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Rapid UPLC-MS/MS Determination of Amantadine and Rimantadine in Human Blood and Urine for Forensic Toxicology

ShengYu Qin[1], Ning Wang[1], Di Liang[2], Xian Ju[2], LiDong Lu[1] & LiuJiao Wu[1]

[1] Guangxi Jingui Forensic Science Institute, China

[2] School of Forensic Medicine, Shanxi Medical University, China

Correspondence: LiuJiao Wu, Guangxi Jingui Forensic Science Institute, Nanning, Guangxi, China. E-mail: 1670552862@qq.com

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Abstract

A rapid UPLC-MS/MS method was developed and validated for quantifying amantadine (AMD) and rimantadine (RIM) in human blood and urine. Sample preparation involved acetonitrile protein precipitation with amantadine-D6 as internal standard. Separation used an ACE C_{18}-PFP column and a mobile phase of 0.1% aqueous formic acid/5 mmol/L ammonium formate-acetonitrile (85:15, v/v). ESI^+-MRM detection was employed. Acetonitrile provided optimal extraction. The method showed excellent linearity (1.0–1000.0 ng/mL; $R^2 > 0.99$), LOD/LOQ , mean recoveries (82.16–105.43%), and precision (RSD 3.57–10.39%). This sensitive, reliable method is suitable for forensic and public health applications.

Keywords: Amantadine, Rimantadine, UPLC-MS/MS, forensic toxicology, method validation

1. Introduction

Amantadine (AMD) and rimantadine (RIM) belong to the class of adamantane derivatives, which are extensively utilized in the livestock and poultry breeding industry due to their potent inhibitory effects against influenza viruses and in the management of Parkinson's syndrome [1]. However, with the continuous expansion of farming scale and the recurrent emergence of various infectious diseases, the usage and frequency of administration of these drugs have shown a consistent upward trend [2]. The escalating use of adamantane drugs can lead to the development of drug resistance in livestock and poultry, as well as excessive accumulation within their bodies, which may subsequently compromise human health through the food chain [3]. AMD and RIM primarily exist in their parent forms within biological systems and are eliminated via glomerular filtration and tubular secretion. Reports indicate that AMD and RIM can induce cortical myoclonus; while symptoms typically resolve upon discontinuation, some patients had a history of renal failure [4]. Therefore, these drugs should be used cautiously in individuals with renal impairment or severe renal insufficiency. The therapeutic dose is relatively close to the toxic dose, and excessive use or co-administration with anticholinergic drugs can readily induce central nervous system symptoms such as hallucinations, dizziness, coma, and mental confusion, potentially leading to fatality in severe cases [5, 6]. Currently, numerous methods exist for detecting AMD and RIM, including gas chromatography (GC), liquid chromatography (LC), liquid chromatography-tandem mass spectrometry (LC-MS/MS), enzyme-linked immunosorbent assay (ELISA), and immunochromatographic assays [7-9]. Most literature focuses on the determination of AMD and RIM residues in animal-derived food products, with relatively few reports on detection methods for amantadine in human biological matrices. To better serve applications in forensic and public health domains, this study established a rapid quantification method for AMD and RIM in human blood and urine based on ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

2. Materials and Methods

2.1 Instruments and Reagents

Liquid chromatography-tandem triple quadrupole mass spectrometer (LC-MS/MS; LCMS-8040, Shimadzu, Japan); Multi-tube vortex mixer (QB-600, Kylin-Bell Instrument, China); Water purification system (Millipore, USA); Pipettes (Eppendorf, Germany).

Amantadine (AMD), Rimantadine (RIM), and Amantadine-D6 (as internal standard; >99% purity; Macklin, Shanghai, China); Formic acid (LC-MS grade), Methanol (LC-MS grade), Acetonitrile (LC-MS grade; Sigma-Aldrich, USA).

2.2 Preparation of Standard Solutions

Stock standard solutions of AMD and RIM (1 mg/mL) were accurately prepared in methanol based on calculated amounts. These solutions were then serially diluted with methanol to obtain working standard solutions at concentrations of 100 μg/mL, 10 μg/mL, etc. The internal standard solution of amantadine-D6 (100 μg/mL) was prepared in methanol. All solutions were stored at -20°C in a refrigerator.

2.3 Sample Preparation

A 100 μL aliquot of blood or urine was pipetted into a 1.5 mL centrifuge tube. Then, 5 μL of the amantadine-D6 internal standard solution (100 μg/mL) and 500 μL of acetonitrile were added. The mixture was vortex-mixed, followed by centrifugation at 13,000 rpm for 10 min at 4°C. Subsequently, the supernatant was collected and filtered through a 0.22 μm organic membrane filter. Finally, 5 μL of the filtrate was injected into the UPLC-MS/MS system for analysis.

2.4 Chromatographic and Mass Spectrometric Conditions

Chromatographic separation was performed on an ACE C18-PFP column (4.6 mm × 150 mm, 5 μm particle size) maintained at 40°C. The injection volume was 5 μL, with a mobile phase flow rate of 0.4 mL/min. The mobile phase consisted of aqueous formic acid (0.1% v/v) containing 5 mmol/L ammonium formate (A) and acetonitrile (B) (85:15, v/v). Ionization was achieved using electrospray ionization (ESI) in positive ion mode. Source parameters were as follows: nebulizing gas flow, 11 L/min; ion spray voltage (IS), 4000 V; drying gas temperature (TEM), 300°C. Detection was performed in positive ion multiple reaction monitoring (MRM) mode. Detailed transition parameters are provided in Table 1.

Table 1. Specific MRM parameters of AMD and RIM

	Rt (min)	Precursor ion (m/z)	Product ion (m/z)	Q1/V	CE/V	Q3/V
AMD	4.153	152	93/135*	-13/-11	-35/-35	-15/-14
RIM	5.473	180.1	163/107.1*	-19/-18	-35/-35	-21/-27
IS	4.152	158	141.1*	-8	-35	-11

*Quantifier ion

3. Results and Discussion

3.1 Selection of Extraction Solvent

The extraction solvent directly influences the extraction efficiency of the target analytes. Four solvents—methanol, acetonitrile, ethyl acetate, and diethyl ether—were compared for their extraction efficiency of AMD and RIM. The results demonstrated that ethyl acetate yielded the lowest extraction efficiency, with recovery rates ranging from 39.71% to 47.63%. Diethyl ether exhibited moderate performance, while both acetonitrile and methanol demonstrated superior recovery rates. However, acetonitrile showed the smallest variability in recovery. Consequently, acetonitrile was selected as the optimal extraction solvent.

3.2 Selection of Mobile Phase

Six mobile phase compositions were compared: methanol-water, methanol-0.1% aqueous formic acid, methanol-0.1% aqueous formic acid containing 5 mmol/L ammonium formate, acetonitrile-water, acetonitrile-0.1% aqueous formic acid, and acetonitrile-0.1% aqueous formic acid containing 5 mmol/L ammonium formate. The results demonstrated that mobile phases containing only water and organic solvent yielded poor peak shapes for the analytes. Upon addition of 0.1% formic acid to the aqueous component, satisfactory peak shapes and appropriate retention times were achieved. When 5 mmol/L ammonium formate was incorporated into the 0.1% aqueous formic acid solution, the analyte peaks exhibited sharp, symmetrical profiles with enhanced sensitivity compared to the previously mentioned mobile phases. Among these, the acetonitrile-0.1% aqueous formic acid (containing 5 mmol/L ammonium formate) system demonstrated optimal peak characteristics. This composition was therefore selected as the mobile phase.

3.3 Chromatographic Column Selection

The separation performance of three columns was compared: Zorbax SB-C_{18} (150 mm × 4.6 mm, 5 µm), Agilent PFP (150 mm × 4.6 mm, 5 µm), and ACE C_{18}-PFP (150 mm × 4.6 mm, 5 µm). Results demonstrated that the Zorbax SB-C_{18} column yielded suboptimal separation efficiency with poor peak symmetry. The Agilent PFP column exhibited improved chromatographic resolution, producing sharp and symmetrical peaks. The ACE C_{18}-PFP column demonstrated superior separation performance and the highest peak area responses among the three columns. Consequently, the ACE C_{18}-PFP column was selected for subsequent analyses (Figure 1).

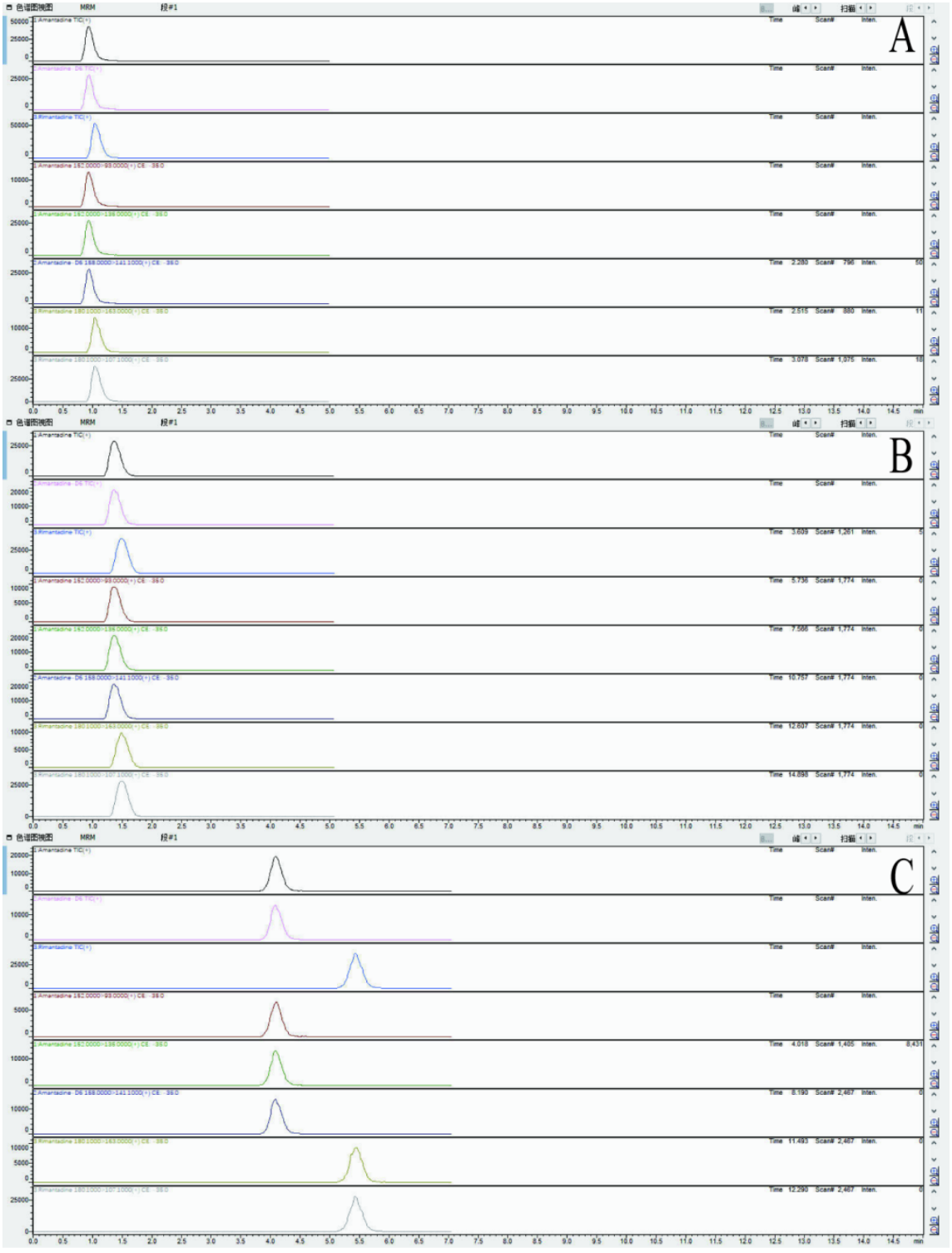

Figure 1. MRM chromatograms of Zorbax SB-C_{18} (A), Agilent PFP (B), and ACE C_{18}-PFP(C)

3.4 Linear Range, Limit of Detection, and Limit of Quantification

A series of mixed standard working solutions were analyzed to construct calibration curves. Linear regression equations and correlation coefficients (R^2) were calculated. The method's limit of detection (LOD) and limit of quantification (LOQ) were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. Both AMD and RIM exhibited excellent linearity over the concentration range of 1.0–1000.0 ng/mL. Relevant parameters are summarized in Table 2.

Table 2. Standard calibration curves for AMD and RIM in blood and urine

	Linearity range (ng/mL)	Linear equation	R2	LOD(ng/mL)	LOQ(ng/mL)
AMD(blood)	1~1000	Y=0.2634X+0.0732	0.994	0.5	1
RIM(urine)	1~1000	Y=0.1824X+0.91	0.998	0.5	1
AMD(blood)	1~1000	Y=0.5312X+0.4821	0.993	0.5	1
RIM(urine)	1~1000	Y=0.7632X+0.6431	0.991	0.5	1

3.5 Recovery and Precision

Blank rabbit blood and urine matrices were fortified with mixed standard solutions of AMD and RIM at three concentration levels: low (10 ng/mL), medium (200 ng/mL), and high (800 ng/mL). Six replicate samples were prepared for each concentration level to evaluate method recovery. The mean recoveries for AMD and RIM ranged from 82.16% to 105.43%, with relative standard deviations (RSDs) between 3.57% and 10.39%. These results demonstrate satisfactory method accuracy and precision.

4. Conclusion

A rapid detection method for amantadine (AMD) and rimantadine (RIM) in human blood and urine was established based on ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). This method demonstrates operational simplicity, high sensitivity, and reliable accuracy, providing efficient and scientifically sound technical support for the rapid detection of AMD/RIM intoxication in forensic toxicological analysis and public health emergencies.

Author Introduction

ShengYu Qin, judicial authenticator,Bachelor degree, research direction: forensic medicine.

Corresponding authors

LiuJiao Wu,judicial authenticator,Bachelor degree, research direction: forensic medicine.

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