African Journal of Biotechnology Vol. 9 (28), pp. 4422-4427, 12 July, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Simultaneous determination of aconitum alkaloids in rat body fluids by high-performance liquid chromatography

Wang Rui[1], Sun Yi-kun[2], Wang Yun[2] and Qiao Yan-jiang[2]*

[1]Shanxi College of Traditional Chinese Medicine, Taiyuan, 030024, China.
[2]Beijing University of Traditional Chinese Medicine, Beijing, 100102, China.

Accepted 6 May, 2010

A sensitive, reliable and accurate high-performance liquid chromatography (HPLC) method coupled with photodiode array detector (DAD) were developed for the simultaneous quantitative determination of aconitine, mesaconitine and hypaconitine in rat plasma and urine by optimizing the extraction, separation and analytical conditions. The analyses were chromatographed on a ZORBAX Eclipse XDB-C_{18} column (150 mm × 4.6 mm i.d.; 5 µm particle size) with gradient elution using solvents of acetonitrile and ammonium acetate buffer (pH 10.0). The detection wavelength was 240 nm. Intra-assay and inter-assay precision of the analyses were less than 10% and the average recovery rates obtained were in the range of 85.63 - 90.94% for all analysis of the three aconitum alkaloids with relative standard deviations (RSD) below 14%. Positive linear relationships were observed in correlation coefficients that exceeded 0.95. The limits of detection (signal-to-noise ratio of 3) were 2.64, 1.58 and 2.75 ng for aconitine, mesaconitine and hypaconitine, respectively. The method can provide a scientific and technical platform to determine the concentration of aconitum alkaloids in plasma during a pilot pharmacokinetic study in rats.

Key words: Aconitum alkaloids, rat body fluids, quantitative analysis, high-performance liquid chromatography.

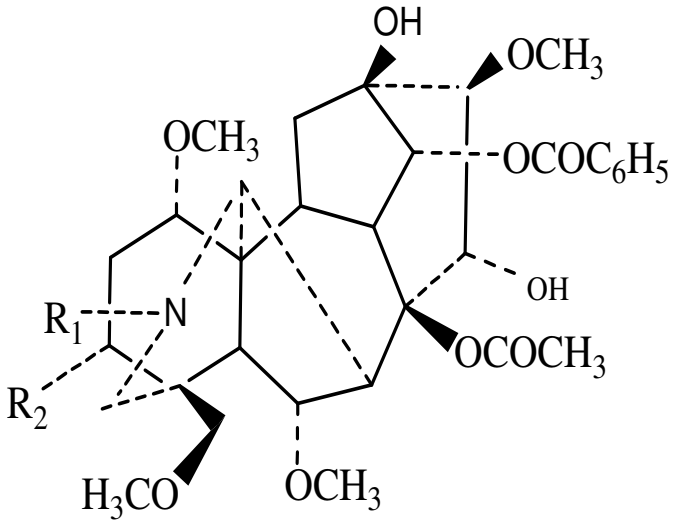
INTRODUCTION

Aconitine, mesaconitine and hypaconitine (Figure 1) are three kinds of highly toxic diester-diterpene *Aconitum* alkaloids, which mainly originated from Chinese medicinal herbs of the genus *Aconitum* in the family of Ranuncucaceae, known as Fuzi (Radix Aconiti Lateralis) or Chuanwu (Radix Aconiti Kusnezoffii). Toxicological studies demonstrated that the lethal dose 50% (LD50) of aconitine, mesaconitine and hypaconitine orally in mice is 1.8, 1.9 and 5.8 mg/kg, respectively (Zhou, 1983). The lethal dose of these alkaloids in humans is estimated to be 2.5 mg (Li and Gao, 1991). Although the aconite roots are highly toxic, they are widely used to treat joint pain, arthritic and rheumatic diseases for over 2,000 years by Chinese doctors. These diester-type alkaloids also have excellent pharmacological effect, such as anti-inflammatory and antinociceptive properties (Ameri, 1998). At present, research works on diester-diterpene *Aconitum* alkaloids is focused on the identification of their poisoning and toxic dose (Zhang et al., 2005). Due to the rapid metabolism of diester-diterpene *Aconitum* alkaloids *in vivo*, the identification of *Aconitum* alkaloids poisoning and the safety research in pharmacology are difficult. Therefore, a simple and reliable determination method of *Aconitum* alkaloids in biological fluid is essential in the field of toxicology, pharmacology and forensic medicine.

Several methods have been reported for detecting aconitine, mesaconitine and hypaconitine, including high-performance liquid chromatography (HPLC) (Wang et al., 2004; Jiang et al., 2005; Wang et al., 2006; Liu and Chao, 2006), liquid chromatography-mass spectrometry (LC-MS) (Ohta et al., 1997; Wang et al., 2001; Kaneko et al., 2006; Zhang et al., 2005), capillary electrophoresis (CE) (Guo et al., 2004; Feng and Li 2002), capillary electrophoresis-mass spectrometry (CE-MS) (Feng et al.,

*Corresponding author. E-mail: yjqiao@263.net. Tel: 010-84738601 Fax: 010-84738611.

Abbreviations: HPLC, High-performance liquid chromatography; **DAD,** photodiode array detector; **RSD,** relative standard deviations; **SPE,** solid phase extraction; **LC-MS,** liquid chromato- graphy-mass spectrometry; **GC-MS,** gas chromatography-mass spectrometry; **CE,** capillary electrophoresis; **CE-MS,** capillary electrophoresis-mass spectrometry.



Name	R_1	R_2
Aconitine	C_2H_5	OH
Meaconitine	CH_3	OH
Hypaconitine	CH_3	H

Figure 1. Chemical structures of aconitine, mesaconitine and hypaconitine.

2003), gas chromatography-mass spectrometry (GC-MS) (Ito et al., 1998) and so forth.

In the current study, we investigated the effect of different alkaline buffer concentrations and pH values and successfully developed a modified HPLC analytical method for the simultaneous quantitative determination of the three diester-diterpene *Aconitum* alkaloids in rat plasma and urine. Furthermore, we investigated the extraction methods, peak confirmation, mobile phase buffer and gradient elution program according to the complexity of biological samples.

MATERIALS AND METHODS

Chemical and reagents

Acetonitrile was of HPLC grade (Sigma-Aldrich, Germany). Solid phase extraction (SPE) columns (Oasis® MCX) were obtained from Waters (Milford, USA). All other chemicals used were of analytical reagent grade and were charged from Beihua Fine Chemicals Co., Ltd (Beijing, China). Deionized water was prepared using a Millipore (Billerica, MA, USA) water purification system. All solvents and solutions were filtered through a Millipore filter (0.45 µm) before using.

Reference standards of aconitine, mesaconitine and hypaconitine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products in China. Their chemical structures were further identified by LC-MS in our laboratory.

Drug-free urine and heparinized plasma were obtained from different young, healthy male SD rats housed in the Laboratory Animal Services Division of the Institute. Urine and plasma samples were stored in glass tube at -20℃ until further use. All experiments were carried out in accordance with the guidelines of the local ethical committee.

Chromatographic system

An Agilent 1100 series LC system (Hewlett-Packard, USA) consisting of a G1311A Quaternary Pumps, a G1379A Degasser, a G1315B Diode-Array Detector and a G1313A auto sampler was employed in this research.

The measurements of the *Aconitum* alkaloids were carried out on a ZORBAX Eclipse XDB-C_{18} column (150 mm × 4.6 mm i.d.; 5 µm particle size; Agilent Technologies Inc., USA) protected by an Eclipse XDB-C_{18} guard column (20 mm × 4.6 mm i.d.; 5 µm particle sizes) at 30℃. The solvents used for HPLC separation of the three alkaloids in samples were carried out with an acetonitrile (A) and buffer solution (B) containing 40 mmol/mL ammonium acetate adjusted with concentrated ammonia to pH 10.0 at a flow rate of 1.0 mL/min. The gradient elution of the mobile phase was 15 - 60% (A) in 0 - 45 min and 60% (A) in 45 - 50 min. Detection was carried out at 240 nm with a reference wavelength of 380 nm.

Preparation of standard and working solution

The standard chemicals of three alkaloids were accurately weighed and dissolved with 0.05% hydrochloric acid (HCl) in methanol as stock standard solutions. These stock solutions were used for the preparation of a mixed working stock solution comprising aconitine (3210 µg/mL), mesaconitine (3060 µg/mL) and hypaconitine (3070 µg/mL), which was prepared with 0.05% HCl in methanol. Analytical standards were prepared from mixed working stock solution by diluting it with reconstituted solution (0.05% HCl solution in methanol) over a concentration range of 1.605 - 160.5 µg/mL, 1.530 - 153.0 µg/mL and 1.535 - 153.5 µg/mL for aconitine, mesaconitine and hypaconitine, respectively. All solutions were stored at 4℃ and remained stable for at least one month (verified by reassaying the standard solutions).

Preparation of urine and plasma standard solutions

Urine and plasma standards were prepared from the mixed working stock solution by diluting with normal rat urine and plasma over a concentration range of 1.605 - 160.5 µg/mL, 1.530 - 153.0 µg/mL and 1.535 - 153.5 µg/mL for aconitine, mesaconitine and hypaconitine, respectively. Then, 0.5 mL of plasma standards and 0.5 mL of urine standards were further spiked with 0.5 mL of acetonitrile; the supernatant was obtained by centrifugation (4℃, 12000 rpm, 20 min), and was lyophilized with dry ice lyophilizer. The dry residue was dissolved in 1 mL of 0.05% HCl in methanol. All

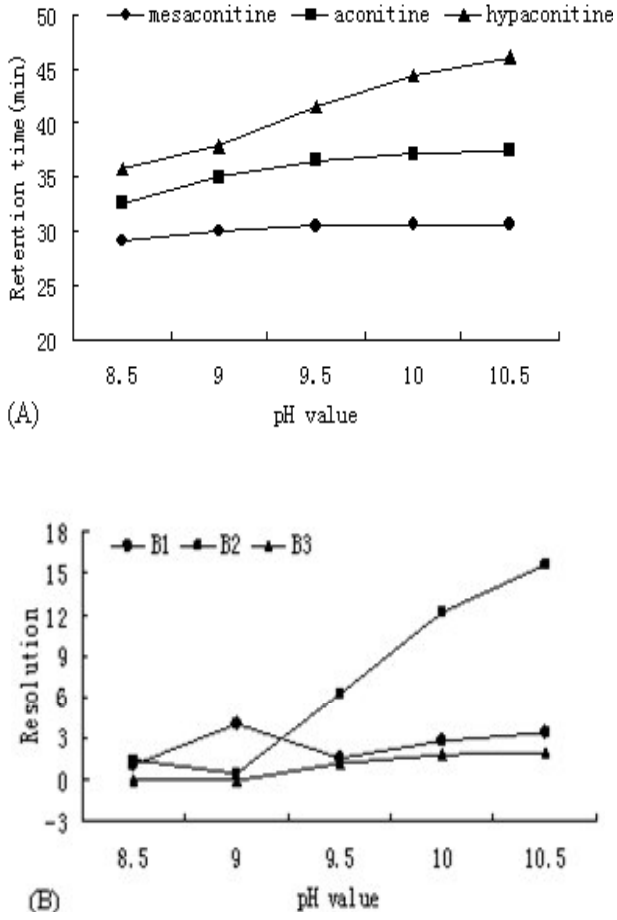


Figure 2. Effect of the pH values on the retention time of the three *Aconitum* alkaloids (A), and on the resolution of the adjacent peaks (B). B1: resolution of unknown compound and mesaconitine; B2: resolution of aconitine and hypaconitine; B3: resolution of unknown compound and hypaconitine.

solutions were filtered through 0.45 µm filter membrane and 10µL filtrate was injected into the HPLC system for quantitation.

Validation of the chromatographic method

Linearity

Calibration curves were established based on six points for each alkaloid with concentrations of 1.605, 3.210, 8.025, 32.10, 80.25 and 160.5 µg/mL for aconitine; 1.530, 3.060, 7.650, 30.60, 76.50 and 153.0 µg/mL for mesaconitine and 1.535, 3.070, 7.675, 30.70, 76.75 and 153.5µg/mL for hypaconitine. The prepared samples were processed in accordance with the preparation of urine and plasma standard solutions (as previously described) and were quantified in accordance with the selected method.

Precision

Precision was evaluated by HPLC analysis with a standard mixture solution of aconitine, meaconitine and hypaconitine under the selected optimal conditions six times in one day for intra-day variation and twice a day in three consecutive days for inter-day variation; it was expressed as the relative standard deviation (RSD).

Repeatability

Six measurements were considered, one of the plasma standard solutions (the concentrations of aconitine, meaconitine and hypaconitine were 16.05, 15.30 and 15.35 µg/mL, respectively). The processing of the measurements was done in accordance with the preparation of urine and plasma standard solutions. The RSD within the six measurements was calculated and used for evaluation of the repeatability.

Recovery

The three different concentration levels for aconitine (8.025, 32.10, 80.25µg/mL), meaconitine (7.65, 30.6, 76.5 µg/mL) and hypaconitine (7.675, 30.70, 76.75 µg/mL) as low, medium and high concentration were used to assess the recovery of the assay method. The prepared samples above (n = 9) were then processed in accordance with the preparation of urine and plasma standard solutions and were quantified under the selected optimal conditions.

RESULTS AND DISCUSSION

Effect of pH value of the mobile phase buffer on the retention time and resolution of HPLC

Mobile phase pH is an important factor in HPLC that can affect analytical results. The *Aconitum* alkaloids in the HPLC chromatograms displayed short retention time in the mobile phase with low- or mid-range pH; therefore, it was difficult to separate them from other alkaloids and from their metabolites. In this study, we employed an alternative method to separate the *Aconitum* alkaloids using a mobile phase pH that was above their pKa. Under such condition, they can exist in free-base forms because their cation charges were neutralized, which improved the peak shape. The retention times of aconitine, meaconitine and hypaconitine were compared with different pH values from 8.5 to 10.5 adjusted by concentrated ammonia solution, in which the ammonium acetate buffer concentration reached 40mmoL/L. When the pH of the mobile phase was increased from 8.5, the retention time of aconitine, mesaconitine and hypaconitine increased (Figure 2A) because of the stronger interaction between these low-polar molecule and hydrophobic-bonded phase.

The results of HPLC analysis showed that the pH values had a distinguished effect on the resolution of the three *Aconitum* alkaloids and other unknown compounds in the samples (Figure 2B). At a pH of 10.0, all these compounds were baseline, separated from each other with resolution values above 1.5. Allowing for the long elution time, the buffer solution for the present studies was finally chosen at a pH of 10.0.

Effect of the mobile phase buffer concentration

To decide an optimal concentration of the mobile phase

Table 1. Regression equations and theirs correlation coefficients of three *Aconitum* alkaloids.

Alkaloids	Linear range (μg)	Plasma		Urine	
		Regression equation	Correlation coefficient	Regression equation	Correlation coefficient
Aconitine	0.0073 - 0.73	$Y = 912.57X + 0.35$	0.972	$Y = 940.97X + 0.93$	0.974
Mesaconitine	0.0077 - 0.77	$Y = 888.72X + 0.28$	0.969	$Y = 966.69X + 2.21$	0.963
Hypaconitine	0.0075 - 0.75	$Y = 943.90X + 0.44$	0.957	$Y = 938.97X + 5.66$	0.964

buffer, four concentrations (10, 20, 30 and 40 mmoL/L) of the ammonia acetate buffer solution with pH 10.0 were used together with acetonitrile as the mobile phase for the HPLC analysis. The results indicated that the retention times of these three alkaloids decreased slightly with increasing concentrations of ammonia acetate in the buffer solution. Their resolutions were slightly affected by ammonia acetate concentration. Therefore, the 40 mmoL/L buffer solution was selected as the HPLC mobile phase.

Urine and plasma standards extraction conditions

In order to optimize extraction conditions of urine and plasma standards, liquid-liquid extraction (LLE) and SPE were carried out. For LLE, two organic solvents (methanol and acetonitrile) were used as extraction solutions. The urine and plasma standards (0.5 mL) were extracted by methanol (1 mL) and acetonitrile (0.5 mL), respectively, in accordance with the preparation of urine and plasma standard solutions. For SPE, the urine and plasma standards (0.5 mL) were directly applied to oasis MCX cartridges (1 cc), which had been conditioned and equilibrated with methanol and deionized water. After washing the cartridges with 1 mL of 2% formic acid and 1 mL of methanol, successively, alkaloids were eluted with 1 mL of solution containing 5% ammonia in methanol. The eluate was lyophilized with dry ice lyophilizer; the residue was dissolved in 1 mL of 0.05% HCl in methanol. The results showed that acetonitrile and SPE are better for the extraction of alkaloids than methanol and LLE, respectively, because more alkaloids can be extracted with less volume solvents. The results of the recovery test also demonstrated that the extraction method was adequate for the analysis.

Validation of HPLC assay

A gradient elution program was developed to separate and quantify the three *Aconitum* alkaloids. Mesaconitine, aconitine and hypaconitine were successfully determined by a single run of HPLC and were eluted at 30.4, 36.9 and 44.1min retention time, respectively. Aconitine, mesaconitine and hypaconitine in plasma and urine standards were identified by comparing both their retention times and the UV spectra with those of reference standards. The peak purity was confirmed by studying the photodiode array detector (DAD) data with peaks of three alkaloids, in which no indication for impurities could be found.

The linearity of the plot concentration (X, μg) for each *Aconitum* alkaloids versus peak areas (Y, mAU) were investigated; the results are expressed as regression equations and their correlation coefficient (R) values are given in Table 1.

The limit of detection, which was defined as the amount of the compounds required to produce a peak with a height three times the level of the average baseline noise (S/N = 3), was determined to be 2.64 ng for aconitine, 1.58 ng for mesaconitine and 2.75 ng for hypaconitine.

The analytical precision from the data of the intra-daily (six times per day) and inter-daily (twice a day for three consecutive days) determinations was indicated by the relative standard deviations, which were less than 10% for all three alkaloids.

The RSDs of the repeatability test were less than 13% for all three alkaloids. The average recovery rates obtained were in the range of 85.63 - 90.94% for all with RSDs below 14%. Therefore, these chromatographic systems for the quantitative determination of the three *Aconitum* alkaloids were appropriate for plasma and urine samples.

Application of the HPLC method for plasma and urine samples

The described method was applied to the analysis of a plasma sample and a urine sample from a male SD rat that had died of aconite root intoxication. As shown in Figure 3, the concentrations of mesaconitine and hypaconitine in plasma were 101 and 129 ng/mL; aconitine could not be detected because of very low levels. The con-

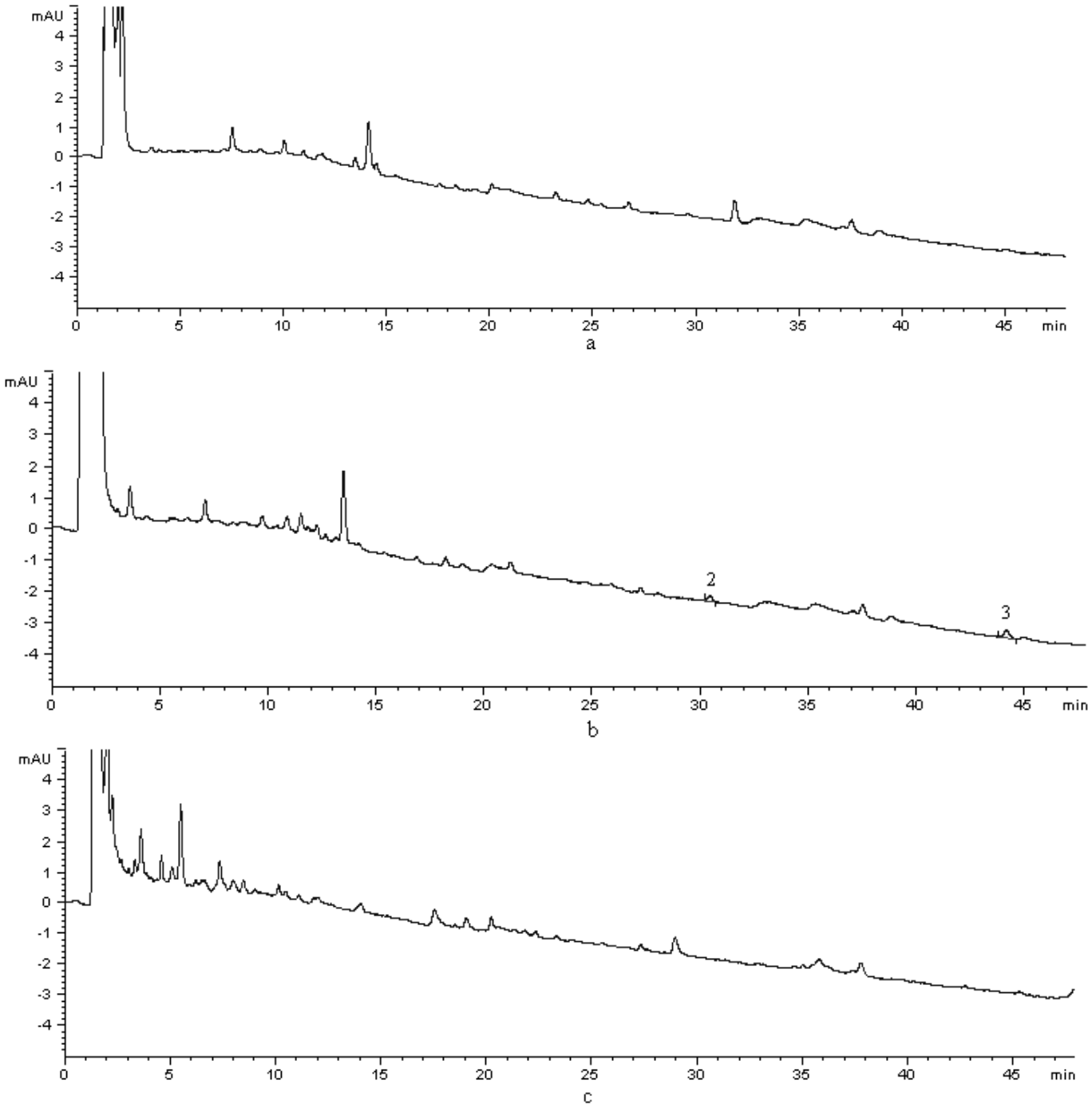


Figure 3. HPLC chromatogram of rat plasma and urine samples: (a) Blank plasma sample; (b) plasma sample obtained from a rat which had died of aconite root intoxication; (c) blank urine sample; (d) urine sample obtained from a rat which had died of aconite root intoxication. (1) Aconitine, (2) meaconitine and (3) hypaconitine.

tents of aconitine, mesaconitine and hypaconitine in urine were 0.418, 1.064 and 8.797 µg, respectively.

Conclusion

Aconitum alkaloids poisoning was usually confirmed by detecting highly toxic diester-diterpene of aconitine, mesaconitine and hypaconitine in rat plasma and urine. By selecting an appropriate alkaline buffer (40mmoL/L ammonium acetate buffers, pH 10.0) with acetonitrile as the mobile phase and suitable gradient elution program, satisfactory chromatographic separation of three *Aconitum* alkaloids with their symmetrical peaks was achieved. The

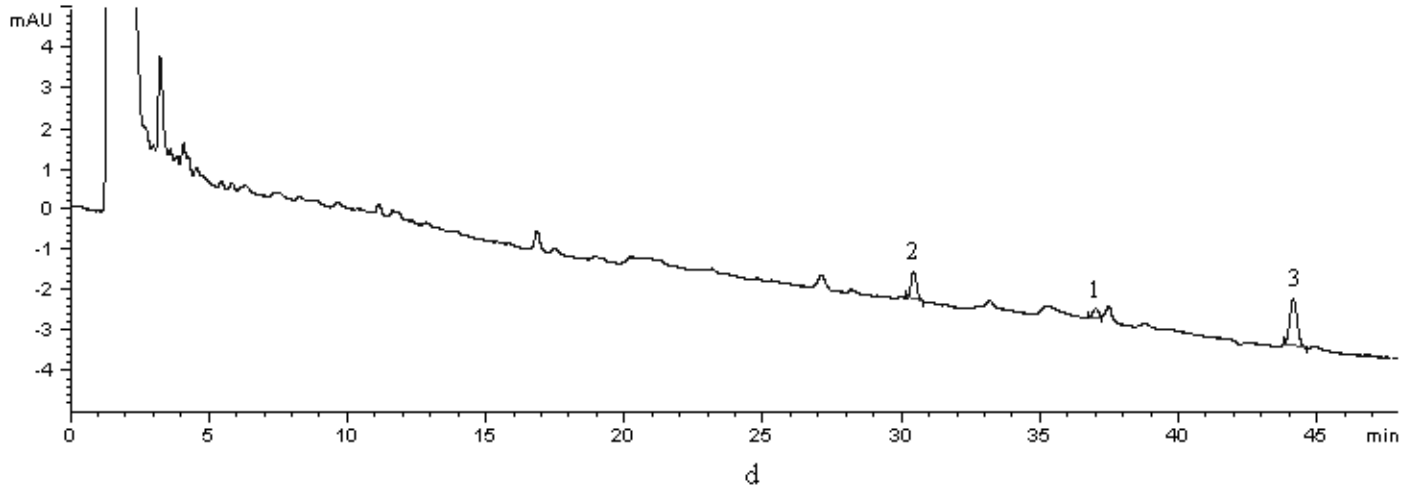


Figure 3. Continued.

effects of ammonium acetate buffer pH values and concentration on the chromatographic behavior of the three alkaloids were investigated. Method validation data indicate that the present method is a reliable, reproducible and accurate HPLC method for the simultaneous determination of the three *Aconitum* alkaloids in rat plasma.

ACKNOWLEDGEMENT

This study is founded by the TCM Special Project of MST of China: Study on quality control technology and quality evaluation with multi- indicators on Chinese herbs, fit for TCM theory and feature. Nos. 2200707009.

REFERENCES

Ameri A (1998). The effects of Aconitum alkaloids on the central nervous system[J].Progress Neurabiol. pp: 211-234.

Feng HT, Li SFY (2002). Determination of five toxic alkaloids in two common herbal medicines with capillary electrophoresis[J]. J. Chromatogr. A. 973:243-247.

Feng HT, Yuan LL, Li SFY (2003). A nalysis of Chinese medicine preparations by capillary electrophoresis–mass spectrometry[J].J. Chromatogr. A. 1014:83-91.

Guo ZS, Pan ZW, Chen XG, Hu ZD (2004). Analysis of the aconitine alkaloids in traditional Chinese medicines by nonaqueous capillary electrophoresis using a new recording mode[J]. Biomed. Chromatogr. 18:381-387.

Ito K, Tanaka S, Konno S, Konishi Y, Mizugaki M (1998). Report on the preparation of deuterium-labelled aconitine and mesaconitine and their application to the analysis of these alkaloids from body fluids as internal standard [J].J. Chromatogr. B. 714:197-203.

Jiang ZH, Xie Y, Zhou H, Wang RJ, Liu ZQ, Wong YF, Cai X, Xu HX, Liu L (2005). Quantification of Aconitum alkaloids in aconite roots by a modified RP-HPLC method[J]. Phytochem. Anal. 16:415-421.

Kaneko R, Hattori S, Furuta S, Hamajima M, Hirata Y, Watanashi K, Seno H, Ishii A (2006). Sensitive analysis of aconitine, hypaconitine, mesaconitine and jesaconitine in human body fluids and Aconitum tubers by LC/ESI-TOF-MS [J]. J. Mass Spectrom, 41:810–814.

Li RJ, Gao XS (1991). Experimental studies on the absorption of aconitine in the upper digestive tract[J].China J. Chin. Mater. Med. 16:46.

Liu XX, Chao RB (2006). Determination of alkaloids in Radix Aconiti Lateralis Preparata by RP-ion-pair HPLC[J].Acta. Pharmaceutica. Sinica. 41:365-369.

Ohta H, Seto Y, Tsunoda N (1997). Determination of aconitum alkaloids in blood and urine sample. High-performance liquid chromatographic separation,solid-phase extraction and mass spectrometric confirmation[J].J. Chromatogr. B. 69:351-356.

Wang ZH, Guo DA, He Y, Hu CH, Zhang JZ (2004). Quantitative Determination of Aconitum Alkaloids in Blood and Urine Samples by High- performance Liquid Chromatography[J].Phytochem. Anal. 15:16-20.

Wang ZH, Wen J, Xing JB, He Y (2006). Quantitative determination of diterpenoid alkaloids in four species of Aconitum by HPLC[J].J. Pharm. Biomed. Anal. 40:1031-1034.

Wang ZJ, He SJ, Xu SQ (2001). Determination of Aconitine alkaloids in Aconitum Plants and related proprietary medicines by liquid chromatography/mass spectrometry/mass spectrometry [J].Anal. Chem. 4:391-395.

Zhang HG, Sun Y, Duan MY, Chen YJ, Zhong DF, Zhang HQ (2005). Separation and identification of Aconitum alkaloids and their metabolites in human urine[J]. Toxicol. 46:500–506.

Zhou YP (1983). Pharmacological action and toxicity of Radix aconiti lateralia and its main components [J].Acta. pharmaceutica. sinica. 18:394-400.