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Easily Operable Quantification Method of 21 Plant-Derived Alkaloids in Human Serum by Automatic Sample Preparation and Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

In this study, we developed an easily operable quantification method for 21 plant-derived alkaloids in human serum by automatic sample preparation and liquid chromatography–tandem mass spectrometry. We designed to perform parallel sample preparation by a developed apparatus, which increased sample throughput. We conducted an automatic sample preparation through de-proteinization with 0.1% formic acid in methanol and achieved recovery rates of 89–107% (2.0–14% RSD) for all targeted analytes, demonstrating its high repeatability. The method validation results were satisfactory as follows: the linearity (r^2) of each calibration curve ranged from 0.978 to 1.000; the inter- and intra-day accuracies were 89.0–125% and 82.1–110%, respectively; the inter- and intra-day precisions were below 13% and 10%, respectively. Additionally, the lower limits of detection and quantification were 0.0044–0.047 and 0.013–0.14 ng/mL, respectively. Finally, the developed method was applied to pseudo-protoveratrine A poisoning serum and pseudo-colchicine poisoning serum, which were prepared by diluting acute-poisoning mice serum with human serum. Our method successfully quantitated protoveratrine A (0.15–0.25 ng/mL) and colchicine (4.8–6.0 ng/mL). Thus, our method is essential for prompt clinical treatment and critical care on patient in acute intoxication cases caused by plant-derived alkaloids.

Keywords Food poisoning · Plant-derived alkaloids · Human serum · Automatic sample preparation · Quantification · LC/MS/MS

Introduction

Food poisoning involving plant toxins occurs worldwide; thus, quick and reliable toxin determination in biological samples is strongly required in clinical treatment and critical care. Most plant toxins contain nitrogen within their molecules (i.e., alkaloids), and they generally have a strong biological activity and are widely used as pharmaceuticals, e.g., atropine [1]. In particular, some plant-derived alkaloids, such as aconitine, show adverse effects due to their intense biological activities [2], which may cause severe acute intoxication owing to overdose.

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To investigate the causative substances in such acute intoxication cases, highly sensitive and selective instruments are necessary for detecting even trace amounts of alkaloids in biological samples, such as blood. To meet these demands, liquid chromatography–tandem mass spectrometry (LC/MS/MS) is used to identify alkaloids in biological samples even in clinical laboratories, and the preparation of biological samples is essential [3–6]. However, analytical skills in sample preparation for biological samples are required to obtain accurate results since the sample preparation is generally manually operated, such as extraction and evaporation.

Recently, automatic sample preparation systems have been developed to minimize the manually operated steps in the sample preparation. Therefore, the automatization of sample preparation can provide accurate analytical results without depending on individual analytical skills. Moreover, new mass spectrometric techniques, called ambient ionization mass spectrometry (AIMS), require little or no sample pretreatment [7]. AIMS, such as desorption electrospray ionization (DESI) [8, 9] and probe electrospray ionization (PESI) [10–13], are defined as ionization techniques for analysis under open-air conditions, allowing high-throughput analysis of toxins to be performed. However, AIMS need special ionization sources and deep knowledge for mass spectrometry; thus, they are not conventional techniques. Therefore, automatic sample preparation systems are the first choice for mass spectrometric identification of plant toxins since they do not need special ionization sources, and beginners in analysis can handle it easily.

Automated systems allow us to save our time and physical fatigue involving manual sample preparation. The systems can also minimize the possibilities of contact infections to viruses, such as COVID-19, and human errors such that a victim's sample will be mistaken for another victim's sample.

Moreover, the manual sample preparation often induces variations, although the automized systems can reduce interlaboratory variations for analytical results. Additionally, after installing the automized systems, they can easily be operated by beginners for instrumental analyses, providing high-reproducible results in any laboratory.

Interesting and practical studies on automatic sample preparation systems have been reported [14–25], and their features are summarized in Table 1. For example, CLAM-2000 supplied by Shimadzu Corporation was used for toxicological screening and quantification of pharmaceutical [15] and illicit drugs in blood [16]. In particular, CLAM-2000 can automatically perform sample preparation, followed by LC/MS/MS. Extrahera™ automated sample processor supplied by Biotage can perform supported-liquid extraction (SLE), solid-phase extraction (SPE), phospholipid depletion (PLD), and protein precipitation (PPT) using a 96-well plate or SPE columns. Extrahera™ has been used to quantify the glyphosate in urine [17] and profile micronutrients in human plasma [18]. Furthermore, Freedom Evo 200 platform supplied by Tecan can be equipped with centrifugation and evaporation devices, and Freedom Evo 200 platform can automatically perform a series of sample preparation [19]. DBS-MS 500 supplied by CAMAG can treat dried blood spot (DBS) sampling, which has been recently used, and it can automatically analyze drugs of abuse from DBS [21].

Based on the aforementioned platforms, the essential mechanical units for analyzing plant-derived alkaloids are as follows: centrifugation unit, evaporation unit, disposable tip system for sampling, and dispensers for solvents and pH adjustment reagents. Such automized systems generally occupy large laboratory space. However, the miniaturization of such systems will be preferable for small examination rooms, such as emergency rooms, especially for plant-derived intoxication cases. Therefore,

Table 1 Automatic sample preparation systems in previous reports

System	Vendor	Analyte	Matrix	Sample preparation	References
CLAM-2000	Shimadzu	Pharmaceutical drugs	Human blood	PPT	[15]
		Illicit drugs and/or metabolites	Human blood	PPT	[16]
Extrahera	Biotage	Glyphosate	Human urine	SPE	[17]
		Micronutrients	Human plasma	SPE	[18]
Freedom Evo 200 platform	Tecan	Illicit, medicinal drugs, and metabolites	Human whole blood	SPE	[19]
		Amphetamines, cocaine, cannabis, opioids, and benzodiazepines	Oral fluid	SLE	[20]
DBS-MS 500	CAMAG	Substances of abuse	Human blood	DBS	[21]
GX-271 ASPEC	Gilson	Antidepressants	Human whole blood	SPE	[22]
MicroLab Star	Hamilton	Epinephrine and norepinephrine	Human plasma	PPT	[23]
RapidFire	Agilent Technologies	Sulfonamides	Honey	SPE	[24]
Versa100	Aurora Biomed	Isoflavones	Biological matrices	LLE	[25]

we have developed a new benchtop apparatus, called ATLAS-LEXT, which can simultaneously perform multiple sample treatments, such as PPT-based extraction, centrifugation, and evaporation of the supernatant. Moreover, disposable tips are available for ATLAS-LEXT, expecting that contamination of low-concentration targeted analytes will be negligible.

Therefore, this study develops and validates the analytical method for the plant-derived alkaloids in human serum using ATLAS-LEXT and liquid chromatograph–tandem mass spectrometer (LC/MS/MS). Three extraction solvents were compared to determine a suitable extraction solvent for the precise quantification of the 21 plant-derived alkaloids. The LC/MS/MS will provide a promising method that improves the sensitivity of trace-level alkaloids in human serum. Finally, the validated method was applied to pseudo-poisoning serum to confirm the method's feasibility.

Materials and Methods

Chemicals and Reagents

Galanthamine, protoveratrine A, veratramine, veratridine, jervine, cyclopamine, cevadine, α-solanine, and α-chaconine were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Colchicine, demecolcine, atropine, scopolamine, aconitine, mesaconitine and hypaconitine were purchased from FUJIFILM Wako Pure Chemical Corporation. Lycorine hydrochloride and protoveratrine B were purchased from Sigma-Aldrich (Tokyo, Japan), sanguinine from Toronto Research chemicals (Toronto, Canada), lycoramine from Carbosynth (Compton, UK), and solanidine from ChromaDex (Los Angeles, CA, US). Yohimbine-[^{13}C, D_3] was purchased from IsoSciences (Ambler, PA, US) and used as the internal standard (IS). The chemical structures of the targeted analytes and IS are shown in Fig. 1. These analytes and IS are categorized in Table 2. High-performance liquid chromatography (HPLC) grade methanol (MeOH), acetonitrile (ACN), and 2-propanol (IPA) were supplied by Kanto Chemical (Tokyo, Japan) and used for extraction solvent. Liquid chromatography–mass spectrometry (LC–MS) grade ACN for mobile phase and HPLC grade 1-mol/L ammonium formate solution were also obtained from Kanto Chemical. LC–MS grade formic acid (FA) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Milli-Q® water (PURELAB® Ultra, ELGA LabWater, High Wycombe, UK) was used throughout the experiments. Pooled human serum was purchased from Biopredic International (Rennes, France).

Preparation of Stock Standard Solutions

Standard stock solutions of lycorine, galanthamine, sanguinine, lycoramine, α-solanine, α-chaconine, solanidine, colchicine, demecolcine, atropine, and scopolamine were prepared using MeOH, and other compounds were dissolved in ACN. Yohimbine-[^{13}C, D_3] was prepared and diluted to 5 ng/mL with 0.1% FA in MeOH. Working mixed standard solution 1 (STD-1) was prepared by mixing the stock standard solutions dissolved with MeOH, and its concentration was adjusted to 10 μg/mL with MeOH. However, working mixed standard solution 2 (STD-2) was prepared by mixing the stock standard solutions dissolved with ACN, and its concentration was adjusted to 10 μg/mL with ACN.

Preparation of Calibrants and Quality Control (QC) Samples

In this study, the IS method was used for the quantitative analysis of plant-derived alkaloids in human serum, except that glycoalkaloids (α-solanine, α-chaconine, and solanidine) were quantitated by the standard addition method. Calibrators and QC samples for the IS method were prepared by spiking 100 μL each appropriately diluted STD-1 and STD-2 into 4.8-mL pooled human serum. Table 3 describes the concentration of calibrators and QC samples.

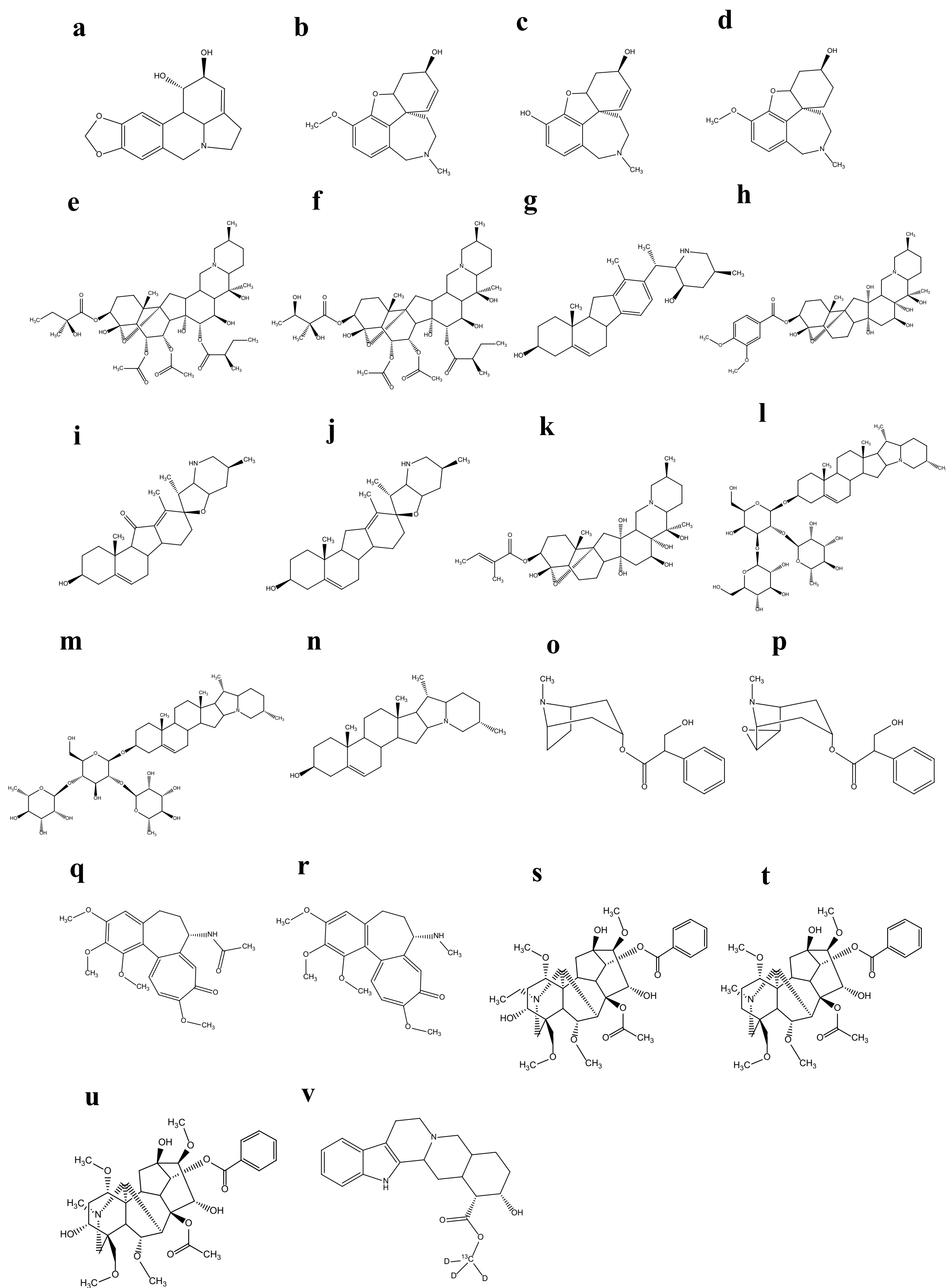
For the standard addition method, 200-μL glycoalkaloids were spiked to 4.8-mL pooled human serum to achieve a five-point calibration curve ($n = 3$ for each calibration point). The absolute amounts of glycoalkaloids spiked into pooled human serum were as follows: 0.25, 0.5, 1.25, 2.5, and 5 μg for α-solanine; 1.25, 2.5, 5, 12.5, and 25 μg for α-chaconine; 2.5, 5, 12.5, 25, and 50 μg for solanidine.

Automated Sample Pretreatment Apparatus

The ATLAS-LEXT (Shimadzu Engineering Inc., Kyoto, Japan) was equipped with the following units: a sample tube rack, a tip rack, a reagent rack, a reagent dispenser, a sample dispenser using disposable tips, a robot arm for transferring sample tubes, a mixing unit, a centrifugal unit, and an evaporation unit (Fig. 2). The sample procedure was customized using Sequence Editor software (Shimadzu Engineering, Ver.1.0.0.0).

Sample Preparation Using ATLAS-LEXT

Figure 2 shows the workflow for automatic sample preparation using ATLAS-LEXT. The spiked or blank human serum samples (0.5 mL each) were pipetted into tubes and set to ATLAS-LEXT. The following operations were performed automatically by ATLAS-LEXT. One hundred microliters of IS (yohimbine-[^{13}C, D_3], 5 ng/mL) was added to each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◂**Fig. 1** Chemical structures of the 21 targeted analytes and the internal standard. **A** Lycorine, **B** galanthamine, **C** sanguinine, **D** Lycoramine, **E** protoveratrine A, **F** protoveratrine B, **G** veratramine, **H** veratridine, **I** jervine, **J** cyclopamine, **K** cevadine, **L** α-solanine, **M** α-chaconine, **N** solanidine, **O** Atropine, **P** scopolamine, **Q** colchicine, **R** demecolcine, **S** aconitine, **T** Hypaconitine, **U** mesaconitine, and **V** yohimbine-[13C, d3]

tube and stirred for 10 s. Subsequently, 0.1% FA aqueous solution (0.5 mL) and 0.1% FA in MeOH (2 mL) were added to each tube, respectively. After stirring for 60 s, the sample tube was centrifuged at 2,000 g for 10 min. The supernatant (0.6 mL) was transferred to a new tube and evaporated to dryness under a gentle nitrogen stream at 85 °C. The atmosphere in the apparatus was kept clean via ventilation by a fan equipped with the apparatus. We designed to perform centrifugation and evaporation of the supernatant parallelly, which increased sample throughput. After evaporation, the residue was reconstituted with 200 µL of 50% MeOH. The reconstituted solution was manually filtered using an Ultrafree®-MC centrifugal device (0.22-µm pore size, hydrophilic PTFE, Merck KGaA, Darmstadt, Germany) by centrifugation at 10,000 g for 2 min (final solution). Finally, 5 µL of the final solution was injected into the LC/MS/MS system.

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

$$\text{Recovery rate (\%)} = \frac{\text{Peak area of analyte obtained from a pre-spiked sample}}{\text{Peak area of analyte obtained from a post-spiked sample}} \times 100 \quad (1)$$

$$\text{Matrix effect (\%)} = \frac{\text{Peak area of analyte obtained from a post-spiked sample}}{\text{Peak area of analyte obtained from a standard solution}} \times 100 \quad (2)$$

LC/MS/MS Conditions

A Nexera X2 LC system coupled with LCMS-8060 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) was used for quantitative analysis. Based on our previous study [6], chromatographic separation was achieved using a Capcell Pak Inert ADME column (2.1 mm i.d. × 150 mm, particle size: 3 µm, Cat.No.95003, OSAKA SODA, Osaka, Japan). The temperature of the column oven was 40 °C. The auto-sampler temperature was set to 10 °C. Mobile phases consisted of 5 mmol/L ammonium formate with 0.1% FA aqueous solution (A) and ACN (B). The gradient conditions were as follows: 0% B for 1 min, 0–90% B (1–11 min, linear gradient), 90% B (11–12 min), and 0% B (12–20 min). The total flow rate was set to 0.3 mL/min.

The mass spectrometer, equipped with an electrospray ionization source, was used in the positive ionization mode. MS parameters were set as follows: nebulizer gas flow rate, 3 L/min; heating gas flow rate, 10 L/min; interface temperature, 300 °C; desolvation line temperature, 250 °C; heat block temperature, 400 °C; drying gas flow rate, 10 L/min. Selected reaction monitoring (SRM) parameters for each analyte were optimized by flow injection analysis of each standard solution and listed in Table 2. Quantifier and qualifier SRM transitions were set for each analyte. LabSolutions software (Shimadzu, Ver.5.99 SP2) was used for the data acquisition and processing. Data acquisition was performed by scheduled SRM mode (data acquisition time was set to the expected retention time ± 1.0 min for each analyte).

Method Validation

For method validation, we applied the IS method to 18 plant-derived alkaloids, except for three glycoalkaloids. Five- or six-point calibration curves ($y = ax + b$) were generated between 0.1 and 25 ng/ mL for each targeted analyte using the IS method. Linear regression with a $1/x^2$ weighting factor was used. Here "x" is the ratio of the analyte concentration to IS concentration, "y" is the ratio of the analyte peak area to IS area, "a" is the slope of the regression line, and "b" is the y-intercept. The inter- and intra-day accuracy and precision were evaluated by analyzing the QC samples ($n = 3$), and accuracy and precision were acceptable below 15% variations. The theoretical lower limits of detection (LLOD) and quantification (LLOQ) were determined as follows: LLODs = 3.29 SD/a, LLOQ = 10 SD/a (SD = the standard deviation of y = intercepts of regression lines and a = slope of the calibration curve). Selectivity was evaluated by analyzing pooled human serum samples.

Animal Experiments

Institute of Cancer Research (ICR) mice (male, 8 weeks old, 34.0–37.8 g) were purchased from Japan SLC Inc (Hamamatsu, Japan). According to our previous study [6], protoveratrine A (25 µg/weight kg) or colchicine (10 mg/weight kg) were administered intraperitoneally to mice ($n = 3$) using a 25 gage needle-disposable syringe (Terumo Corporation, Tokyo, Japan). After 5 min of their administration, blood samples were collected from their abdominal aorta using a 25 gage needle-disposable syringe (Terumo Corporation) under isoflurane anesthesia using an inhalation apparatus

Table 2 Optimized SRM parameters for the targeted analytes and the internal standard

Examples of plants	Targeted analytes	Retention time (min)	Precursor Ion (*m/z*)	Transitions (*m/z*)	Collision energy (eV)
N. tazetta L. var. *chinensis* Roemer	Lycorine	4.9	288.1	**147.0**[a]	30
N. pseudonarcissus L.				119.0	37
Leucojum aestivum L.	Galanthamine	5.1	288.2	**213.3**	24
				198.3	33
	Sanguinine	4.6	274.2	**199.0**	24
				184.1	38
	Lycoramine	5.1	290.2	**233.2**	20
				215.2	26
Veratrum album L. subsp. *oxypetalum* Hult é n	Protoveratrine A	8.4	794.4	**776.4**	42
Veratrum stamineum Maxim				658.4	54
	Protoveratrine B	7.6	810.4	**792.4**	42
				658.4	55
	Jervine	7.6	426.3	**114.1**	33
				109.1	34
	Veratramine	7.9	410.3	**295.2**	30
				84.1	32
	Veratridine	8.0	674.4	**456.4**	54
				474.3	47
	Cyclopamine	8.2	412.3	**114.1**	31
				109.1	33
	Cevadine	8.2	592.4	**456.3**	50
				474.3	44
Solanum tuberosum L.	α-Solanine	6.9	868.5	**398.4**	75
				722.4	70
	α-Chaconine	7.0	852.5	**706.3**	70
				398.2	70
	Solanidine	8.9	398.4	**98.0**	47
				126.3	44
Datura metel L.	Atropine	5.9	290.2	**124.2**	25
Scopolia japonica				93.1	32
	Scopolamine	5.5	304.2	**138.2**	24
				156.2	17
Colchicum autumnale L.	Colchicine	7.6	400.2	**358.2**	24
Gloriosa superba				310.2	28
	Demecolcine	6.4	372.3	**310.1**	30
				340.2	20
Aconitum japonicum ssp.*subcuneatum*	Aconitine	8.7	646.3	**586.3**	38
				105.1	55
	Hypaconitine	8.8	616.3	**556.3**	34
				338.2	42
	Mesaconitine	8.2	632.3	**572.3**	35
				354.2	44
Internal standard	Yohimbine-[^{13}C, d_3]	6.6	359.2	**144.1**	34

[a]Bold type is used for quantifiers and normal type for qualifiers

Table 3 Validation results of the developed method ($n = 3$)

Targeted analytes	LOD (ng/mL)	LOQ (ng/mL)	Range (ng/mL)	Calibrators levels (ng/mL)	Regression equation	Linearity (r^2)	QC conc (ng/mL)	Intraday		Interday	
								accuracy (%)	Precision (%CV)	accuracy (%)	Precision (%CV)
Lycorine	0.023	0.071	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.291x + 0.00228$	0.997	0.25	100	2.9	101	3.1
							20	88.3	2.7	99.7	4.0
Galanthamine	0.030	0.092	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.309x + 0.000983$	0.999	0.25	98.1	4.6	104	5.3
							20	94.1	2.1	107	1.8
Sanguinine	0.028	0.084	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.196x - 0.000403$	0.998	0.25	92.9	10	89.0	7.1
							20	91.0	4.1	91.8	13
Lycoramine	0.045	0.14	0.25–25	0.25, 0.5, 1, 5, 10, 25	$y = 0.291x - 0.00308$	0.999	0.75	97.1	2.7	104	1.3
							20	93.2	1.5	107	6.1
Protoveratrine A	0.019	0.058	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.318x - 0.00154$	0.999	0.25	99.0	7.8	106	4.5
							20	92.1	2.6	105	6.5
Protoveratrine B	0.0044	0.013	0.1–25	0.1, 0.5, 5, 10, 25	$y = 0.220x - 0.0147$	0.978	0.25	82.1	7.9	92.7	7.0
							20	106	2.2	113	4.2
Jervine	0.047	0.14	0.25–25	0.25, 0.5, 1, 5, 10, 25	$y = 0.0676x - 0.0000539$	0.999	0.75	99.1	7.5	102	6.2
							20	93.9	2.5	99.2	3.4
Veratramine	0.0093	0.028	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.750x - 0.0117$	0.998	0.25	100	1.5	125	1.1
							20	100	1.4	112	3.7
Veratridine	0.029	0.087	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.167x - 0.00647$	0.991	0.25	109	6.5	117	3.2
							20	103	1.3	106	2.9
Cyclopamine	0.028	0.084	0.25–25	0.25, 0.5, 1, 5, 10, 25	$y = 0.233x - 0.00327$	0.997	0.25	110	3.4	119	1.4
							20	96.0	0.6	104	4.0
Cevadine	0.023	0.071	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.168x - 0.00425$	0.991	0.25	110	4.0	110	3.8
							20	101	2.1	112	3.4
Atropine	0.023	0.069	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.639x - 0.00519$	0.994	0.25	103	2.8	113	3.9
							20	97.0	1.5	107	3.8
Scopolamine	0.028	0.085	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.408x + 0.00151$	1.000	0.25	93.9	6.1	105	3.1
							20	96.1	5.0	109	2.6
Colchicine	0.013	0.041	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.190x + 0.000315$	0.997	0.25	93.2	1.0	101	3.8
							20	98.6	1.9	101	2.9
Demecolcine	0.010	0.032	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.0751x - 0.000186$	0.999	0.25	104	3.9	109	11
							20	94.3	3.4	108	5.3
Aconitine	0.024	0.073	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.342x - 0.00105$	0.997	0.25	103	5.5	109	1.0
							20	93.1	4.5	108	5.1
Hypaconitine	0.021	0.062	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.362x - 0.00707$	0.997	0.25	102	3.7	111	1.7
							20	95.2	1.3	109	4.0
Mesaconitine	0.029	0.086	0.1–25	0.1, 0.5, 1, 5, 10, 25	$y = 0.292x - 0.000695$	0.998	0.25	105	6.8	113	1.9
							20	93.5	1.7	109	4.0

(NARCOBIT-E, Natsume Seisakusho, Tokyo, Japan). The obtained blood was moved to a 1.5 mL Eppendorf tube from the syringe, and it was kept on an ice bath. Serum samples were prepared through blood sample centrifugation at 10,000*g* for 30 min and stored at − 80 °C until analysis. Then, the serum samples were diluted with pooled human serum to obtain pseudo-poisoning samples (50- or 500-fold dilution). All animal experiments were approved by the Animal Experimental Committee of Nagoya University Graduate School of Medicine (No. 20406). We executed animal experiments in accordance with the Regulations on Animal Experiments in Nagoya University and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Notice No. 71 of the Ministry of Education, Culture, Sports, Science and Technology in Japan, 2006).

Results and Discussion

Selection of Targeted Analytes

In this study, we selected toxic plants and their components, which recently cause food poisoning based on the statistical information of the Japanese Ministry of Health, Labor, and Welfare. Since 2010, 190 cases of food poisonings caused by toxic plants have been reported in Japan, resulting in 793 patients and 14 deceased. Figure 3 shows the details of the plant species involved in those cases and the percentage of each group, where the plant species that commonly contain the main toxic alkaloids are classified into the same group. Since more than 80% of the cases were due to groups 1–6, the main toxic 21 alkaloids in groups 1–6 were carefully selected as the targeted analytes (Table 2).

Optimization of LC/MS/MS Conditions

The MS parameters were optimized by flow injection analysis for each targeted analyte to determine the most abundant product ion of the analytes. Here, the protonated molecule $[M+H]^+$ was selected as the precursor ion. The SRM transitions were set using LabSolutions software (Table 2).

As shown in Fig. 1, the chemical structures of the targeted analytes were varied. For instance, sanguinine, one of the phenanthrene alkaloids, is highly polar. However, solanidine, the common aglycon of α-solanine and α-chaconine, is non-polar due to its steroid skeleton. The ADME column, where the adamantyl group is introduced as a functional group, was selected for LC separation to retain the targeted analytes with such different polarities based on our previous study [6].

The peak shape of each targeted analyte was satisfactory (Fig. S1). The targeted analytes could be successfully differentiated through each SRM transition, although most compounds' chromatographic separation was achieved, except for jervine and protoveratrine B, mesaconitine, cyclopamine, and cevadine.

Optimization of Sample Preparation Using ATLAS-LEXT

ATLAS-LEXT can perform automatic sample preparation of biological samples through PPT or LLE. We selected PPT as the sample pretreatment of human serum since it was not easy to efficiently extract the analytes with different polarities by LLE.

In the preliminary experiments, PPT conditions were optimized using different extraction solvents: 0.1% FA in MeOH, 0.1% FA in ACN, and 0.1% FA in IPA. The spiked human serum (1.0 ng/mL) was used. In our previous study, FA was added to each extraction solvent to prevent protoveratrine A and B from deacetylation [6]. The recovery rates of each extraction solvent were evaluated.

As shown in Fig. 4 and Table S1, the recovery rates were 89–107% (0.1% FA in MeOH), 88–113% (0.1% FA in ACN), and 79–100% (0.1% FA in IPA), demonstrating that all extraction solvents were acceptable. However, the relative standard deviations (RSD%) of the recovery rates were different: 2.0–14% for 0.1% FA in MeOH, 3.1–21% for 0.1% FA in ACN, and 2.1–18% for 0.1% FA in IPA. For instance, the recovery rate of aconitine showed higher RSD% for 0.1% FA in ACN (21%) and 0.1% FA in IPA (9.4%) than that for 0.1% in MeOH (7.4%). As shown in Table S1, there were no remarkable differences in matrix effects among the three extraction solvents. Based on these results, 0.1% FA in MeOH was selected as the extraction solvent for automatic sample preparation.

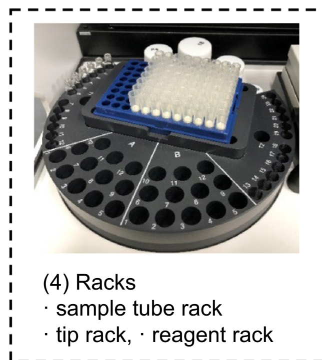
The spiked human sera (0.75 or 7.5 ng/mL) were also used for evaluating 0.1% FA in MeOH as the extraction solvent (Table S2). The recovery rates were 80.0–115% (0.75 ng/mL) and 78.0–129% (7.5 ng/mL), respectively, and the RSD% of the recovery rates were 1.8–11% (0.75 ng/mL) and 0.8–6.3% (7.5 ng/mL), respectively, demonstrating that 0.1% FA in MeOH were acceptable as the extraction solvent.

Furthermore, the following experiments were performed to confirm a carryover of targeted analytes, except for glycoalkaloids. Two spiked human sera with high concentrations of the target analytes (200 ng/mL) were treated by ATLAS-LEXT, followed by two blank human serum samples. As a result, no targeted analytes were detected in the two blank samples (data not shown). Thus, there was no carryover during the automatic sample preparation.

a

(1) Exterior of ATLAS-LEXT

(2) Robot arms for transferring sample tubes
(3) Dispensers
· reagent dispenser
· sample dispenser using disposable tips



(4) Racks
· sample tube rack
· tip rack, · reagent rack

(5) Mixing unit
(6) Centrifugal unit

(7) Evaporation unit

b

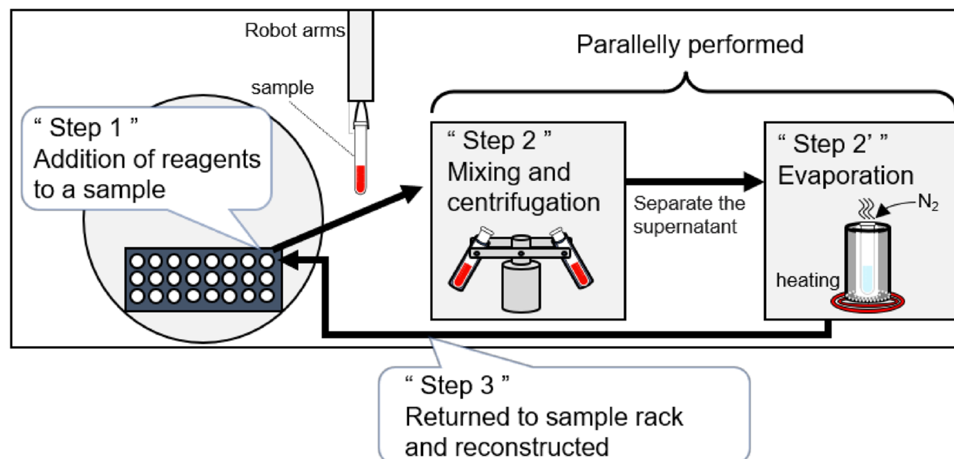


Fig. 2 **a** Photographs of (1) exterior of ATLAS-LEXT (600 mm × 585 mm × 592 mm), (2) robot arms for transferring sample tube, (3) dispensers, (4) trash box for tips, (5) racks, (6) mixing unit, (7) centrifugal unit, and (8) evaporation unit. **b** Workflow for automatic sample preparation using ATLAS-LEXT

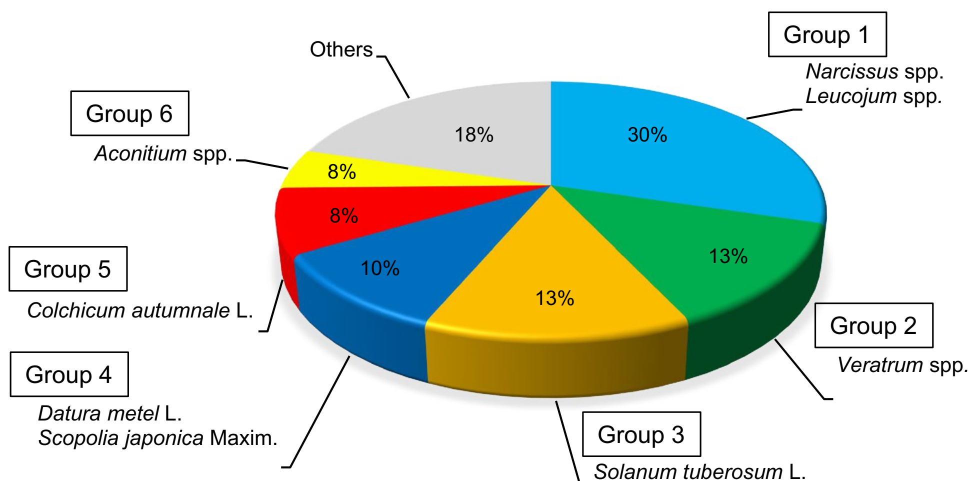


Fig. 3 Pie chart of toxic plants involved in food poisoning in Japan (2010–2019)

Comparison of Automatic and Manual Sample Preparation

To further evaluate the performance of ATLAS-LEXT, the recovery rates obtained using ATLAS-LEXT were compared with those obtained from the manual sample preparation conducted by an experienced analyst. The automatic sample preparation recovery rates ranged from 89 to 107%, whereas those of manual sample preparation were 81.5–118%. Overall, biases from 100% recovery rate (i.e., ideal value) were smaller in automatic sample preparation than those in manual sample preparation for some targeted analytes, such as galanthamine, α-solanine, atropine, and demecolcine (Fig. S2 and Table S3), demonstrating that almost the same recovery rates were observed between automatic sample preparation by ATLAS-LEXT and manual sample preparation.

Method Validation

In our previous study [6], we used yohimbine-[^{13}C, d_3] as IS. Before the validation of our method, the variation of dispensing solvents was evaluated because the dispensing accuracy of IS is essential for assuring the high quantitativity of the method. To confirm the dispensing accuracy of IS, 100-μL IS in 0.1% FA in MeOH was dispensed into blank human serum ($n = 4$) using the reagent dispenser equipped with ATLAS-LEXT, and sample preparations for the serum using PPT were performed. IS were detected in each serum, and the RSD% of the peak areas of IS was 6.0%, proving satisfactory dispensing accuracy of IS.

Subsequently, ATLAS-LEXT and LC/MS/MS-based methods were validated for 18-targeted analytes, except for glycoalkaloids, and the results are presented in Table 3. Each calibration curve exhibits good linearity ($r^2 > 0.978$) over the range. The inter- and intra-day accuracies were good

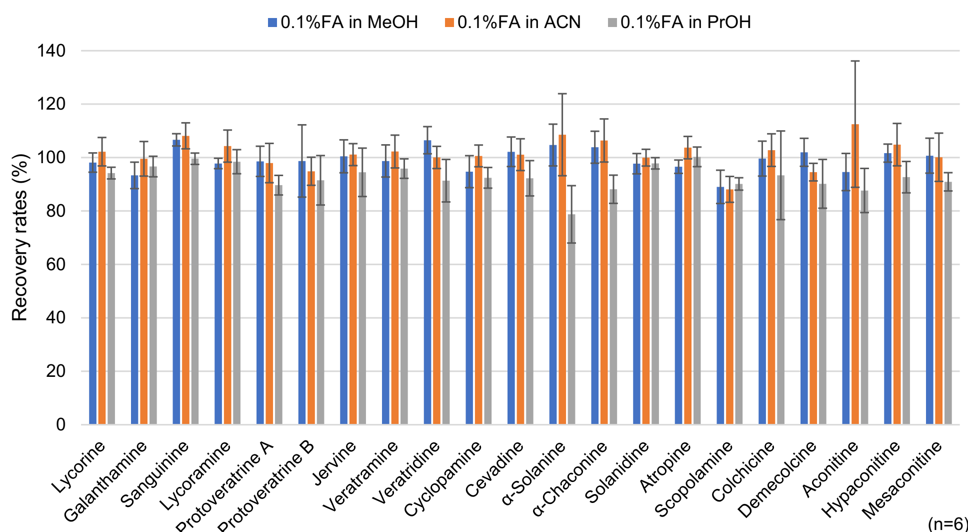


Fig. 4 Recovery rates for all targeted analytes in human serum prepared by PPT using each organic solvent

values of 89.0–125% and 82.1–110%, respectively. The inter- and intra-day precisions were also acceptable, below 13% and 10%, respectively. The accuracies at the lowest points of each calibration curve (0.1 or 0.25 ng/mL) were also satisfactory (99.0–105%), and their precisions were below 9%. The LLOD and LLOQ were 0.0044–0.047 and 0.013–0.14 ng/mL, respectively. Thus, our method showed sufficient quantitativity for 18 analytes in human serum.

To compare our results with other studies [5, 6, 26], the validation data in the previous studies were listed in Table S4, where common analytes among the previous studies and the present study were shown. To compare the results appropriately, the validation data for almost the same QC concentration were extracted. Table 3 and S4 showed that the validation results in this study were comparable to those reported previously.

Moreover, to the best of knowledge, the LLOQs in our method were below the blood or serum concentration levels in acute toxic states of protoveratrine A, protoveratrine B, veratridine, cevadine, α-solanine, α-chaconine, aconitine, hypaconitine, and mesaconitine [27–31], demonstrating the sensitivity of our method was satisfactory. Moreover, no endogenous interference peaks were observed for targeted analytes and IS, except for glycoalkaloids, at the corresponding retention times of each analyte in their SRM chromatograms. As described earlier, the matrix effects of the method were 40–145%.

Application to the Standard Addition Method

Glycoalkaloids, including *Solanum tuberosum* L. (potato), an essential source of nutrition, are detected in human serum due to ingestion of potato as a daily diet. *Solanum tuberosum* L. contains an mg-order amount of glycoalkaloids in the germ and periderm and trace amounts in the tuber. The trace amounts of glycoalkaloids were detected in any human serum used in our preliminary experiments. Since true "blank" human serum for glycoalkaloids is not readily available, we applied a standard addition method to quantify glycoalkaloids in pooled human serum.

Six-point calibration curves ($y_s = a_s x_s + b_s$) were constructed for each glycoalkaloid, and linear regression was used. Here, "x_s" is the absolute amount of each glycoalkaloid spiked into blank serum, "y_s" is the peak area of each glycoalkaloid, "a_s" is the slope of the regression line, and "b_s" is the y_s-intercept. Using the standard addition method, the extrapolated curves for each glycoalkaloid exhibited good linearity ($r^2 > 0.999$) over the calibration range. The regression equations are $y_s = 14{,}122x_s + 4{,}736.6$ for α-solanine, $y_s = 11{,}272x_s + 18{,}540$ for α-chaconine, and $y_s = 121{,}310x_s + 362{,}306$ for solanidine, respectively (Fig. S3). The concentrations of each glycoalkaloid in pooled human serum were 0.067 ± 0.013 ng/mL for α-solanine, 0.33 ± 0.11 ng/mL for α-chaconine, and 0.60 ± 0.17 ng/mL for solanidine (Table S5). The concentrations of these glycoalkaloids were significantly lower than those in the blood of glycoalkaloid poisoning cases in previous studies [29, 32], suggesting that the glycoalkaloids detected in the pooled human serum used in this study would be derived from a daily diet. These results confirmed that our method could detect glycoalkaloids even at sub-ppb concentration levels.

Applications

Real patient serum in acute intoxication cases is necessary to confirm the feasibility of the developed method, though it is difficult to obtain it during this research period. Alternatively, pseudo-poisoning serum was prepared based on our previous study [6]. The targeted analytes-administrated mice sera were diluted with the pooled human serum to change the matrix of the mice sera to be similar to that of human serum. Two pseudo-poisoning sera were prepared as model samples and subjected to the developed method. Low-dilution pseudo-serum: protoveratrine A-administered mice sera were 50-fold diluted with human serum. High-dilution pseudo-serum: colchicine-administered mice sera were 500-fold diluted with human serum.

Results of Low-Dilution Pseudo-Serum

As shown in Fig. 3, food poisonings caused by *Veratrum* alkaloids occurred frequently. Additionally, the quantification of *Veratrum* alkaloids in serum is essential when acute toxic cases involving *Veratrum* alkaloids occur. However, *Veratrum* alkaloids were detected from the patient's serum at very low concentration levels (ppb order) [28, 33]. For instance, protoveratrine A is known to be a high-toxic *Veratrum* alkaloid [34]. Moreover, to examine the applicability of our method, protoveratrine A was selected as a low-dilution pseudo-serum prepared by protoveratrine A-administered (25 μg/weight kg i.p.) mice serum. Our method was applied to the pseudo-serum ($n = 3$), successfully detecting protoveratrine A in the pseudo-serum and quantitating their concentrations (0.15–0.25 ng/mL). These quantitative results were consistent with our previous study, where the same serum samples were quantitated using the validated manual extraction method (Table S6) [6]. Thus, the feasibility of our method was demonstrated, especially for *Veratrum* alkaloids.

Results of High-Dilution Pseudo-Serum

Colchicine, an alkaloid of *Colchicum autumnale* L. and related species, is an old and well-known drug for treating Familial Mediterranean fever and gout [35]. Recently,

food poisonings caused by *Colchicum autumnale* L. have frequently occurred in Japan due to accidental ingestion of *Colchicum autumnale* L. Additionally, suicide cases using colchicine have also been reported [36]. Thus, the quantification of colchicine in serum has high priority in food poisoning cases involving plant-derived alkaloids.

To examine the applicability of our method to the colchicine poisoning cases, pseudo-poisoning sera of colchicine were prepared as high-dilution pseudo-serum. Here, colchicine-administered (10 mg/weight kg i.p.) mice sera were diluted with human serum.

Our method was applied to the high-dilution pseudo-serum ($n = 4$), successfully detecting colchicine and quantitating their concentrations (4.8–6.0 ng/mL, Table S6). These quantitative values are almost the same as the mean maximum concentration (6.0 ± 3.4 ng/mL) of colchicine in plasma obtained from 20 male volunteers, ingested a single 2.0-mg colchicine tablet on the pharmacokinetic study [37]. Furthermore, Saito et al. reported that the serum concentration of colchicine in an actual poisoning case was 6.1 ng/mL [26]. Additionally, Abe et al. reported that the colchicine concentration in patient plasma in a fatal case of colchicine self-poisoning was 60 ng/mL [38], remarkably higher than the LLOQ level of our method. Thus, our method could be applied to such poisoning cases and quantification of colchicine at its therapeutic dose, demonstrating our method's practicality.

Thus, our method will be helpful for prompt clinical treatment and critical care on a patient in acute intoxication cases caused by plant-derived alkaloids. Therefore, demonstration experiments using our method for real patient sera will be performed in future. Moreover, the other natural toxins such as marine and mushroom toxins will be added into the present analytical method, and it is under planning.

Conclusion

We developed an easily operable quantification method for 21 plant-derived alkaloids in human serum by automatic sample preparation and LC/MS/MS. We designed to perform parallel sample preparation by ATLAS-LEXT, which increased sample throughput. The recovery rates for all targeted analytes were 89–107%, and the RSD of the recovery rates ranged from 2.0 to 14%. The method validation showed that the linearity (r^2) of each calibration curve ranged from 0.978 to 1.000. The inter- and intra-day accuracies were 89.0–125% and 82.1–110%, respectively, and the inter- and intra-day precisions were below 13% and 10%, respectively. Additionally, LLOD and LLOQ were 0.0044–0.047 and 0.013–0.14 ng/mL, respectively, indicating the method's high sensitivity. Furthermore, the developed method was applied to two pseudo-serum to demonstrate its practicality. ATLAS-LEXT can easily be operated by beginners for instrumental analyses, and it would be useful to eliminate bothersome work and physical fatigue involving manual sample preparation. This method is useful for preventing infections to viruses and human errors. In other words, just installing the automized, our method will provide high-reproducible results in any laboratory without analytical experts.

Our method is essential for prompt clinical treatment and critical care on a patient in acute intoxication cases caused by plant-derived alkaloids, and demonstration experiments using our method in clinical laboratories are underway.

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Author contributions MT: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing—Original Draft, Writing—Review and Editing, Visualization, Supervision, and Project administration. NT: Software and Resources. TW: Software and Resources. RI: Visualization. SC: Visualization. AM: Visualization. HM: Visualization. HT: Software and Resources. KZ: Conceptualization, Methodology, Resources, Writing—Original Draft, Writing—Review and Editing, Supervision, and Project administration.

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Availability of Data and Material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The authors have no conflicts of interest to declare.

Animal Experiments The animal study was approved by the Animal Experimental Committee of Nagoya University Graduate School of Medicine (No. 20406). We executed animal experiments in accordance with the Regulations on Animal Experiments in Nagoya University and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Notice No. 71 of the Ministry of Education, Culture, Sports, Science and Technology in Japan, 2006).

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