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A simple method for the determination of glyphosate, glufosinate and their metabolites in biological specimen by liquid chromatography/tandem mass spectrometry: an application for forensic toxicology

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ABSTRACT

Glyphosate (GLYP) and glufosinate (GLUF) are phosphorus-containing amino acid type herbicides that are used worldwide. With their rising consumptions, fatal intoxication cases due to these herbicides, whether accidental or intentional, cannot be ignored. Both compounds are difficult to detect, and their pretreatment for instrumental analysis are complicated and time-consuming. Our aim was to develop a simple and rapid quantification method for the two herbicides and their metabolites with liquid chromatography/tandem mass spectrometry (LC/MS/MS). We also compared 2-amino-4-phosphonobutyric acid and DL-2-amino-5-phosphonopentanoic acid as alternative internal standards (IS) to GLYP$^{13}C_2{}^{15}N$. Herbicide-containing specimens were highly diluted, evaporated to dryness, and derivatized with acetate/acetic anhydride and trimethyl orthoacetate for 30 min. at 120°C. Our optimized LC conditions successfully separated the target analytes, with acceptable linearities (R^2>0.98) and matrix effects (65%–140%). Accuracy and precision ranged from 80.2 % to 111 %, and from 1.3 % to 13 % at the higher concentration, respectively.The concentration of the herbicides and their metabolites were investigated in a postmortem case of suspected herbicide poisoning cases, in which we detected GLYP and its metabolites. Using one of the three ISs, the GLYP concentrations ranged from 3.1 to 3.5 mg/mL, and 3.3 to 4.5 mg/mL in plasma and urine, respectively; GLYP metabolite concentrations in plasma and urine were 18 to 20 μg/mL and 44 to 54 μg/mL. We thus succeeded in developing a rapid method without extraction for measuring GLYP and GLUF along with their metabolites, and demonstrated its practical applicability.

Keywords: glyphosate, glufosinate, liquid chromatography/tandem mass spectrometry, forensic toxicology, postmortem specimens

Abbreviations:
AMPA: aminomethylphosphonic acid
AP-4: (±)-2-amino-4-phosphonobutyric acid
AP-5: DL-2-amino-5-phosphonopentanoic acid
APPA: DL-2-amino-3-phosphonopropionic acid
GLUF: glufosinate
GLYP: glyphosate
IS: internal standard

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LC/MS/MS: liquid chromatography/tandem mass spectrometry
LC-MS/MS: liquid chromatograph-tandem mass spectrometer
MPPA: 3-methylphosphinicopropionic acid

INTRODUCTION

Glyphosate [2-(phosphonomethylamino) acetic acid] (GLYP) and glufosinate {2-amino-4-[hydroxy (methyl) phosphoryl] butanoic acid} (GLUF) are phosphorus-containing amino acid type herbicides. Both GLYP and GLUF are categorized as non-selective herbicides[1,2] and are used globally. Consumption of both herbicides are increasing: the total worldwide GLYP use (agricultural and non-agricultural) rose more than 12-fold from about 67 million kg in 1995 to 826 million kg in 2014,[3] and GLUF use in the US arose from less than 0.1 million kg in 1997 to approximately 4.5 million kg in 2016.[4] Although both herbicides are regarded as relatively safe, some researchers have raised grave concern that chronic exposure to GLYP may cause human health hazards.[5,6] Given their extensive use and possible adverse influences to the environment, human and animal health, GLYP and GLUF have drawn much interest in the field of environmental toxicology.

GLYP and GLUF are also important compounds in the field of forensic toxicology. A substantial number of cases due to GLYP and GLUF intoxications have been reported.[7-10] According to the reports by the National Research Institute of Police Science in Japan, the numbers of fatal intoxication cases due to GLYP and GLUF were almost consistent in the range of 29 and 72 from 1997 to 2017, while the number of fatal intoxication cases due to organophosphates and paraquat (including diquat), decreased from 182 and 274 in 1997 to 55 and 42 in 2017, respectively.[11] Moreover, there were no specific findings reported in the autopsies of fatal intoxication cases due to GLYP and GLUF. It is thus crucial to detect and identify these herbicides in suspected cases.

Having high polarity and poor volatility, GLYP, GLUF and their main metabolites, aminomethylphosphonic acid (AMPA) and 3-methylphosphinicopropionic acid (MPPA), are difficult to separate by liquid chromatography or analyze by gas chromatography without derivatization. Many studies still have reported on the determination of these compounds mainly in crops, surface water, or soil[5,12-14]; some methods for biological specimen have also been reported.[8,15-20] Previously reported methods have adapted liquid-liquid extraction using chloroform,[15] solid-phase extraction using anion exchange column (Oasis MAX),[16] and a method based on the Quick Polar Pesticides Method (QuPPe) approach which combined liquid-liquid extraction and solid-phase extraction.[17] For derivatization, *N*-acetyl, *O*-methyl derivatization using acetic acid and trimethyl orthoacetate,[18] derivatization with *N*-Methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide[8] or *p*-*toluenesulfonyl* chloride[19] have been reported. These extraction and derivatization procedures, however, are generally time-consuming and complicated.

To circumvent such cumbersome sample pretreatment processes, we have developed a simple and rapid quantification method using liquid chromatography/tandem mass spectrometry (LC/MS/MS) for determining GLYP, GLUF, AMPA, and MPPA in a small volume of biological samples without extraction process.

MATERIALS AND METHODS

Materials

Authentic compounds of GLYP, GLUF, and AMPA were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan); MPPA, (±)-2-amino-4-phosphonobutyric acid (AP-4), and DL-2-amino-5-phosphonopentanoic acid (AP-5) were purchased from Merck KGaA (Darmstadt, Germany); DL-2-amino-3-phosphonopropionic acid (APPA) was purchased from Tocris Bioscience (Minneapolis, MN, USA); stable isotope-labeled GLYP ($GLYP^{13}C_2{}^{15}N$) was purchased from LGC labor GmbH (Augsburg, Germany). The chemical structures of GLYP, GLUF, AMPA, MPPA, $GLYP^{13}C_2{}^{15}N$, AP-4, AP-5, and APPA are shown in Fig. 1A; the *N*-acetyl *O*-methyl derivatized compounds are shown in Fig. 1B. Acetic anhydride, ammonium formate, and acetonitrile were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan); acetic acid was from Merck KGaA (Darmstadt, Germany); trimethyl orthoacetate was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Blank human serum and urine samples were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan).

Stock solutions of the target analytes (100 ng/μL) and internal standard (IS) (50 ng/μL) were dissolved in 10% methanol and stored at –20°C.

Instrumentation

In order to optimize the LC-MS/MS conditions, we tried to measure the compounds under three conditions. In brief, each condition was a combination of an LC-MS/MS system and an LC column as follows: Condition 1: LC-MS/MS (Waters, Milford, MA, USA) and ACQUITY UPLC BEH C18 column (Waters), Condition 2: LC-MS/MS (Waters) and SM-C18 column (Imtakt Corporation, Kyoto, Japan), and Condition 3: LC-MS/MS (Shimadzu Corporation, Kyoto, Japan) and SS-C18 column (Imtakt). Details of these conditions are presented in Table 1. In Conditions 1 and 2, we used APPA as the IS.[8] The MS/MS parameters for GLYP, GLUF, AMPA, MPPA, $GLYP^{13}C_2{}^{15}N$, AP-4, and AP-5 are shown in Table 2. Data were acquired in the positive electrospray ionization mode, with selected reaction monitoring (SRM) to measure the area of product ion peaks.

Optimization of derivatization

Derivatization was performed based on the methods using an acetic acid–acetic anhydride mixture and trimethyl orthoacetate adapted by Sato et al and Z. H. Kudzin et al[18,21] with slight modification. GLYP, GLUF, AMPA, MPPA, $GLYP^{13}C_2{}^{15}N$ (IS), AP-4 (IS), and AP-5 (IS) were spiked into 100-fold diluted plasma or urine to a final concentration of 1 μg/mL. A 200 μL aliquot was evaporated under nitrogen flow at 80°C. The residue was dissolved in 200 μL of acetate/acetic anhydride (98/2, v/v) and 200 μL of trimethyl orthoacetate. The derivatization conditions were evaluated under the following: incubation temperature was at 110°C, 120°C, and 130°C, and incubation time was for 15 min, 30 min, 60 min, and 90 min. After evaporation, the residue was reconstituted with 50 μL of acetonitrile and 450 μL of 10 mM ammonium formate. Finally, five microliters of the reconstituted samples were injected into the LC-MS/MS system. Derivatization efficiency was evaluated by comparing the area of product ion peaks.

Sample preparation

Plasma or urine specimens were diluted 100 fold and added a mixture of GLYP, GLUF, AMPA, MPPA, $GLYP^{13}C_2{}^{15}N$ (IS), AP-4 (IS), and AP-5 (IS). After a 200 μL aliquot was evaporated, the residue was derivatized with 200 μL of acetate/acetic anhydride (98/2, v/v) and 200 μL of trimethyl orthoacetate at 120°C for 30 min. Then the dried residue was reconstituted

with 50 μL of acetonitrile and 450 μL of 10 mM ammonium formate, of which 5 μL were injected into the LC-MS/MS system.

Linearity

For the preparation of calibration standards, appropriate amounts of GLYP, GLUF, AMPA, and MPPA were spiked to plasma or urine of which final concentrations were in the range of 0.05 to 5 μg/mL, except for MPPA in urine (0.05 to 1 μg/mL). The three IS compounds were spiked at 25 ng/mL.

Evaluation of matrix effect

Matrix effects were assessed by comparing the peak areas of two different groups of authentic analytes (final concentrations: 1 μg /mL) dissolved in 200 μL of 10% methanol (A), and in the same volume of plasma (B) or urine (C). Matrix effects were defined by the following equation: [B/A or C/A ×100(%)]. Analyses were carried out in sextuplets.

Accuracy and precision

The quality control samples were analyzed within the same day to study intra-day accuracy and precision or over 5 consecutive days to study inter-day accuracy and precision. Samples were analyzed in sextuplet at two concentrations.

Stability of derivatized samples

Derivatized compounds in plasma or urine specimens were incubated for three days, one week or two weeks at room temperature, 4°C or –20°C. Following incubation period, samples were evaporated to dryness and reconstituted before analysis. Data are expressed as mean ±standard deviation. Statistical significance was determined by Welch's *t*-test using Excel. Significant difference between two groups was considered by p-value of < 0.05.

Postmortem sample analysis

Case history: A male in his seventies with no significant medical history, assaulted a family member. He fainted in the restroom, frothed at the mouth, and had a seizure while under investigation. He was transferred to an emergency hospital, where he lost consciousness and went to asystole. Although cardiopulmonary resuscitation was performed, his death was confirmed approximately 1 hour after his arrival to the hospital. It was assumed that he may have been intoxicated by some poison(s) based on the clinical manifestations and autopsy findings. However, the deceased had no history, and no trace of drugs or poisons was observed. In the screening of psychoactive drugs and psychopharmaceuticals by LC-MS/MS, only atropine, administered at the emergency room, was identified.

Plasma and urine specimens were stored in –20°C until prior to analysis. The study was approved by the Institute Review Board of Nagoya University Graduate School of Medicine (2015-0500).

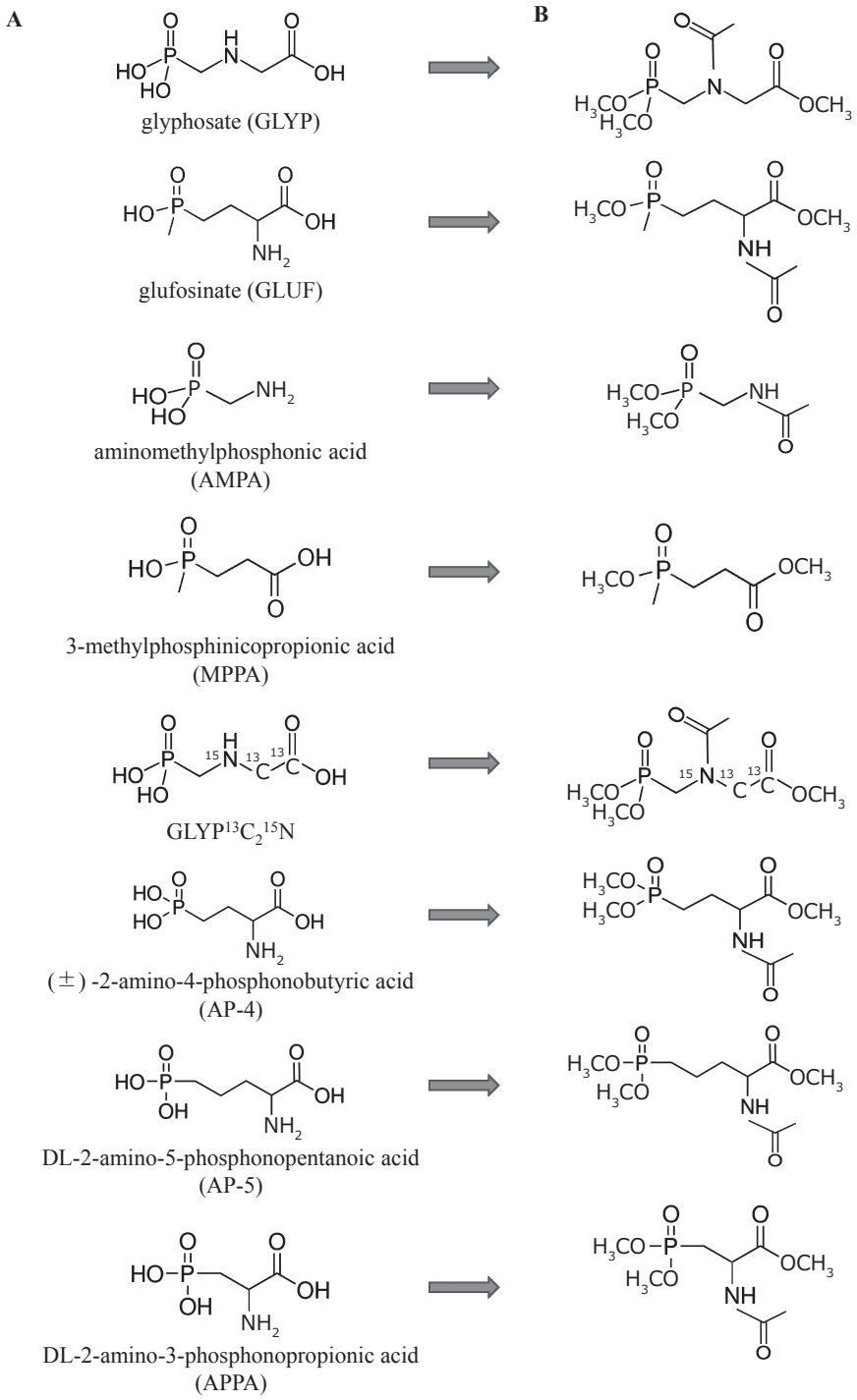


Fig. 1 Chemical structures of target analytes
Fig. 1A: Chemical structures of GLYP, GLUF, AMPA, MPPA, GLYP$^{13}C_2{}^{15}N$, AP-4 and AP-5.
Fig. 1B: Chemical structures of the *N*-acetyl *O*-methyl derivatized compounds.

Table 1 LC-MS/MS conditions using ACQUITY UPLC BEH C18 column (Condition 1), Scherzo SM-C18 column (Condition 2) and Scherzo SS-C18 column (Condition 3)

	Condition 1	Condition 2	Condition 3
LC	ACQUITY UPLC system (Waters)	ACQUITY UPLC system (Waters)	Nexera X2 (Shimadzu)
Column	ACQUITY UPLC BEH C18 (Waters) (2.1mm × 100 mm i.d., particle size 1.7 μm)	Scherzo SM-C18 (Imtakt) (2 mm × 100 mm i.d., particle size 3 μm)	Scherzo SS-C18 (Imtakt) (150 mm × 2 mm i.d., particle size 3 μm)
Mobile phase	[A] 0.1% formic acid, [B] acetonitrile (A/B 80:20)	[A] 0.1% formic acid, [B] acetonitrile (A/B 80:20) A: B 95:5→70:30 (10min)→ 95:5 (2min)	[A] 10 mM ammonium formate, [B] 200 mM ammonium formate/acetonitrile 50/50, v/v A:B 95:5→75:25 (0.5min)→ 75:25 (4.5 min)
Flow rate	0.5 mL/min	0.5 mL/min	0.5 mL/min
MS	ACQUITY TQD system (Waters)	ACQUITY TQD system (Waters)	LCMS-8050 (Shimadzu)
Measurement mode	ESI positive scan (50 to 300)	ESI positive scan (50 to 500), SRM	ESI positive SRM

ESI: electrospray ionization
SRM: selected reaction monitoring

Table 2 Optimized LC-MS/MS parameters

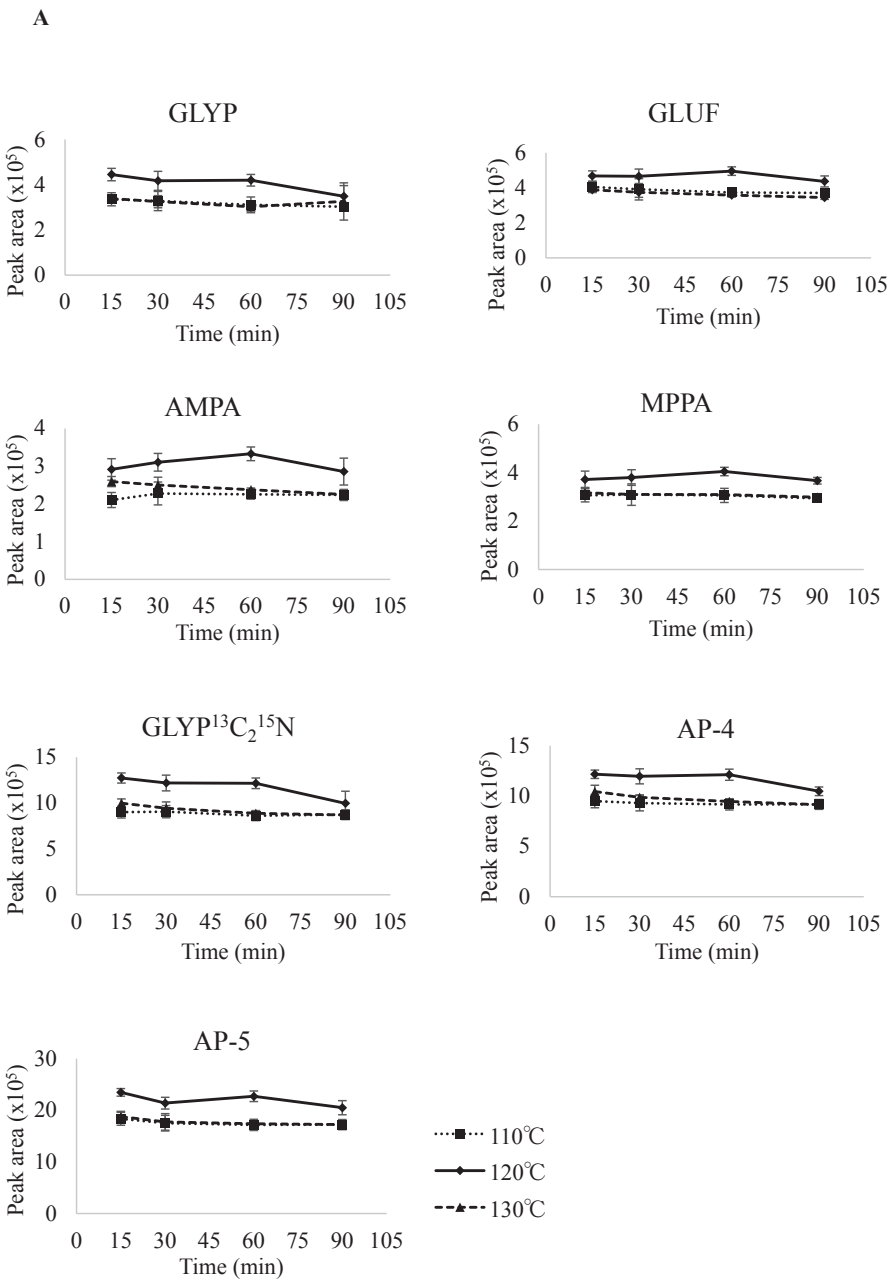
	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Q1 pre bias (V)	Collision energy (eV)	Q3 pre bias (V)
GLYP	2.05	254	102	18	17	20
GLUF	1.73	252	210	10	14	15
AMPA	1.46	182	111	22	18	22
MPPA	1.91	181	149	13	16	28
GLYP$^{13}C_2{}^{15}N$	2.04	257	105	18	17	21
AP-4	2.09	268	166	20	19	11
AP-5	2.59	282	180	11	21	12

RESULTS

Optimization of pretreatment

In order to optimize the derivatization conditions, we derivatized the compounds in plasma or urine specimens at 110, 120, and 130°C for 15, 30, 60, and 90 min per temperature setting. In plasma specimens, the values of derivatization efficiency were higher at 120°C for almost all compounds than other temperatures (Fig. 2A). On the other hand, the values of derivatization ef-

ficiency in urine specimens were slightly higher at 110°C than 120°C (Fig. 2B). For convenience purposes of derivatizing plasma and urine specimens simultaneously, we thus selected 120°C as the optimized derivatization temperature. There was almost no difference in derivatization efficiency between 15 and 30 min for most compounds. In general, longer incubation results in more stable derivatized efficiency, thus 30 min was selected as the incubation time for this derivatization method. The final derivatization condition was thus optimized at 120°C for 30 min.



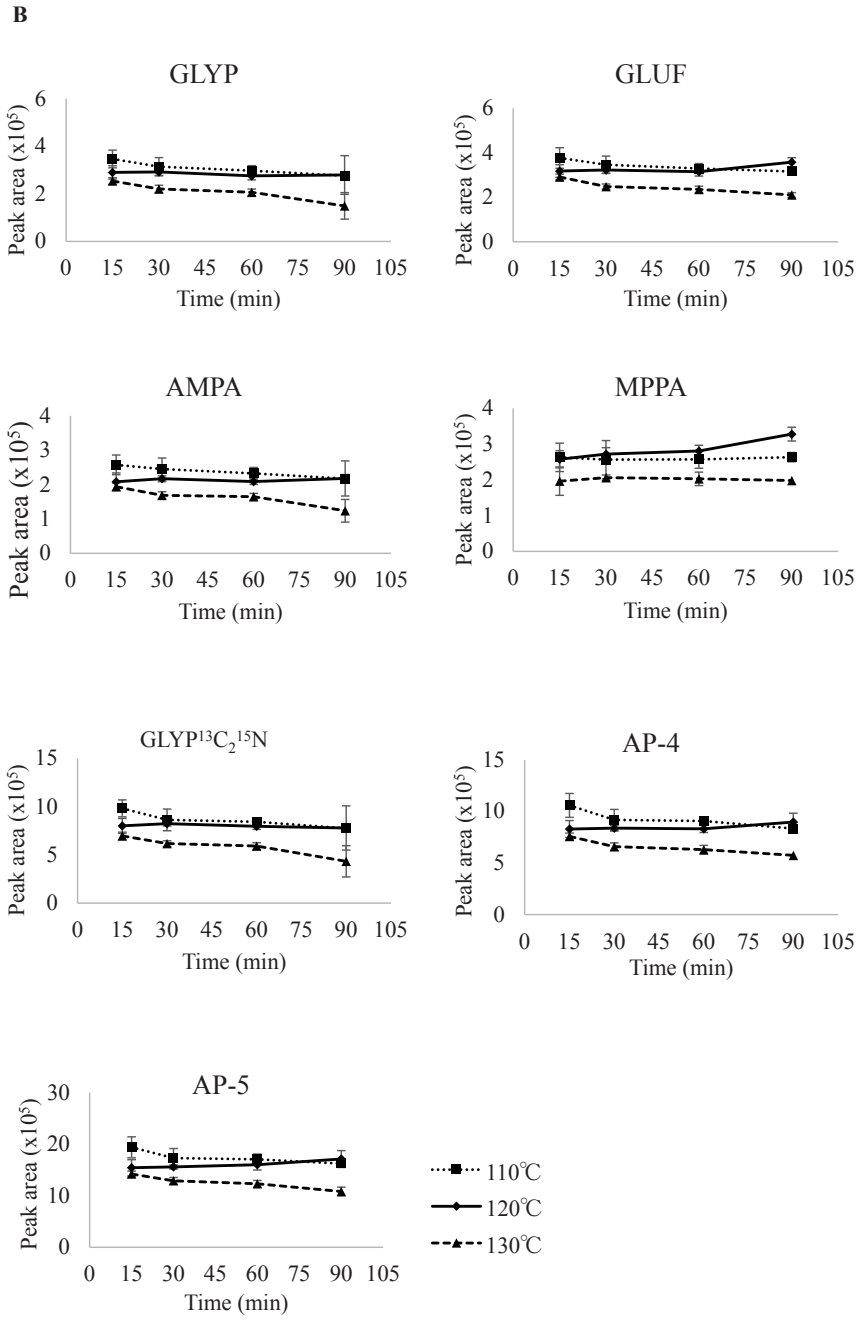


Fig. 2 Optimization of derivatization temperature and time

Fig. 2A: Results of derivatization temperature and time opmitmization in plasma.

Fig. 2B: Results of derivatization temperature and time optimization in urine.

Peak areas of derivatized compounds at 110°C (closed squares and dotted line), 120°C (closed diamonds and solid line), or 130°C (closed triangles and broken line) are plotted. Peak area values are means ±standard deviation (n=5).

Optimization of LC-MS/MS conditions

Using an ACQUITY UPLC BEH C18 column and an ACQUITY UPLC system, the analytes could not be separated in the total ion chromatogram (Supplementary Fig. 1); a Scherzo SM-C18 column achieved slightly better separation (Supplementary Fig. 2). We thus tried to obtain SRM chromatograms of GLYP, GLUF, AMPA, and APPA with the SM-C18 column (Supplementary Table 1); however, the results were not satisfactory. GLYP and APPA peaks nearly coeluted, and the fragment ions of APPA were not distinguishable from those of GLYP. Given the above reasons, we concluded that this condition was inadequate for quantitative analysis of our target analytes. Finally, by adopting the LCMS-8050 system with an SS-C18 column, we were able to achieve sufficient separation and sharp peaks shapes for GLYP, GLUF, AMPA, and MPPA (Fig. 3).

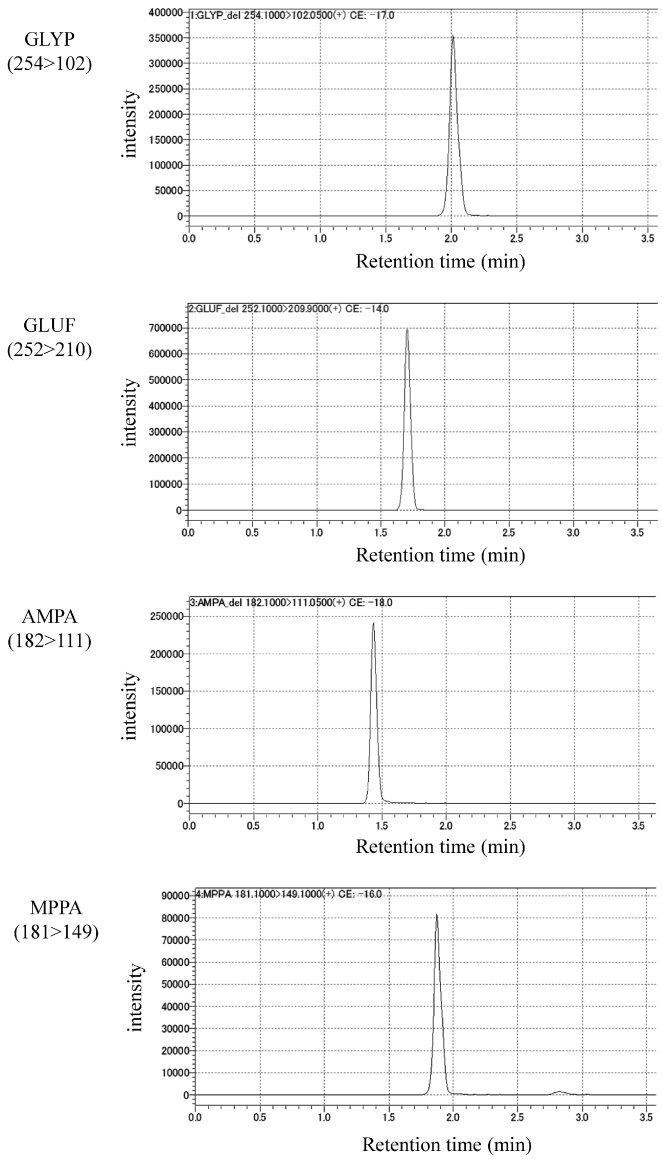


Fig. 3 SRM chromatograms obtained for authentic standards of GLYP, GLUF, AMPA, and MPPA using an LCMS-8050 LC-MS/MS and Scherzo SS-C18 separation column

Validation of the method

Linearity, limits of detection, limits of quantification, matrix effect, and the accuracy and precision values for intra-day and inter-day experiments were determined as described in the Methods section. Linearity, defined by R^2, was above 0.989 for all analytes (Tables 3 and 4). Matrix effects ranged from 95.4% to 140% in plasma (Table 3) and from 64.9% to 89.8% in urine (Table 4). All analytes exhibited ion suppression in urine specimens.

Tables 5–8 summarize the intra-day and inter-day accuracy and precision in plasma and urine specimens. Accuracy ranged from 80.2% to 121%, and precision ranged from 1.3% to 26%.

Table 3 Linear range, determination coefficients, LODs, LOQs and matrix effects of target analytes in plasma

	IS	Linear range (μg/ml)	Linear equation	Linearity (R^2)	LOD (μg/ml)	LOQ (μg/ml)	Matrix effect (%) (n=6)
	$GLYP^{13}C_2{}^{15}N$		y = 0.3388x – 0.0005	0.999			
GLYP	AP-4	0.05–5	y = 0.2973x + 0.0045	0.999	0.02	0.05	109
	AP-5		y = 0.249x + 0.0029	0.997			
	$GLYP^{13}C_2{}^{15}N$		y = 0.246x + 0.0067	0.998			
GLUF	AP-4	0.05–5	y = 0.2157x + 0.0096	0.999	0.02	0.05	104
	AP-5		y = 0.1806x + 0.0074	0.999			
	$GLYP^{13}C_2{}^{15}N$		y = 0.1101x + 0.0065	0.997			
AMPA	AP-4	0.05–5	y = 0.0965x + 0.0075	0.998	0.02	0.05	116
	AP-5		y = 0.0809x + 0.006	0.996			
	$GLYP^{13}C_2{}^{15}N$		y = 0.2801x – 0.0007	0.997			
MPPA	AP-4	0.05–5	y = 0.2459x + 0.0033	0.993	0.02	0.05	140
	AP-5		y = 0.2061x + 0.002	0.989			
$GLYP^{13}C_2{}^{15}N$							106
AP-4							105
AP-5							95.4

LOD: limits of detection
LOQ: limits of quantification

Table 4 Linear range, determination coefficients, LODs, LOQs and matrix effects of target analytes in urine

	IS	Linear range (μg/ml)	Linear equation	Linearity (R^2)	LOD (μg/ml)	LOQ (μg/ml)	Matrix effect (%) (n=6)
GLYP	$GLYP^{13}C_2{}^{15}N$	0.05–5	y = 0.2863x – 0.007	0.997	0.02	0.05	77.5
	AP-4		y = 0.269x + 9E-05	0.998			
	AP-5		y = 0.1509x + 0.0055	0.997			
GLUF	$GLYP^{13}C_2{}^{15}N$	0.05–5	y = 0.3349x – 0.0285	0.998	0.02	0.05	82.1
	AP-4		y = 0.3148x – 0.0202	0.999			
	AP-5		y = 0.1769x – 0.006	0.998			
AMPA	$GLYP^{13}C_2{}^{15}N$	0.05–5	y = 0.2645x + 0.0063	0.996	0.02	0.05	64.9
	AP-4		y = 0.2485x + 0.0126	0.995			
	AP-5		y = 0.1393x + 0.0126	0.995			
MPPA	$GLYP^{13}C_2{}^{15}N$	0.05–1	y = 0.3754x + 0.0199	0.993	0.02	0.05	89.8
	AP-4		y = 0.3491x + 0.0211	0.992			
	AP-5		y = 0.2098x + 0.0142	0.995			
$GLYP^{13}C_2{}^{15}N$							78.8
AP-4							77.8
AP-5							68.6

LOD: limits of detection
LOQ: limits of quantification

Table 5 Intra-day accuracy and precision of GLYP, GLUF, and their metabolites in plasma

	IS	$GLYP^{13}C_2{}^{15}N$		AP-4		AP-5	
	μg/mL	Acc. (%)	Pre. (%)	Acc. (%)	Pre. (%)	Acc. (%)	Pre. (%)
GLYP	0.2	108	2.0	106	1.5	103	5.6
	4	99.8	2.0	102	3.4	103	6.1
GLUF	0.2	92.7	3.9	93.5	3.2	90.3	5.1
	4	98.6	4.5	101	3.1	102	4.5
AMPA	0.2	83.7	12	83.7	12	80.5	12
	4	92.4	9.1	94.3	7.5	95	7.2
MPPA	0.2	118	6.3	120	6.4	99.8	11
	4	105	7.1	108	8.1	109	8.1

Acc: accuracy
Pre: precision

Table 6 Inter-day accuracy and precision of GLYP, GLUF, and their metabolites in plasma

	IS	GLYP$^{13}C_2{}^{15}N$		AP-4		AP-5	
	μg/mL	Acc. (%)	Pre. (%)	Acc. (%)	Pre. (%)	Acc. (%)	Pre. (%)
GLYP	0.2	95.5	6.6	96.7	2.8	99.7	2.2
	4	99.3	4.1	96.6	2.5	93.1	4.1
GLUF	0.2	99.0	4.5	104	2.5	106	3.4
	4	109	2.4	105	3.7	102	1.8
AMPA	0.2	121	26	90.7	23	91.3	22
	4	111	4.0	108	4.6	104	3.9
MPPA	0.2	103	5.6	101	2.1	106	2.6
	4	110	5.5	106	6.7	102	5.4

Acc: accuracy
Pre: precision

Table 7 Intra-day accuracy and precision of GLYP, GLUF, and their metabolites in urine

	IS	GLYP$^{13}C_2{}^{15}N$		AP-4		AP-5	
	μg/mL	Acc. (%)	Pre. (%)	Acc. (%)	Pre. (%)	Acc. (%)	Pre. (%)
GLYP	0.2	108	3.1	106	5.2	103	6.2
	4	91.4	1.3	91.0	3.6	89.8	4.3
GLUF	0.2	92.7	7.5	93.5	4.2	90.3	5.3
	4	108	3.4	108	4.8	106	4.6
AMPA	0.2	83.7	9.1	83.7	7.4	80.5	8.9
	4	83.4	5.0	83.0	6.8	81.9	6.3
MPPA	0.08	118	22	120	24	99.8	22
	0.8	105	9.1	105	13	105	13

Acc: accuracy
Pre: precision

Table 8 Inter-day accuracy and precision of GLYP, GLUF, and their metabolites in urine

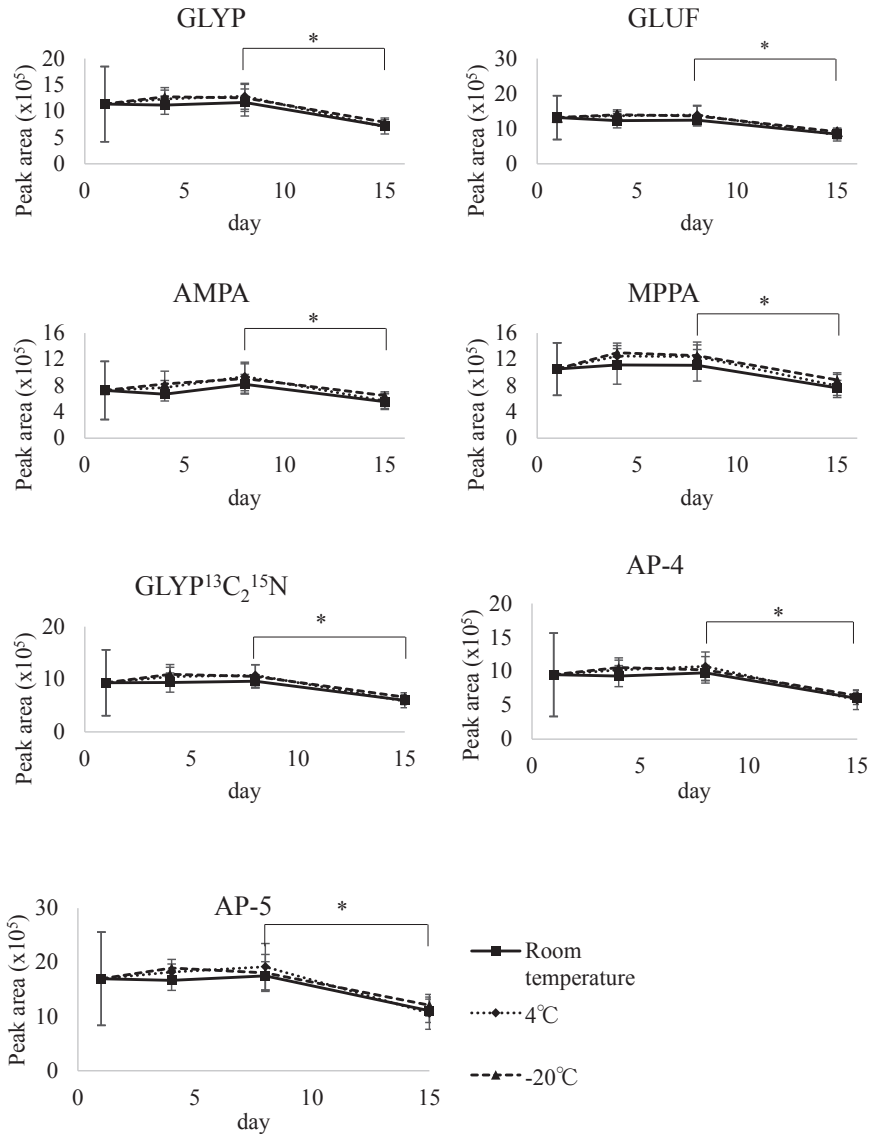
	IS	GLYP$^{13}C_2{}^{15}N$		AP-4		AP-5	
	μg/mL	Acc. (%)	Pre. (%)	Acc. (%)	Pre. (%)	Acc. (%)	Pre. (%)
GLYP	0.2	95.9	5.8	93.7	3.3	87.6	12
	4	89.3	1.3	90.8	2.0	88.4	2.5
GLUF	0.2	121	18	114	15	101	10
	4	95.1	5.0	96.8	5.3	94.2	5.4
AMPA	0.2	104	15	100	7.5	91.8	10
	4	88.8	6.0	90.2	4.9	87.8	5.0
MPPA	0.08	94.7	8.7	96.9	7.7	102	6.5
	0.8	86.4	8.7	84.2	9.3	80.2	9.5

Acc: accuracy
Pre: precision

Stability of derivatization

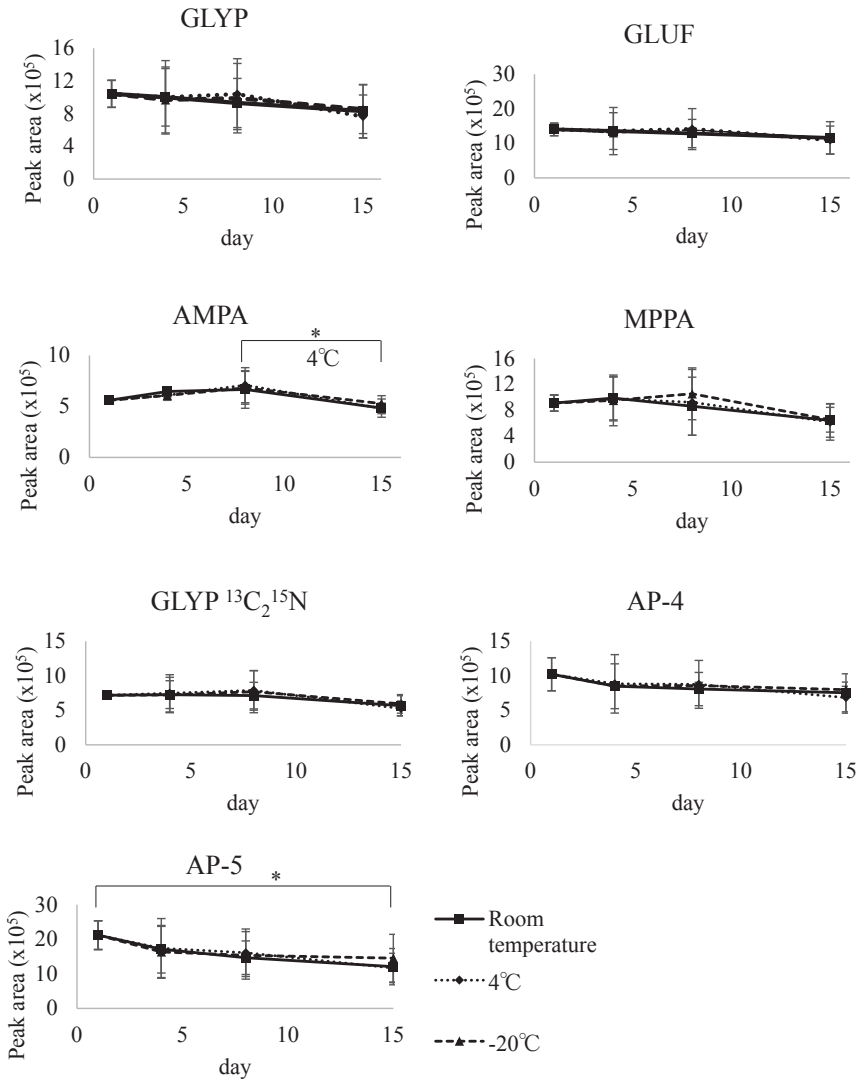
Next, we evaluated the stability of the derivatized compounds in plasma and urine. In plasma, derivatized GLYP, GLUF, AMPA and MPPA were stable within one week under all conditions. After a week, peak areas of the compounds decreased significantly (Fig. 4A). In urine, derivatized GLYP, GLUF, and MPPA were stable for two weeks. Peak area of derivatized AMPA decreased significantly after one week (Fig. 4B).

A



*p<0.05

B



*p<0.05

Fig. 4 Stability of derivatized compounds

Fig. 4A: Stability of derivatized compounds in plasma specimens.

Fig. 4B: Stability of derivatized compounds in urine specimens.

Peak areas of derivatized compounds incubated at room temperature (closed squares and solid line), 4ºC (closed diamonds and dotted line), or –20ºC (closed triangles and broken line) are plotted. Peak area values are means ±standard deviation (n=6). Welch's *t*-test was used to compare two groups. Asterisk indicates a significant difference ($p<0.05$).

Application to postmortem specimens

The present method was applied to a fatal intoxication case. As shown in Table 9, GLYP and AMPA were successfully detected and quantified in plasma and urine; GLUF and MPPA were not detected in the specimens. Concentration of GLYP ranged from 3.1 to 3.5 mg/mL in plasma and from 3.3 to 4.5 mg/mL in urine; AMPA concentration ranged from 18 to 20 μg/mL in plasma and from 44 to 54 μg/mL in urine.

Table 9 Quantitative results of GLYP and AMPA in plasma and urine specimens of a fatal intoxication case

n=5	GLYP (mg/ml)			AMPA (μg/ml)		
IS	GLYP$^{13}C_2{}^{15}N$	AP-4	AP-5	GLYP$^{13}C_2{}^{15}N$	AP-4	AP-5
plasma	3.5±0.03	3.1±0.22	3.1±0.14	20±4.73	18±4.12	20±4.72
urine	4.5±0.09	3.5±0.91	3.3±0.88	55±19.7	50±15.9	44±14.6

Mean±standard deviation. GLUF and MPPA were not detected.

DISCUSSION

In this study, we developed a rapid quantitative method for GLYP, GLUF, AMPA, and MPPA in urine and plasma. Sample extraction processes are generally required to analyze target compounds in crude biological specimens when analyzing with analytical instruments such as LC-MS/MS. Although useful for removing contaminants such as sugars, lipids, proteins, and nucleotides present in biological specimens, there is a possibility that the sample extraction step also decreases recovery rates of the target compounds. Furthermore, some extraction methods such as solid-phase extraction are complicated and time-consuming. On the other hand, since our method does not require any extraction step, decrease in the recovery rate due to extraction can be prevented. Moreover, our method is very simple since only sample dilution is required to prepare the crude biological specimens such as urine and plasma. For derivatization, it takes several hours in previously reported methods.[21,22] On the other hand, our method takes only 30 min for derivatization. Furthermore, since our method does not require any extraction step, have successfully developed a rapid analysis method for GLYP, GLUF, and their metabolites.

In our method validation studies, precision values varied more at the lower concentration compared to those at the higher concentration. In intoxication cases, GLUF concentrations have been reported from 0.6 to 440 mg/mL in serum and 91 to 39,701 mg/mL in urine.[9,23] The concentration of MPPA is reported to be about 10 % of the parent compound in serum.[24] The mean GLYP and AMPA concentrations are reported as 4.15 mg /mL and 61.6 μg/mL in postmortem blood specimens, respectively.[19]

To quantify the concentrations of target compounds, ISs are commonly used to correct the extraction rates and intensities of analytes. Stable isotope compounds with the same or similar structure, such as GLYP$^{13}C_2{}^{15}N$ in our case, are generally used. However, stable isotope compounds are expensive and laboratories may not have the financial luxury of purchasing the appropriate stable isotope-labeled compounds; applying a cheaper and more conveniently available ISs is therefore desired for practical purposes. In this study, we compared AP-4 and AP-5 as ISs alternative to GLYP$^{13}C_2{}^{15}N$. AP-4 and AP-5 performed just as well as ISs compared with GLYP$^{13}C_2{}^{15}N$ in the linearity of calibration curves for urine and plasma. Furthermore, the matrix effects of the ISs were not inferior to the stable isotope IS. These results suggest that AP-4 and AP-5 can be applicable as ISs for correcting GLYP, GLUF, and their metabolites in quantitative

LC-MS/MS analysis.

In our presented case, the concentrations of GLYP and AMPA in postmortem plasma ranged from 3.1 to 3.5 mg/mL and 18 to 20 μg/mL, respectively. In postmortem urine, the concentrations of GLYP and AMPA were found to be from 3.3 to 4.5 mg/mL and 44 to 55 μg/mL, respectively. Considering the concentrations in previously reported cases and the results from our present study, our method seems sufficient to analyze urine and plasma specimens in fatal intoxication cases of GLYP and/or GLUF. However, it should be noted that background GLYP and AMPA concentrations in biological specimens must be lower than those in intoxication cases. In the US, GLYP and AMPA concentrations in urine ranged from 0.1 to 9.4 μg/mL for non-farm families and 0.02 to 18 μg/mL in farm families.[25] Considering the above report, while our method is applicable for fatal intoxication cases, further improvement is needed to apply our method for occupational and environmental health studies.

Recently, Usui et al reported a method for identifying and quantifying GLYP and GLUF in human serum by probe electrospray ionization-tandem mass spectrometry (PESI-MS/MS).[20] Usui's method has potential to be useful in emergency medicine for its simplicity and rapid analysis time, where time is a critical issue in acute poisoning cases, but requires a unique instrumentation setup. While our present method still requires derivatization, the advantage of our method is the use of cheap and commercially available IS for the quantitative analysis. Most forensic laboratories already employ LC-MS/MS for routine drug analysis; our method can be easily adopted in forensic laboratories by obtaining the column and IS compounds presented in this manuscript. Furthermore, our method can also detect and quantify the metabolites, AMPA and MPPA, which is critical in forensic toxicology where the presence of metabolites help to identify if the drugs were ingested before death or not.

In this report, we have developed a rapid and simple quantitation method for herbicides GLYP and GLUF as well as metabolites AMPA and MPPA applicable for analyzing fatal intoxication cases. With LC-MS/MS, some very hydrophilic compounds such as these herbicides are difficult to be analyzed. The SS-C18 column has been reported to give sharp peaks of hydrophilic compounds such as acetylcholine, creatinine, ipratropium, and fluticasone, which all commonly separately poorly using conventional LC columns, and thus enabled these compounds to be more precisely analyzed.[26-28] Likewise, our present method using SS-C18 column can efficiently separate hydrophilic herbicides and metabolites, therefore could be applicable for analyzing other hydrophilic poisons and/or psychoactive drugs that are difficult to be measured in conventional columns; further study is underway.

CONCLUSION

We have developed a method in which GLYP, GLUF, AMPA, and MPPA were detected in a small volume of plasma and urine specimens with simple derivatization, without any extraction steps. For this method, AP-4 and AP-5 were found to perform equally well as IS in comparison to using the stable isotope labeled GLYP$^{13}C_2{}^{15}N$. Applicability to real case sample was demonstrated by quantifying GLYP and AMPA in the plasma and urine specimens of a fatal intoxication case.

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CONFLICTS OF INTEREST

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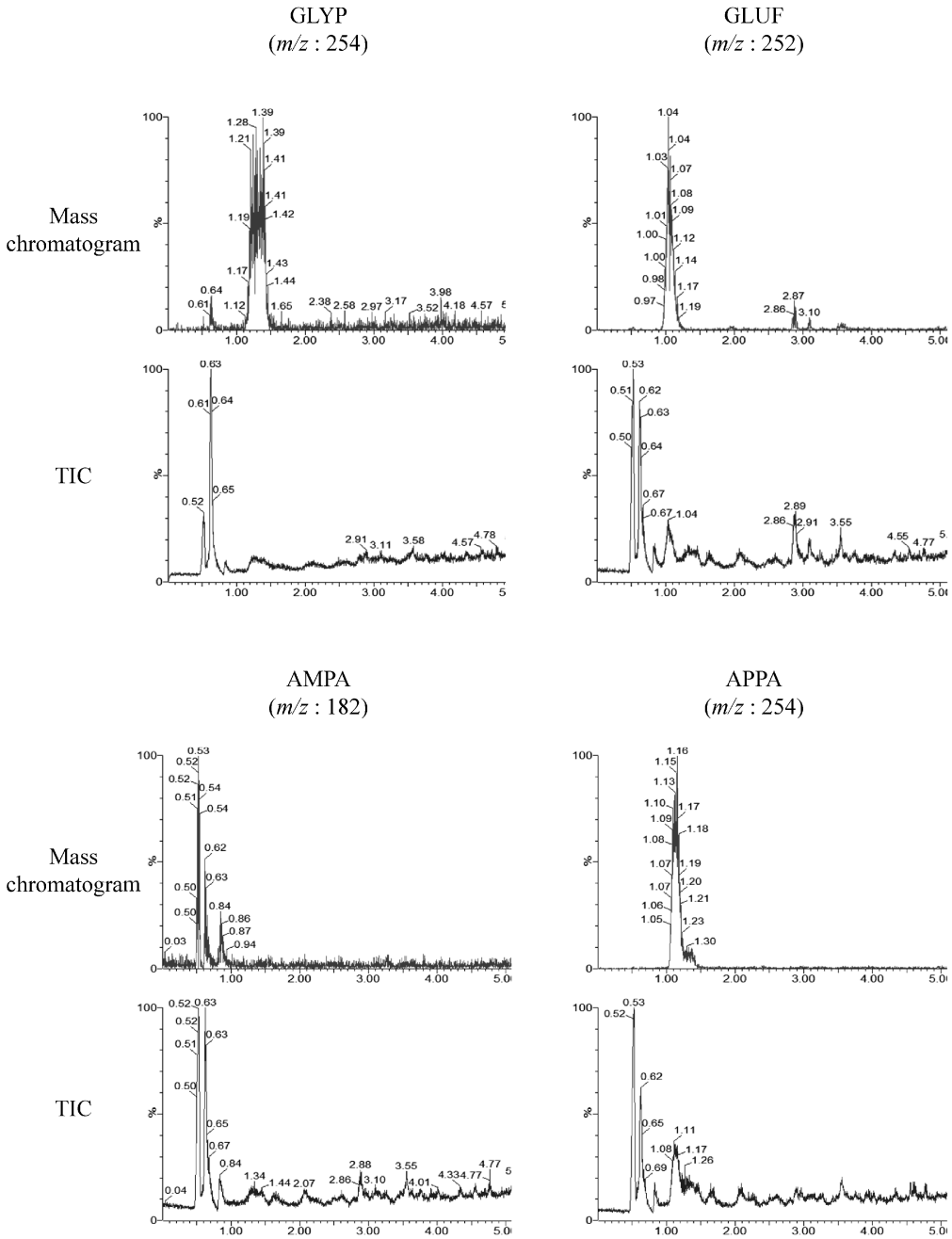
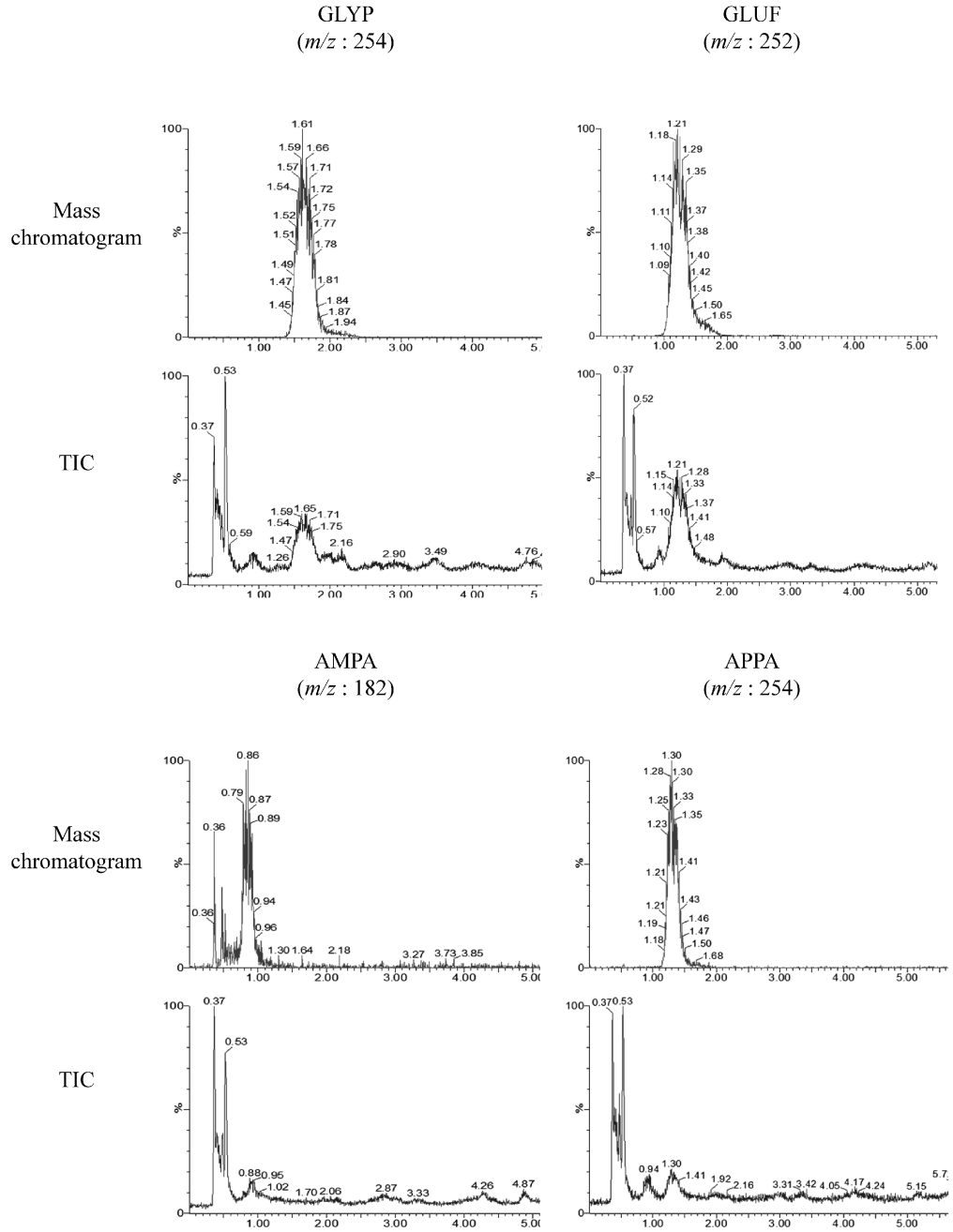
Appendix



Supplementary Fig. 1 Total ion chromatograms and mass chromatograms obtained from LC/MS/MS of GLYP, GLUF, AMPA, and APPA (authentic standards) using an ACQUITY UPLC BEH C18 column



Supplementary Fig. 2 Total ion chromatograms and mass chromatograms obtained from LC/MS/MS of GLYP, GLUF, AMPA and APPA (authentic standards) using a Scherzo SM-C18 column

Supplementary Table 1 Selected reaction monitoring mode parameters with Condition 2

	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)	Cone voltage (V)
GLYP	254	102	15	20
GLUF	252	210	12	25
AMPA	182	111	16	15
APPA	254	212	12	25