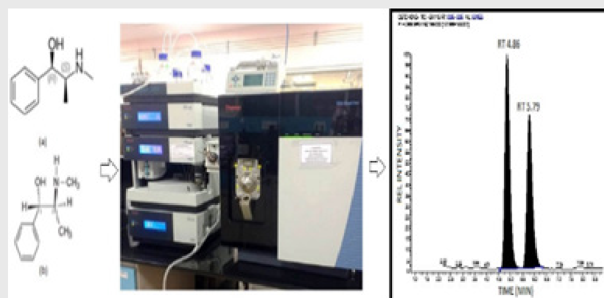
PICTORIAL ABSTRACT



SUMMAY

Liquid Chromatography-Tandem Mass spectrometry obviously has a crucial and focal role in the screening and confirmation analysis of medications of misuse at present and later on. The improvement toward more sensitive methodology will always a continuous process to avoid false reporting accompanied by reliable results. Based on the utility of this methodology, the stereoisomers can be easily discriminated, this analytical method offers substantial regulatory and scientific advantages over the conventional urine testing methods.

Cite this article: Maurya AK, Sahu PL, Jethi S, Gosain H, Sahu K. Method Development and Validation of Ephedrine and Pseudoephedrine in Horse Urine by Liquid Chromatography-Tandem Mass Spectrometry. Indian J of Pharmaceutical Education and Research. 2020;54(3s):s671-s676.