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Screening Method for 23 Alkaloids in Human Serum Using LC/MS/MS with a Pentafluorophenyl Column in Dynamic Multiple Reaction Monitoring Mode

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Abstract

Alkaloids are nitrogen-containing organic compounds, generally basic, and found in plants, fungi, and bacteria. Some alkaloids are used in medicine, but some compounds are highly toxic. Accidental ingestion, homicide, and suicide have occurred due to plants containing alkaloids. The identification of toxic components in biological samples is important for the diagnosis and/or treatment of poisoning cases in forensic and emergency medicine. Alkaloids have a wide variety of structures, such as isoquinoline alkaloid, indole alkaloid, tropane alkaloid, and diterpene alkaloid; therefore, there are few reports of simultaneous analysis methods. We have established a method for the simultaneous analysis of 23 alkaloids in human serum with a liquid chromatograph-tandem mass spectrometer (LC/MS/MS). A liquid-liquid extraction which was modified from the first step of the QuEChERS AOAC method was used for serum pretreatment. The separation of the compounds was performed using a pentafluorophenyl (PFP) column, CAPCELL CORE PFP (2.1 mm I.D. × 100 mm, 2.7 μm) in gradient mode. Mobile phase A consisted of 10 mM ammonium formate and 0.1% formic acid in ultrapure water, and mobile phase B was 10 mM ammonium formate and 0.1% formic acid in methanol. Simultaneous analysis was performed in dynamic multiple reaction monitoring mode. The separation of 23 alkaloids was satisfactory, as PFP columns exhibited different retention behaviors than alkyl phase columns. The PFP column effectively retained polar aromatic compounds; therefore, it was suitable for alkaloid analysis. The validated method was applied to a forensic case of aconite poisoning. The present method was useful in LC/MS/MS screening for 23 alkaloids in human serum.

Keywords

Alkaloid, Pentafluorophenyl Column, LC/MS/MS, QuEChERS, Simultaneous Analysis

1. Introduction

Alkaloids are nitrogen-containing organic compounds, which are generally basic, and found in plants, fungi, and bacteria [1]. Although often used as medicines, the use of some alkaloids has been reported in homicide and suicide cases owing to their strong toxicity [2] [3] [4] [5] [6]. In addition, there have been many cases of alkaloid poisoning caused by ingesting plants containing toxic alkaloids that were mistaken as edible [7]-[22]. In poisoning instances, it is important to identify the causative compounds to diagnose toxic symptoms and/or determine the cause of death. Screening tests for toxicants in biological samples are required, particularly where the identity of the ingested plant is unknown. Therefore, we developed a screening method for alkaloids contained in plants posing the risk of accidental ingestion. There are various types of alkaloids, such as isoquinoline alkaloid, indole alkaloid, tropane alkaloid, and diterpene alkaloid. Many reports of analytical methods exist for detecting individual alkaloids [3] [4] [21]-[31], but there have been few reports of their simultaneous analysis [32] because of their numerous chemical structures. Simultaneous analysis of alkaloids in human urine and herbal samples by liquid chromatograph-tandem mass spectrometer (LC/MS/MS) using a C8 column was reported in one study [32]. In forensic medicine, the results of toxicology tests in blood or serum are important for diagnosing poisoning symptoms at the time of death, so we developed an analysis method using serum as the specimen. The analytical column commonly used for medicinal and toxic substance analysis is alkyl-silica columns; however, peak tailing occurs with some basic compounds, including alkaloids. Therefore, we validated an analytical method using a pentafluorophenyl (PFP) analytical column, which efficiently separated polar aromatic compounds. In recent years, PFP columns have been applied to various analyses [33] [34] [35] [36] [37], as they exhibit different retention behavior from columns of alkyl phases, which retain polar aromatic compounds, demonstrate excellent stereoselectivity, and effectively separate halogen compounds.

The target compounds of the present method were 23 alkaloids found in plants. **Table 1** shows the classification of the alkaloids, families, and representative plants for each compound. The measurement mode employed was dynamic multiple reaction monitoring (dMRM). The dMRM mode only operates when the target compounds are eluted from liquid chromatograph (LC) and are more sensitive than the multiple reaction monitoring (MRM) mode. The serum was pretreated using a modified QuEChERS AOAC method [38]. We established a screening method for 23 alkaloids in human serum using dMRM with LC/MS/MS

Table 1. Classification of alkaloids, family, and representative plants of target compound.

Classification of alkaloid	Family	Representative plants	Compound
Colchicum alkaloid	*Colchicaceae*	*Colchicum autumnale*	Colchicine
Diterpene alkaloid	*Ranunculaceae*	*Aconitum*	14-Anisoylaconine
			Aconitine
			Benzoylhypaconine
			Benzoylmesaconine
			Hypaconitine
			Jesaconitine
			Mesaconitine
Imidazole alkaloid	*Rutaceae*	*Pilocarpus microphyllus*	Pilocarpine
Indole alkaloid	*Apocynaceae*	*Rauwolfia serpentina*	Reserpine
	Loganiaceae	*Strychnos nux-vomica*	Strychnine
Isoquinoline alkaloid	*Amaryllidaceae*	*Lycoris radiata, Narcissus*	Galantamine
			Lycorine
	Papaveraceae	*Lamprocapnos spectabilis*	(+)-Bicuculline
			Protopine
		Papaver somniferum	Morphine
			Papaverine
Phenethylamine alkaloid	*Ephedraceae*	*Ephedra sinica*	Ephedrine
Pyridine alkaloid	*Solanaceae*	*Nicotiana tabacum*	(-)-Cotinine
			(-)-Nicotine
Tropane alkaloid	*Erythroxylaceae*	*Erythroxylum coca*	Cocaine
	Solanaceae	*Datura metel*, *Scopolia japonica*	Atropine
			Scopolamine

equipped with a pentafluorophenyl column. This validated method was successfully applied to a forensic case of aconite poisoning. To the best of our knowledge, the present study is the first to describe LC/MS/MS screening method for alkaloids in human serum using a PFP column and the application of this technique to cases of poisoning.

2. Materials and Methods

2.1. Reagents

(+)-Bicuculline (>98.0% purity), atropine (>99.0% purity), galantamine hydrobromide (98.0% purity), lycorine hydrochloride monohydrate (98.0% purity), papaverine hydrochloride (>99.0% purity), pilocarpine hydrochloride (>98.0% purity), protopine (>98.0% purity), reserpine (>98.0% purity), and scopolamine hydrobromide trihydrate (>98.0% purity) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). (-)-Cotinine (97.0+% purity), (-)-nicotine

(Wako 1st grade, 97.0+% purity), colchicine (Wako 1st grade, 95.0+% purity), strychnine nitrate (Wako special grade, 98.0+% purity), aconitum diester alkaloids standard (for the Japanese pharmacopoeia crude drugs test, aconitine 0.05 mg, hypaconitine 0.15 mg, jesaconitine 0.05 mg, and mesaconitine 0.1 mg), and aconitum monoester alkaloids standard (for the Japanese pharmacopoeia crude drugs test, 14-anisoylaconine hydrochloride 0.1 mg, benzoylhypaconine hydrochloride 0.05 mg, and benzoylmesaconine hydrochloride 0.1 mg) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Cocaine hydrochloride and morphine hydrochloride hydrate were purchased from Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). Ephedrine "NAGAI" (ephedrine hydrochloride 40 mg/mL) was purchased from Nichi-Iko Pharmaceutical Co., Ltd. (Toyama, 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 and Quality Control Samples

Aconitum diester alkaloids standard and aconitum monoester alkaloids standard were dissolved in 5 mL of acetonitrile and stored at −20˚C prior to use. Stock standard solutions of 500 μg/mL of reserpine were prepared in acetonitrile and stored at −20˚C prior to use. Stock solutions of other compounds (500 μg/mL) were prepared by dissolving the corresponding standards in methanol and stored at −20˚C prior to use. 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. To prepare the quality control samples, each stock solution was diluted to 60, 600, and 6000 ng/mL for hypaconitine; 40, 400, and 4000 ng/mL for mesaconitine; 38, 378, and 3782 ng/mL for 14-anisoylaconine; 19, 188, and 1880 ng/mL for benzoylhypaconine; 38, 377, and 3767 ng/mL for benzoylmesaconine; and 20, 200, and 2000 ng/mL for the other compounds with acetonitrile at the time of use, and an aliquot of each diluted solution (10 μL) was added to the blank serum (0.2 mL).

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 10 mM ammonium formate and 0.1% formic acid in ultrapure water, mobile phase B consisted of 10 mM ammonium formate and 0.1% formic acid in methanol. The gradient elution program was as follows: starting conditions of 10% B, increasing to 100% B (0 - 10 min), holding at 100% B (10 - 5 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, 4000 V; nebulizer gas (N_2) pressure, 50 psi; drying gas (N_2) flow rate, 10 L/min at 300˚C; sheath gas (N_2) flow rate, 12 L/min at 350˚C. The optimal chromatographic and mass spectrometric conditions for the analysis of all the compounds 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 retention time. Simultaneous analysis was performed in dMRM mode for 1 min before and after the retention time of each compound. The determination of the MS/MS parameters and data acquisition was performed using the MassHunter Workstation Software (version B.07.00, Agilent Technologies).

2.4. Sample Preparation

The samples were prepared using a modified version of the procedure described by Kudo *et al.* [38]. 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, 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 acetonitrile (100 μL) and centrifuged at 15,000 rpm for 10 min. The supernatant was separated into a 2 mL amber glass vial fitted with 100 μL volume insert, and an aliquot (1 μL) of the solution was injected into the LC/MS/MS system.

2.5. Method Validation

The matrix effect, extraction recovery, and lower limit of detection (LOD) were verified. The matrix effect and extraction recovery were estimated at three analyte concentrations (3, 30, and 300 ng/mL for hypaconitine, 2, 20, and 200 ng/mL for mesaconitine, 2, 19, and 189 ng/mL for 14-anisoylaconine, 1, 9, and 94 ng/mL for benzoylhypaconine, 2, 19, and 188 ng/mL for benzoylmesaconine, and 1, 10, and 100 ng/mL for the other compounds) in the serum. The following solutions and extracts were prepared (n = 6): acetonitrile solution with added standard and IS (A), blank matrix extract with added standard and IS (B), and serum extract of the quality control sample pretreated with the present method

(C). The matrix effect and recovery rate were calculated using the following equations.

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

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

The LOD was defined as the lowest concentration that yielded a reproducible peak with a signal-to-noise ratio of 3 or more.

2.6. Application to Forensic Case

The validated screening method was used to analyze a sample that had been previously qualitatively tested using another method. The serum used was residual samples that had been stored at −30˚C.

Case: The deceased was a female in her late teens. She was found deceased, lying on her back, on the floor in her home. The vomit on the floor was mixed with leaves and roots shaped like aconite. It was assumed that the deceased ate aconite for suicidal purposes. The deceased's specimen was analyzed using another method dedicated to aconite at the time of appraisal in order to investigate the cause of death, and aconitine and jesaconitine was detected.

3. Results and Discussion

3.1. LC/MS/MS Conditions

The optimized MS/MS parameters for the compounds are shown in Table 2. Figure 1 shows the dMRM chromatograms of the serum extracts prepared by adding the compounds to the blank serum at a concentration of 150 ng/mL for hypaconitine, 100 ng/mL for mesaconitine, 95 ng/mL for 14-anisoylaconine, 47 ng/mL for benzoylhypaconine, 94 ng/mL for benzoylmesaconine, and 50 ng/mL for other compounds using the pretreatment procedure. Satisfactory separation of all compounds was observed with no interference between the peaks in the dMRM chromatograms of the serum extracts. The CAPCELL CORE PFP column is a reverse-phase core shell column in which a PFP group is added to the silica gel. PFP columns allow for dipole-dipole, π-π, charge transfer, and ion-exchange interactions, in addition to the dispersive interactions available on alkyl-silica columns [39]. When methanol was used as the mobile phase organic solvent, the peak areas were larger than those when acetonitrile was used. It was speculated that the π-π interactions functioned by using methanol. Hence, we employed methanol as the organic solvent for mobile phase B.

3.2. Sample Preparation, Matrix Effect, Extraction Recovery, and LOD

Table 3 shows the retention time, matrix effect, extraction recovery, and LOD of the compounds. The matrix effects of aconitum diester alkaloids including aconitine, hypaconitine, jesaconitine, and mesaconitine were 53.1% - 65.2%. The retention times of these compounds were longer than 11 min, and it was estimated that ion suppression occurred due to overlap in retention time with

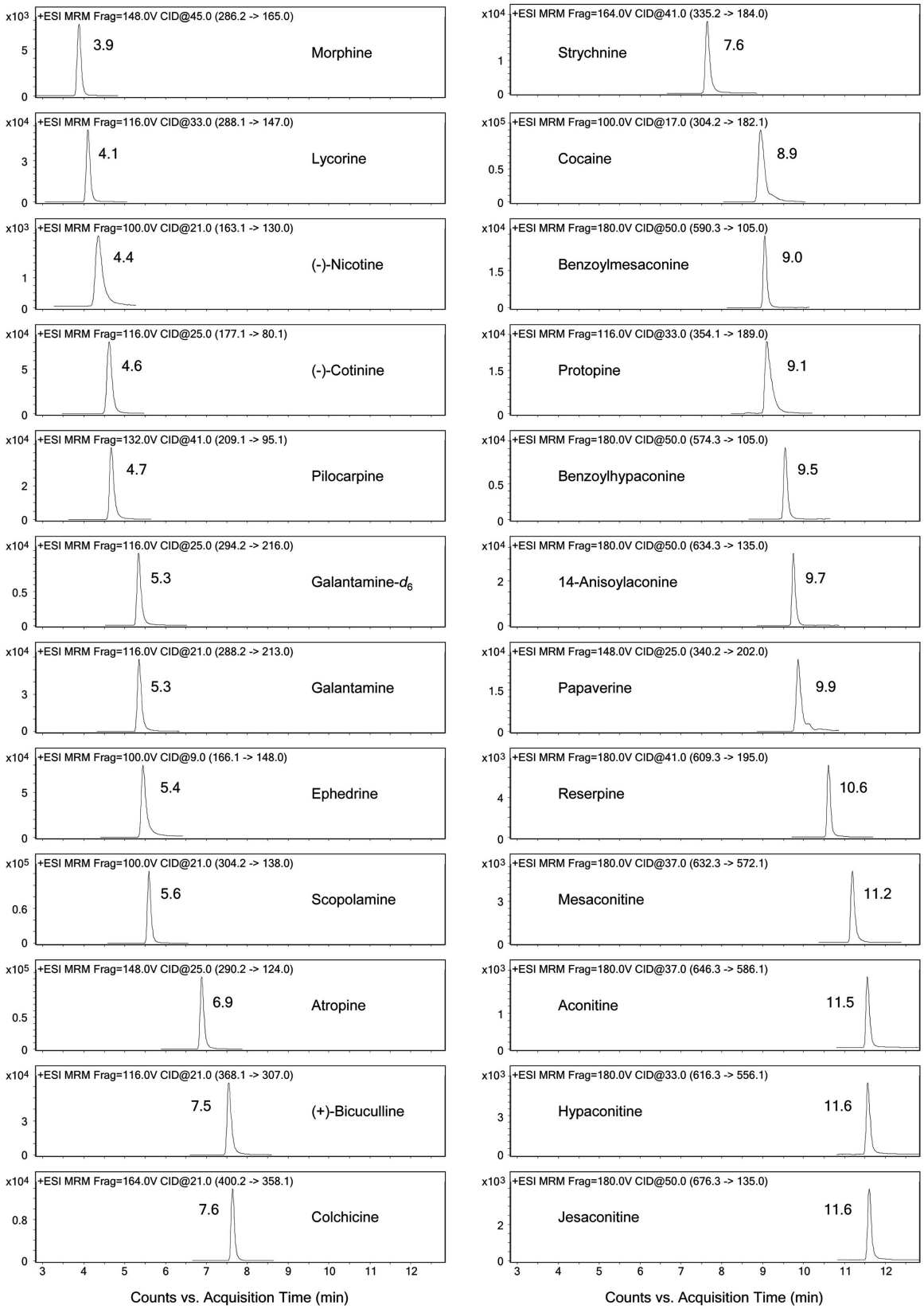

Figure 1. Dynamic multiple reaction monitoring chromatograms of serum samples spiked with the target compounds, and galantamine-d_6 standards.

Table 2. Tandem mass spectrometry parameters of analytes and internal standard (IS).

Compound	Molecular formula	Monoisotopic mass	Precursor ion $[M + H]^+$	Product ion (m/z)	Fragmentor voltage (V)	Collision energy (V)	Polarity
(-)-Cotinine	$C_{10}H_{12}N_2O$	176.09	177	80	116	25	Positive
(-)-Nicotine	$C_{10}H_{14}N_2$	162.12	163	130	100	21	Positive
(+)-Bicuculline	$C_{20}H_{17}NO_6$	367.11	368	307	116	21	Positive
14-Anisoylaconine	$C_{33}H_{47}NO_{11}$	633.31	634	135	180	50	Positive
Aconitine	$C_{34}H_{47}NO_{11}$	645.31	646	586	180	37	Positive
Atropine	$C_{17}H_{23}NO_3$	289.17	290	124	148	25	Positive
Benzoylhypaconine	$C_{31}H_{43}NO_9$	573.29	574	105	180	50	Positive
Benzoylmesaconine	$C_{31}H_{43}NO_{10}$	589.29	590	105	180	50	Positive
Cocaine	$C_{17}H_{21}NO_4$	303.15	304	182	100	17	Positive
Colchicine	$C_{22}H_{25}NO_6$	399.17	400	358	164	21	Positive
Ephedrine	$C_{10}H_{15}NO$	165.12	166	148	100	9	Positive
Galantamine	$C_{17}H_{21}NO_3$	287.15	288	213	116	21	Positive
Galantamine-d_6 (IS)	$C_{17}H_{15}D_6NO_3$	293.19	294	216	116	25	Positive
Hypaconitine	$C_{33}H_{45}NO_{10}$	615.3	616	556	180	33	Positive
Jesaconitine	$C_{35}H_{49}NO_{12}$	675.33	676	135	180	50	Positive
Lycorine	$C_{16}H_{17}NO_4$	287.12	288	147	116	33	Positive
Mesaconitine	$C_{33}H_{45}NO_{11}$	631.3	632	572	180	37	Positive
Morphine	$C_{17}H_{19}NO_3$	285.14	286	165	148	45	Positive
Papaverine	$C_{20}H_{21}NO_4$	339.15	340	202	148	25	Positive
Pilocarpine	$C_{11}H_{16}N_2O_2$	208.12	209	95	132	41	Positive
Protopine	$C_{20}H_{19}NO_5$	353.13	354	189	116	33	Positive
Reserpine	$C_{33}H_{40}N_2O_9$	608.27	609	195	180	41	Positive
Scopolamine	$C_{17}H_{21}NO_4$	303.15	304	138	100	21	Positive
Strychnine	$C_{21}H_{22}N_2O_2$	334.17	335	184	164	41	Positive

Table 3. Retention times, matrix effect (n = 6), extraction recovery (n = 6), and lower limit of detection (LOD) for analyte in human serum.

Compound	Retention time (min)	Quality control concentration (ng/mL)	Matrix effect (%)	Extraction recovery (%)	LOD (ng/mL)
(-)-Cotinine	4.6	1	97.9	102.7	0.1
		10	98.9	73.6	
		100	100.2	79.4	
(-)-Nicotine	4.4	1	97.0	69.1	0.2
		10	95.5	73.0	
		100	100.4	70.9	

Continued

(+)-Bicuculline	7.5	1	93.2	106.1	0.02
		10	95.2	101.3	
		100	93.5	97.7	
14-Anisoylaconine	9.7	2	83.8	81.8	0.4
		19	76.8	70.5	
		189	74.5	74.5	
Aconitine	11.5	1	56.0	59.8	0.2
		10	59.2	55.2	
		100	59.4	51.8	
Atropine	6.9	1	102.4	94.8	0.01
		10	101.2	86.7	
		100	100.4	84.5	
Benzoylhypaconine	9.5	1	85.0	85.3	0.5
		9	78.8	84.7	
		94	76.1	88.8	
Benzoylmesaconine	9.0	2	90.9	84.9	0.4
		19	83.8	88.2	
		188	80.5	94.1	
Cocaine	8.9	1	93.1	81.8	0.05
		10	92.5	89.4	
		100	95.3	85.2	
Colchicine	7.6	1	94.2	94.0	0.05
		10	94.1	85.4	
		100	90.8	78.1	
Ephedrine	5.4	1	102.7	72.6	0.2
		10	102.0	77.7	
		100	99.9	79.6	
Galantamine	5.3	1	96.3	102.1	0.02
		10	98.1	100.4	
		100	98.9	99.4	
Galantamine-d_6	5.3	-	-	-	-
Hypaconitine	11.6	3	56.9	68.0	0.6
		30	56.7	52.4	
		300	55.6	50.4	
Jesaconitine	11.6	1	58.8	57.8	0.5
		10	65.2	50.3	
		100	63.5	48.5	

Continued

Lycorine	4.1	1	99.4	95.6	0.02
		10	97.6	93.5	
		100	99.1	90.5	
Mesaconitine	11.2	2	53.1	65.2	0.4
		20	56.2	68.2	
		200	55.4	69.6	
Morphine	3.9	1	113.3	90.6	0.2
		10	99.3	83.4	
		100	104.0	75.8	
Papaverine	9.9	1	72.6	62.2	0.02
		10	79.2	53.4	
		100	79.4	53.5	
Pilocarpine	4.7	1	95.6	98.4	0.1
		10	98.9	89.5	
		100	98.2	91.3	
Protopine	9.1	1	87.8	85.1	0.2
		10	91.1	81.9	
		100	91.4	85.2	
Reserpine	10.6	1	72.0	40.0	0.2
		10	70.6	54.5	
		100	71.5	51.3	
Scopolamine	5.6	1	95.9	111.6	0.01
		10	97.8	106.6	
		100	98.2	103.6	
Strychnine	7.6	1	103.0	102.2	0.05
		10	101.0	98.0	
		100	97.8	92.1	

phospholipids that were not fully removed from the serum during extraction. The matrix effect of other compounds was 70.6% - 113.3% and showed less significant ion suppression or enhancement. The extraction recovery rates of aconitum diester alkaloids were 48.5% - 69.6%. Since these compounds were unstable and susceptible to hydrolysis [40] [41], it was assumed that they were degraded during the extraction. The recovery rates of papaverine were 53.4% - 62.2%. Papaverine was considered to have oxidized during the extraction owing to its susceptibility to oxidation [42]. The recovery rates of reserpine were 40.0% - 54.5%, and it was thought to be photodegraded during extraction because the compounds decompose under light [43]. The recovery rates of other compounds were 69.1% - 111.6%, confirming that the target substances were sufficiently ex-

tracted. Aconitum diester alkaloids, papaverine, and reserpine were detected with an LOD lower than their intoxication ranges [44] [45]. Therefore, the present method was determined to be compatible with the screening analysis for 23 alkaloids in the serum.

The QuEChERS method is a pretreatment procedure used to extract pesticide residues, such as those found in vegetables [46] [47]. 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 [38]. Since aconitines are degraded by methanol [48], we modified the method to use acetonitrile as the redissolving solution. This simple method which is performed using the dehydration and the salting-out effects of magnesium sulfate and sodium acetate, respectively, was suitable for the simultaneous extraction of compounds with diverse chemical structures. In dMRM mode, analytes are only monitored while they are eluting from the LC, and the MS duty cycle is not wasted by monitoring for the analytes when they are not expected. Hence, the peak areas of the compounds using dMRM are larger than those obtained using MRM. Thus, the present method was able to detect the compounds at an LOD of 0.01 - 0.6 ng/mL (**Table 3**).

By using the present pretreatment method and LC/MS/MS analysis with the PFP analytical column, we succeeded in simultaneously analyzing 23 alkaloids that had previously been analyzed using each different method.

3.3. Application to Forensic Cases

The serum of the deceased, who was presumed to have ingested aconite, was analyzed using the validated method. The dMRM chromatogram of the extract obtained from the serum of the deceased is shown in **Figure 2**. Aconitine and jesaconitine were detected, with the result being the same as that of another

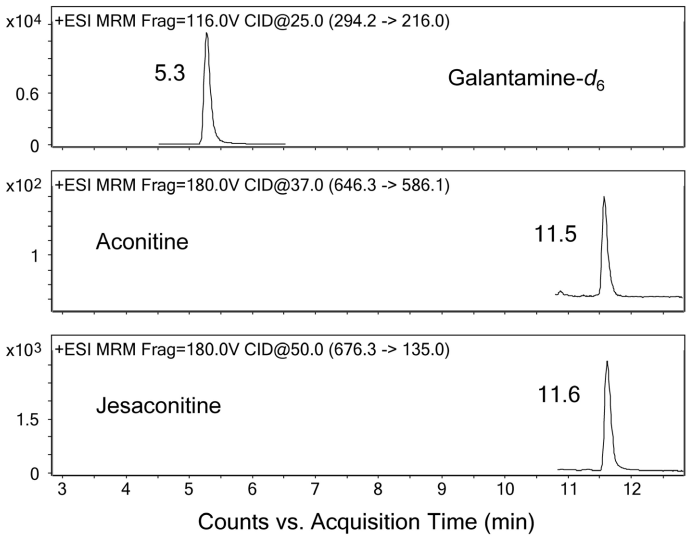

Figure 2. Dynamic multiple reaction monitoring chromatograms of serum of forensic case.

analysis method for aconite performed during autopsy. The present method was successfully applied to the LC/MS/MS screening of serum for forensic cases. As the compounds and composition of the aconite toxin vary by species and region [49], several compounds must be included in the screening analysis to avoid missing the causative agent of poisoning. The present method was found to be effective for alkaloid screening in human serum.

4. Conclusion

In this study, we established an LC/MS/MS simultaneous analysis method for 23 alkaloids in human serum, which were difficult to analyze simultaneously. The pretreatment method was a modification of the first step of the QuEChERS AOAC method. Twenty-three alkaloids were efficiently separated by using a PFP analytical column. Simultaneous analysis using the dMRM mode improved the sensitivity. The validated method was applied to a forensic case and successfully screened alkaloids in human serum. Hence, the present LC/MS/MS simultaneous analysis method was effective for the screening of alkaloids in human serum in forensic and clinical toxicology.

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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