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LC-MS/MS Analysis of Lycorine and Galantamine in Human Serum Using Pentafluorophenyl Column

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Abstract

Lycorine and galantamine are natural alkaloids found in Amaryllidaceae plants, such as narcissus. Narcissus leaves and roots are sometimes accidentally ingested because they resemble vegetables. Lycorine and galantamine are toxic and cause such effects as nausea, vomiting, and abdominal pain, when accidentally ingested. In a case of narcissus poisoning, the detection of lycorine and galantamine in biological samples is vital to determine whether they have been ingested. This study establishes a liquid chromatography-tandem mass spectrometry (LC/MS/MS) method to measure the lycorine and galantamine content of human serum, which can be used for mild to fatal poisoning cases. A serum pretreatment procedure was performed using acetonitrile and QuEChERS AOAC powder. The separation of the compounds was conducted using a pentafluorophenyl column, CAPCELL CORE PFP (2.1 mm I.D. × 100 mm, 2.7 μm). Lycorine, galantamine, and galantamine-d_6 (internal standard) were identified by the transitions of *m/z* 288 → 147, *m/z* 288 → 213, and *m/z* 294 → 216, respectively. The calibration curves were linear in the ranges of 0.05 to 5 ng/mL and 5 to 100 ng/mL, with $R^2 > 0.999$. The precision and accuracy were within the permissible range. The matrix effects of lycorine and galantamine were 94.3% - 98.4% and 87.8% - 91.1%, respectively. The extraction recovery rates of lycorine and galantamine were 101.9% - 112.7% and 95.6% - 107.1%, respectively. The present method detected lycorine and galantamine in the sera of three patients with mild poisoning that had accidentally ingested. This method is applicable in cases of lycorine and galantamine poisoning.

Keywords

Lycorine, Galantamine, Narcissus, Pentafluorophenyl Column, LC/MS/MS

1. Introduction

Narcissus is a plant of the Amaryllidaceae family that is commonly used for ornamental purposes. Narcissus has many similarities with vegetables, its leaves resemble those of Chinese chive (*Allium tuberosum*), while their bulbs are similar to those of onions (*Allium cepa*) and garlic (*Allium sativum*). Narcissus contains alkaloids, including lycorine and galantamine, which are toxic when ingested. The effects of narcissus poisoning include nausea, vomiting, abdominal pain, diarrhea, chills, and fever [1] [2] [3] [4]. Cases of narcissus poisoning have been identified in both humans and animals [3]-[9]. For example, a family in Japan ate narcissus bulbs mistaken for onions and suffered from nausea and vomiting [7]. Several cases have been reported in the United Kingdom wherein patients accidentally ingested narcissus, as it resembled a vegetable. These narcissus plants were purchased from a supermarket, where they were displayed next to the vegetables [8]. In case of narcissus poisoning, the detection and quantification of lycorine and galantamine in serum are essential to determine the degree of poisoning. Although some existing methods analyze raw or cooked plants [10] [11] [12], few methods have been developed for the analysis of biological samples [9] [13]. An analytical method for the analysis of human biological samples is therefore required.

Lycorine and galantamine induce an emetic effect upon ingestion, leading to the expulsion of toxic components from the body. In mild to moderate cases of narcissus poisoning, relatively low concentrations of lycorine and galantamine are expected in the blood; hence, the analytical method must have sufficient sensitivity to detect these alkaloids at even low concentrations. We validated a liquid chromatography-tandem mass spectrometry (LC/MS/MS) method to detect a wide range of lycorine and galantamine concentrations in human serum. It has been reported that a case analyzes lycorine and galantamine in deceased's biological samples using the hydrophilic interaction liquid chromatography (HILIC) [9]. In the HILIC mode, the column requires long equilibration time to achieve reproducible chromatographic results [14]. It is necessary to develop a method that can be analyzed in a short time in order to treat patients. Thus, we investigated an analytical method using a reverse-phase column with shorter equilibration time than HILIC mode. Octadecylsilyl (ODS) analytical columns are commonly used for drug analysis in forensic and emergency medicine; however, these columns may not achieve an efficient separation of basic compounds, including alkaloids. We therefore employed a pentafluorophenyl (PFP) column, which retains polar aromatic compounds, to ensure an efficient separation.

In the present study, an LC/MS/MS method for the analysis of lycorine and

galantamine in human serum samples was established, and the method was applied to clinical cases. To the best of our knowledge, the present study describes the first analysis of lycorine and galantamine in the human serum using a PFP column and the application of this technique to cases of poisoning.

2. Materials and Methods

2.1. Reagents

Lycorine hydrochloride monohydrate (98.0% purity) and galantamine hydrobromide (98.0% purity) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Galantamine-O-(methyl-d_3)-N-(methyl-d_3) (Galantamine-d_6) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Acetonitrile (LC/MS grade, 99.9+% purity), methanol (LC/MS grade, 99.7+% purity), formic acid (guaranteed reagent) and ammonium formate (Wako special grade, 95.0+% purity) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Ultrapure water was obtained using a Direct-Q UV3 system (Merck, Darmstadt, Germany). QuEChERS Extract Pouches, AOAC method (containing magnesium sulfate (6.0 g) and sodium acetate (1.5 g)) were purchased from Agilent Technologies (Santa Clara, CA, USA). L-Consera® I EX Nissui (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), a freeze pool serum, was used as the blank serum. The blank serum was confirmed to be negative for the analytes of interest before use.

2.2. Preparation of Standard Solutions, Calibrators, and Quality Control Samples

Stock solutions of lycorine and galantamine (0.5 mg/mL) were prepared by dissolving the corresponding standards in methanol and stored at −20˚C prior to use. To prepare the calibration curve samples, each stock solution was diluted to 1, 2, 10, 20, 50, 100, 200, 500, 1000, 1500, and 2000 ng/mL with acetonitrile at the time of use, and an aliquot of each diluted solution (10 µL) was added to blank serum (0.2 mL). To prepare the quality control (QC) samples, each stock solution was diluted to 10, 100, 1000, and 2000 ng/mL with acetonitrile at the time of use and an aliquot of each diluted solution (10 µL) was added to blank serum (0.2 mL). The internal standard (IS), galantamine-d_6, was dissolved in methanol to prepare a 1.0 mg/mL stock solution, diluted to 200 ng/mL with acetonitrile, and stored at −20˚C prior to use.

2.3. LC/MS/MS Conditions

An Agilent 1290 liquid chromatograph, equipped with a CAPCELL CORE PFP column (2.1 mm I.D. × 100 mm, 2.7 µm, Osaka Soda Co, Ltd., Osaka, Japan), in combination with a 6460 triple quadrupole mass spectrometer with an Agilent Jet Stream electrospray ionization (ESI) source (Agilent Technologies, Santa Clara, CA, USA) was used for the analysis. Mobile phase A consisted of ammonium formate buffer (10 mM) containing 0.1% formic acid, while mobile phase

B was acetonitrile. The gradient elution program was as follows: starting conditions of 10% B, increasing to 40% B (0 - 6 min) and to 100% B (6 - 10 min), returning to 10% B, and equilibrating for 6 min before the next analysis. The flow rate was 0.2 mL/min. The autosampler was maintained at 4˚C. The column oven temperature was 40˚C. The ESI parameters were: capillary voltage of 4000 V, nebulizer gas (N_2) pressure of 50 psi, drying gas (N_2) flow rate of 10 L/min at 300˚C, sheath gas (N_2) flow rate of 12 L/min at 350˚C. Measurements were performed in multiple reaction monitoring (MRM) and product ion scan modes. The optimal chromatographic and mass spectrometric conditions for the analysis of lycorine, galantamine and galantamine-d_6 were obtained by injecting pure standard solutions into the LC/MS/MS system. Each pure standard solution was measured in product ion scan mode to confirm the product ion spectrum. The determination of the MS/MS parameters and the acquisition of data was performed by the MassHunter Workstation Software (version B.07.00, Agilent Technologies).

2.4. Sample Preparation

Samples were prepared using a modified version of the procedure reported by Kudo *et al.* [15]. Serum (0.2 mL), ultrapure water (0.4 mL), galantamine-d_6 IS solution (10 μL, 200 ng/mL) and a stainless bead (diameter 4 mm) were added to a 5 mL screw cap vial before, acetonitrile (0.6 mL) was added, and the sample solution was mixed using a vortex mixer. QuEChERS powder (0.2 g) was subsequently added, and the solutions was stirred and centrifuged at 3000 rpm for 10 min. The supernatant was separated, and the solvent was evaporated to dryness under a nitrogen stream at 60˚C. The resulting residue was dissolved in methanol (100 μL) and centrifuged at 15,000 rpm for 10 min. Subsequently, an aliquot (1 μL) of the supernatant was injected into the LC/MS/MS system.

2.5. Method Validation

The method was validated in accordance with the guidelines of Scientific Working Group for Forensic Toxicology [16]. The matrix effect, extraction recovery, lower limit of detection (LOD), lower limit of quantification (LOQ), calibration curve linearity, precision, accuracy, and carryover were each verified.

2.5.1. Matrix Effect and Extraction Recovery

The matrix effect and extraction recovery were estimated at four concentration levels of analyte (0.5, 5, 50, and 100 ng/mL) in the serum. The following solutions and extracts were prepared (n = 6): a methanol solution with added standard and IS (A); a blank matrix extract with added standard and IS (B); an extract prepared by adding standard and IS to blank matrix (C). The matrix effect and recovery rate were calculated using the following equations:

$$\text{Matrix effect (\%)} = (B/A) \times 100$$

$$\text{Recovery rate (\%)} = (C/B) \times 100$$

2.5.2. Calibration, LOD, and LOQ

To prepare the calibration curve samples, the serum-added samples with lycorine and galantamine concentrations of 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 75, and 100 ng/mL in the serum, were extracted using the sample preparation procedure and measured using the MRM mode (n = 6). A calibration curve was plotted using the ratio of the area of the peak of the target substance to that of the peak of the IS. A regression line was obtained, and the coefficient of determination (R^2) was calculated. The calibration curve was plotted in the range of 0.05 to 5 ng/mL and 5 to 100 ng/mL.

The LOD was evaluated based on a signal-to-noise ratio (S/N) of three or more. The LOQ was defined as the lowest calibration curve concentration at which the deviation of accuracy and precision was within ±20% and the S/N was more than 20.

2.5.3. Precision and Accuracy

The precision and accuracy of the method was determined by analyzing the QC samples on the same day (n = 6, intra-day) and on six different days (inter-day). These samples had analyte concentrations 0.5, 5, 50, and 100 ng/mL in the serum. The coefficients of variation (CV) and bias were calculated. A CV and bias within ±15% were considered permissible.

2.5.4. Carryover

For carryover, the blank serum-extracted samples were measured after analyzing the serum-added samples having 100 ng/mL concentration, the highest point in the calibration curve range (n = 6). The chromatograms of the blank samples were checked for the presence of interference peaks at the same retention times of the lycorine, galantamine, and IS.

2.6. Cases in Emergency Medicine

The validated method was employed to measure the serum of the three patients that accidentally ingested narcissuses and were transported to a critical care center. The patients were a man and woman in their 60s and a woman in her 30s. These three patients cooked and ingested 80 g of narcissuses that grew naturally in their gardens, mistaking them for Chinese chives. Figure 1 shows the leaves that the patients accidentally ate and brought to the hospital. The patients were rushed to the hospital because two patients developed nausea and vomiting approximately 30 minutes after ingestion, and one patient developed abdominal bloating. All three patients were alert and had normal vital signs. After a follow-up of approximately 3 h, the symptoms of the patients improved, and they returned home. Their serum, when transported to the hospital, was analyzed to investigate the cause of their symptoms.

3. Results and Discussion

3.1. LC/MS/MS Conditions

Optimized MS/MS parameters for lycorine, galantamine, and IS are shown in

Table 1. Figure 2 shows chromatograms and product ion spectra of 1 μg/mL standard solutions of lycorine, galantamine, and IS measured in the product ion scan mode. Lycorine, galantamine, and IS was satisfactorily separated on the PFP column.

The CAPCELL CORE PFP column is a reverse-phase core shell column wherein a PFP group is added to the silica gel. In recent years, analytical methods using PFP columns for pharmaceuticals, illicit drugs, and genotoxic impurities have been reported [17] [18] [19] [20] [21]. PFP columns effectively retain polar aromatic compounds, demonstrate excellent stereoselectivity, and effectively separate halogen compounds. Thus, a PFP column was employed for the analysis of the basic aromatic compounds lycorine and galantamine.

Table 1. Tandem mass spectrometry parameters of the analytes and internal standard (IS).

Compounds	Molecular weight	Precursor ion $[M + H]^+$	Product ion (m/z)	Fragmentor voltage (V)	Collision energy (V)	Polarity
Lycorine	287.12	288	147	116	33	Positive
Galantamine	287.15	288	213	116	21	Positive
Galantamine-d_6 (IS)	293.19	294	216	116	25	Positive

Figure 1. Leaves accidentally ingested by the patients.

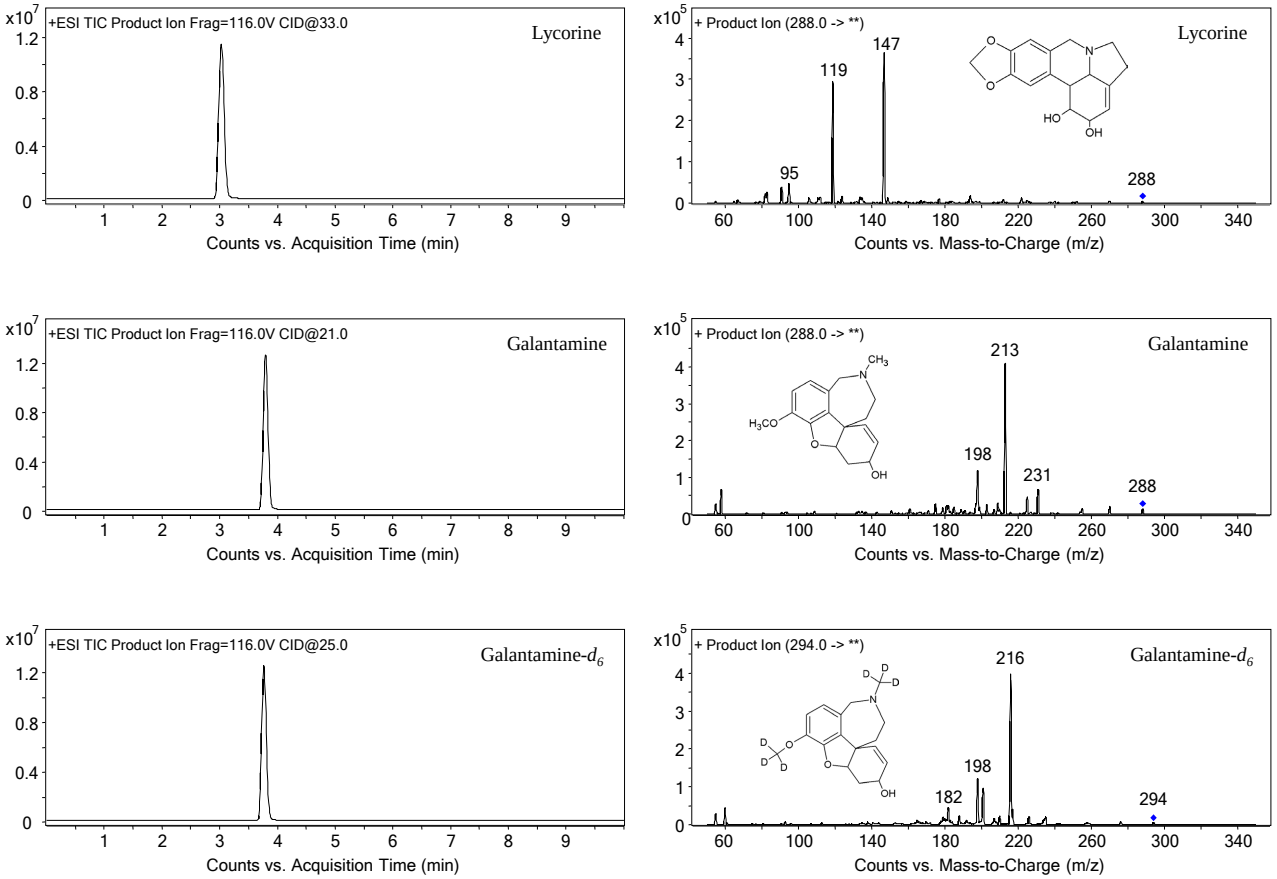


Figure 2. Product ion chromatograms and product ion spectra of lycorine, galantamine, and galantamine-d_6 standard solutions, along with the structures of lycorine, galantamine, and galantamine-d_6.

3.2. Sample Preparation, Matrix Effect, and Extraction Recovery

Figure 3 shows the MRM chromatograms of the extracts, prepared by adding lycorine, galantamine, and IS to the blank serum and using the pretreatment procedure. The retention times of lycorine, galantamine, and IS were 3.1, 3.9, and 3.9 min, respectively. Satisfactory separation of lycorine, galantamine, and IS was observed with no interference between the peaks in the MRM chromatograms of the serum extracts.

Lycorine exhibited a matrix effect of 94.3% - 98.4%, while that of galantamine was 87.8% - 91.1% (**Table 2**). Lycorine and galantamine showed less significant ion suppression or enhancement, suggesting the efficient and thorough removal of impurities during sample preparation. The extraction recovery rates of lycorine and galantamine were 101.9% - 112.7% and 95.6% - 107.1%, respectively (**Table 2**), confirming that the target substances were efficiently extracted.

The concentrations of natural poisons in the biological samples are expected to be low in mild cases of narcissus poisoning owing to the emetic effects; therefore to increase the concentration of the target compounds in the extract, we selected an extraction method that can concentrate the samples. The QuEChERS method is a pretreatment procedure developed by Anastassiades *et al.* to analyze

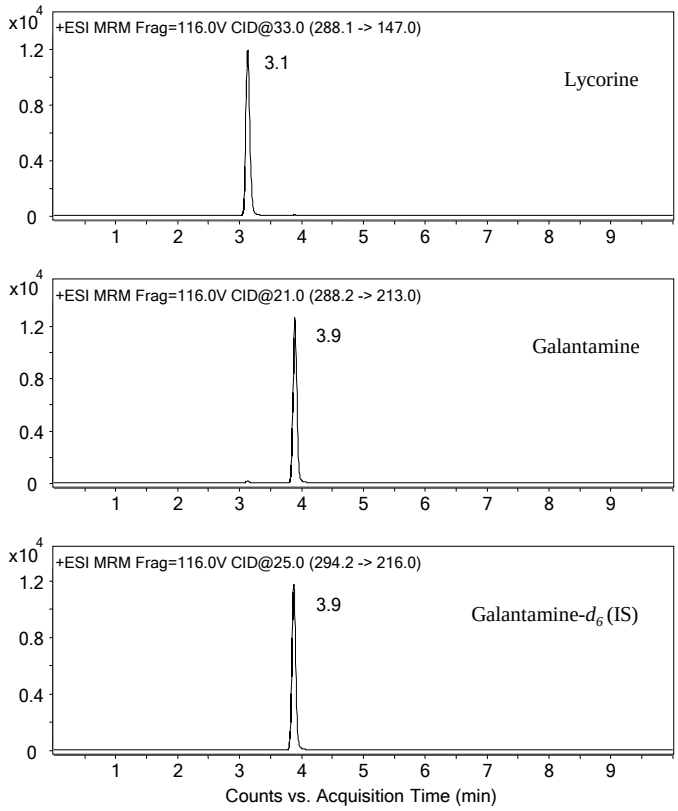


Figure 3. Multiple reaction monitoring chromatograms of serum samples spiked with the lycorine, galantamine, and galantamine-d_6 standards.

Table 2. Matrix effects and extraction recovery rates of the analytes in the human serum.

Compounds	QC concentration (ng/mL)	Matrix effect (%)	Extraction recovery (%)
Lycorine	0.5	98.4	101.9
	5	94.7	105.0
	50	95.6	109.8
	100	94.3	112.7
Galantamine	0.5	91.1	95.6
	5	88.9	101.0
	50	88.5	105.3
	100	87.8	107.1

pesticide residues, such as those found in vegetables, wherein liquid-liquid extraction is followed by dispersion solid-phase extraction [22]. This allows the facile extraction of a sample with a smaller amount of reagent than the conventional method used to inspect pesticides. Lehotay reported a method to simultaneously salt-out and dehydrate a target substance [23]. Moreover, the QuECh-

ERS method has been applied in the extraction of animal and human biological samples [24] [25] [26]. Kudo *et al.* applied the liquid-liquid extraction method, the first step of the QuEChERS method, in the analysis of human biological samples, including blood [15]. We modified the pretreatment procedure reported by Kudo *et al.* [15] to effectively extract samples with a simple operation. The liquid-liquid extraction was performed using the dehydration effect and the salting-out effect of magnesium sulfate and sodium acetate, the components of the QuEChERS AOAC powder. This simple method can simultaneously detect acidic and basic compounds, and yields a cleaner extract than the typical liquid-liquid extraction method. This extraction method can therefore be extended to detect natural toxins other than lycorine and galantamine in the future.

3.3. LOD, LOQ, Calibration, Precision, and Accuracy

Table 3 shows the LOD, LOQ, calibration curve range, calibration curve equiations, and linearity (R^2) of lycorine and galantamine. The LOD and LOQ of both lycorine and galantamine were 0.01 ng/mL and 0.05 ng/mL, respectively. Linearity was observed in both the low range of 0.05 to 5 ng/mL and the high range of 5 to 100 ng/mL, with an R^2 of more than 0.999 in serum for lycorine and galantamine. The present method was able to quantify a wide range of concentrations.

The intra-day and inter-day precisions of lycorine were 0.9% to 1.8% CV, and 0.5% to 2.2% CV, respectively, while those of galantamine were 0.7% to 2.0% CV and 0.4% to 1.7% CV, respectively (Table 4). The intra-day and inter-day accuracies of lycorine were −3.9% to 0.7% bias and −5.6% to 0.1% bias, respectively, while those of galantamine were −9.7% to −2.8% bias and −11.3% to −3.3% bias, respectively (Table 4). Both the intra-day and inter-day precision and accuracy of the present method are within the acceptance criteria. We suggest that the present method is sufficiently reliable and reproducible for the quantitative analysis of lycorine and galantamine in human serum.

3.4. Carryover

The blank samples injected after the analysis of the serum containing 100 ng/mL concentration showed no signals corresponding to lycorine, galantamine, or IS; thus, carryover was not observed.

Table 3. Lower limit of detection (LOD), lower limit of quantification (LOQ), calibration curve equations, coefficient of determination (R^2) for the analytes in the human serum.

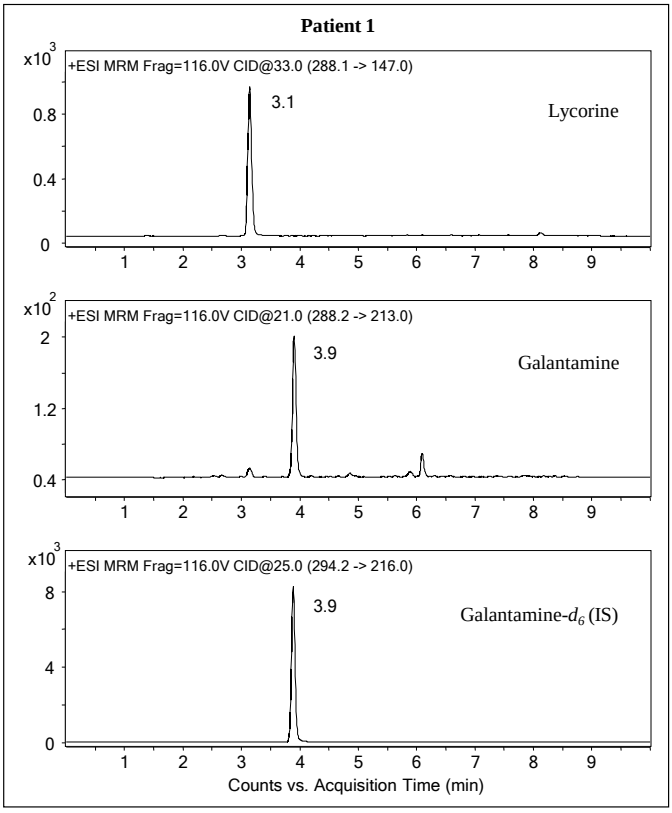
Compounds	LOD (ng/mL)	LOQ (ng/mL)	Concentration range (ng/mL)	Calibration curve equations	Linearity (R^2)
Lycorine	0.01	0.05	0.05 - 5	y = 0.1071x + 0.0012	0.9995
			5 - 100	y = 0.12x − 0.0924	0.9991
Galantamine	0.01	0.05	0.05 - 5	y = 0.0947x + 0.001	0.9996
			5 - 100	y = 0.1034x − 0.0811	0.9995

3.5. Cases in Emergency Medicine

The sera of the three patients that accidentally ingested narcissus were analyzed using the validated method. The MRM chromatograms of the sera collected from the three patients during the hospital visit reveal that lycorine and galantamine were present in the serum of all three patients [Figure 4]. The concentrations of each analyte are listed in Table 5.

Table 4. Intra- and inter-day precision and accuracy of the method in the detection of the analytes in the human serum.

Compounds	QC concentration (ng/mL)	Intra-day		Inter-day	
		Precision (% CV)	Accuracy (% bias)	Precision (% CV)	Accuracy (% bias)
Lycorine	0.5	1.4	−3.9	1.5	−5.6
	5	1.3	0.03	2.2	0.1
	50	1.8	0.7	1.3	−0.2
	100	0.9	−0.4	0.5	−0.6
Galantamine	0.5	2.0	−9.7	1.7	−11.3
	5	0.8	−4.3	0.9	−3.9
	50	1.3	−2.8	1.2	−3.3
	100	0.7	−3.0	0.4	−3.3



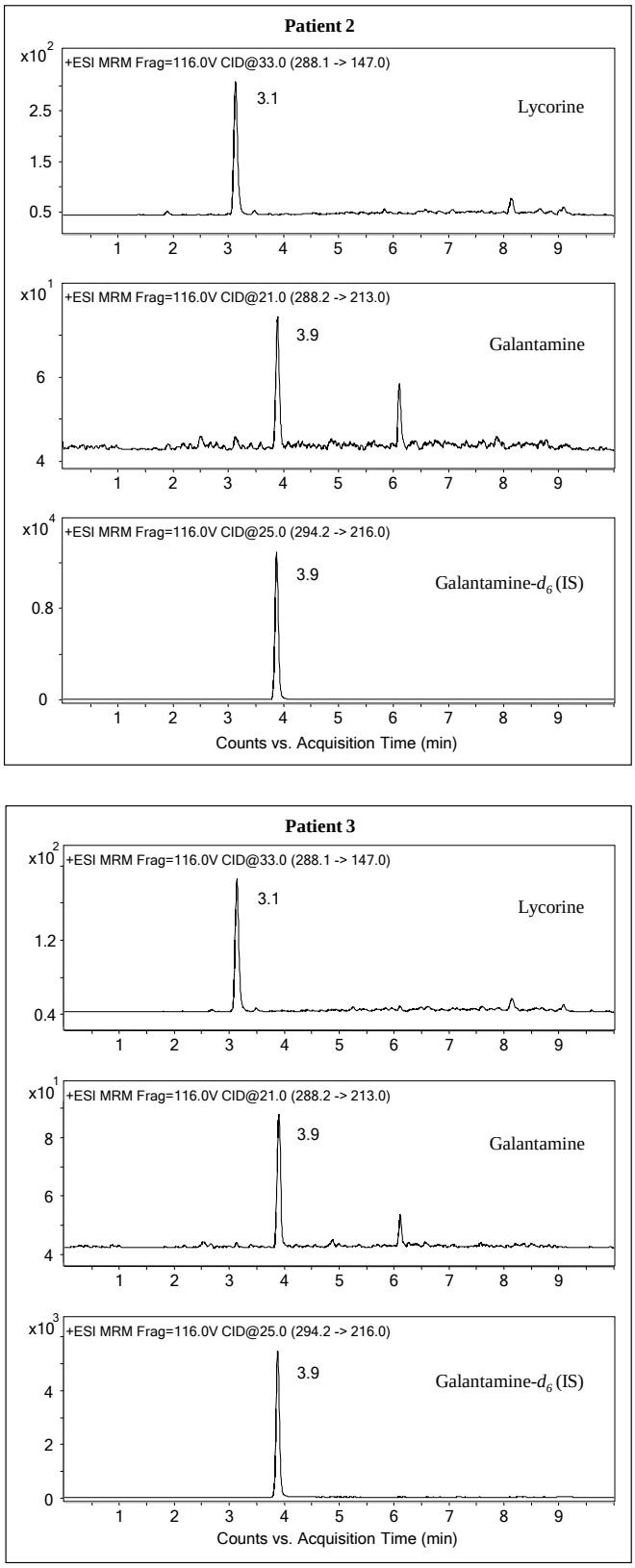


Figure 4. Multiple reaction monitoring chromatograms of the sera of the present three patients. Patient 1: Man in his 60s, Patient 2: Woman in her 60s, Patient 3: Woman in her 30s.

Table 5. Lycorine and galantamine concentrations in the sera of the present three patients.

Patient No.	Age	Sex	Concentrations in serum (ng/mL)	
			Lycorine	Galantamine
1	60's	M	1.13	0.19
2	60's	F	0.20	0.02
3	30's	F	0.25	0.07

The ingestion of narcissus causes the rapid onset of gastrointestinal symptoms including vomiting, nausea, diarrhea, and acute abdominal pain that may persist for several hours [7]. All three patients in the present study displayed vomiting symptoms approximately 30 min after the meal, similar to the symptoms of narcissuses poisoning. In a previously reported fatal case in which bacteremia worsened after ingestion of narcissus, the concentrations of lycorine and galantamine in the blood of the deceased at the time of emergency transportation were 24.7 ng/mL and 94 ng/mL, respectively [9]. The concentrations of lycorine and galantamine in the serum of the three patients in the present study were much lower than the previously reported cases. Because vomiting, one of the toxic effects of narcissus, was observed in the present cases, we concluded that lycorine and galantamine were excreted from the body before large quantities could be absorbed into the blood, thereby resulting in relatively low blood concentrations of these alkaloids. All three patients were awake and recovered after a few hours, suggesting that the observed concentrations of these alkaloids in their serum were consistent with their symptoms. The present method is expected to be applicable to the analysis of sera from patients with narcissus poisoning.

4. Conclusion

An LC/MS/MS method for the detection of lycorine and galantamine in human serum was established. Efficient separation was achieved using a PFP column. A high sensitivity was obtained by applying a pretreatment procedure involving the first step of the QuEChERS method. The present method quantified the lycorine and galantamine concentration in human serum samples in the ranges of 0.05 to 5 ng/mL and 5 to 100 ng/mL.

Ethical Approval

This study has been approved by the Hospital Ethics Committee of the Kitasato University School of Medicine (B20-082).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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