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Determination of glyphosate, glyphosate metabolites, and glufosinate in human serum by gas chromatography–mass spectrometry

Megumi Motojyuku, Takeshi Saito*, Kazuki Akieda, Hiroyuki Otsuka, Isotoshi Yamamoto, Sadaki Inokuchi

Department of Emergency and Critical Care Medicine, Tokai University School of Medicine, Isehara, Kanagawa 259-1143, Japan

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ABSTRACT

This paper describes an assay for the determination of glyphosate (GLYP), glyphosate metabolites [(aminomethyl) phosphonic acid] (AMPA), and glufosinate (GLUF) in human serum. After protein precipitation using acetonitrile and solid-phase extraction, serum samples were derivatized and analyzed by gas chromatography–mass spectrometry (GC–MS). The assay was linear over a concentration range of 3–100.0 μg/ml for GLYP, AMPA, and GLUF. The overall recoveries for the three compounds were >73%. The intra- and inter-day variations were <15%. Precision and accuracy were 6.4–10.6% and 88.2–103.7%, respectively. The validated method was applied to quantify the GLYP and AMPA content in the serum of a GLYP-poisoned patient. In conclusion, the method was successfully applied for the determination of GLYP and its metabolite AMPA in serum obtained from patient of GLYP-poisoning.

1. Introduction

Glyphosate [*N*-(phosphonomethyl)glycine] (GLYP) and glufosinate (GLUF) [DL-Homoalanin-4-yl-(methyl)phosphinate ammonium salt] (Fig. 1) are phosphonic and amino acid group-containing chemicals that constitute an important category of pesticides and are extensively used as herbicides in many countries including Japan. In Japan, GLYP and GLUF were registered as pesticides in 1980 and 1984, respectively. Thereafter, many cases of accidental and suicidal poisoning due to the ingestion of these herbicides have been reported [1–4]. GLUF-poisoning is characterized by various neurological symptoms such as disturbances of consciousness, convulsions and apnea that appear several hours after ingestion [1,2]. The mortality rate due to suicidal poisoning with GLYP is usually low compared with the rates in the case of paraquat and organophosphate poisoning. Farly diagnosis and appropriate treatment of GLYP is often life saving [5]. Most fatalities occurred within 72 h after the ingestion of a large volume of GLYP herbicide. The mortality rate due to GLYP-poisoning is reported to be between 7.5% and 16.7% [5–7]. The mortality rates due to paraquat and organophosphate poisoning are 69.2% and 33.3%, respectively [8,9].

GLYP and GLUF herbicides contain surfactants [10] that probably cause poisoning [1,7,10]. However, these surfactants are not disclosed by the manufacturers. Therefore, a rapid and reliable screening method is required to identify GLYP and GLUF in biological samples. GLYP and GLUF disintegrate in the body into (aminomethyl) phosphonic acid (AMPA), (Fig. 1) and 3-(methylphosphinico) propionic acid (3-MPPA), respectively. GLYP and GLUF are extremely polar compounds, and in most cases a derivatization of the ionic character is required to enable chromatographic separation via gas chromatography (GC) [11], GC–MS [12–14], and liquid chromatography (LC) [15–17]. However, the previously reported sample preparation method, especially the derivatization procedure is time-consuming.

We have developed an analytical method for the identification of GLYP, GLUF, and AMPA. Although 3-MPPA is the main metabolite of GLUF, its levels are considerably lower than those of GLUF. This method is based on a rapid derivatization procedures an analysis is carried out using GC–MS.

2. Drugs and chemicals

GLYP was obtained from Wako (Osaka, Japan). DL-2-Amino-3-phosphonopropionic acid and AMPA were purchased from Sigma (St. Louis, MO, USA). GLUF was purchased from AccuStandard (New Heaven, CT, USA). Sep-Pak® Plus PS-2 cartridges (JJAN2013) were used in preparing serum samples for the analysis of each compound (Waters Corporation, Milford, MA, USA). The derivatization reagent *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) + 1% *tert*-butyldimethylchlorosilane (TBDMCS) was purchased from Pierce (Rockford, IL, USA). All other chemicals and

* Corresponding author. Tel.: +81 463 93 1121; fax: +81 463 95 5337.
E-mail address: saito@is.icc.u-tokai.ac.jp (T. Saito).

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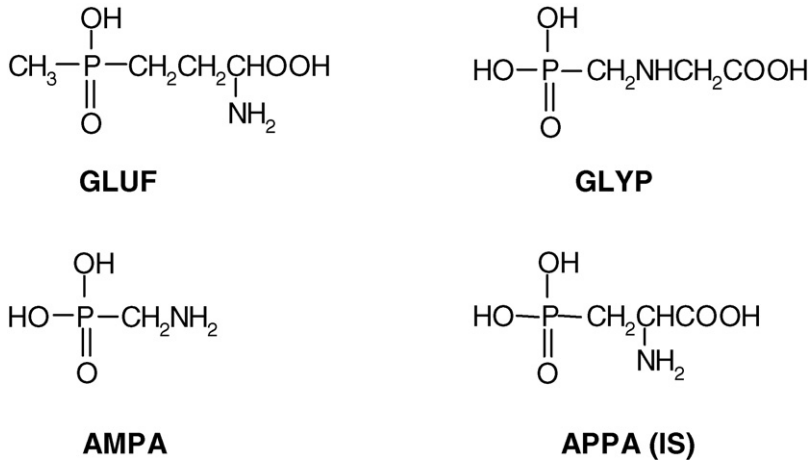

Fig. 1. Chemical structures of (aminomethyl) phosphonic acid (AMPA), glyphosate (GLYP), glufosinate (GLUF), and internal standard (IS). Chemical name.

reagents were of analytical grade (Wako, Japan). The serum sample for the validation study was collected from a volunteer.

3. Calibration curve and quality control samples

AMPA, GLYP, and GLUF (1 mg/ml) were prepared in 10% methanol. These solutions were further diluted with 10% methanol to concentrations of 100 and 10 μg/ml and stored for a maximum of 3 months at 4 °C. These solutions were used as calibrators. A stock solution of DL-2-amino-3-phosphonopropionic acid (1 mg/ml) was prepared in 10% methanol and used as an internal standard (IS). The IS stock solution was further diluted with 10% methanol to a concentration of 100 μg/ml. Blank serum samples (0.2 ml) were spiked with 2 μl of calibrator (10 μg/ml), 1 and 2 μl of each calibrator (100 μg/ml) and 1, 2, 5, 10 and 20 μl of each solution (1 mg/ml), resulting in calibration samples containing 0.02 μg (0.1 μg/ml), 0.1 μg (0.5 μg/ml), 0.2 μg (1 μg/ml), 0.6 μg (3 μg/ml), 1 μg (5 μg/ml), 2 μg (10 μg/ml), 5 μg (25 μg/ml), 10 μg (50 μg/ml), and 20 μg (100 μg/ml) of each compound, i.e., AMPA, GLYP, and GLUF, respectively.

Each quality control (QC) solution was prepared in the same manner as mentioned above. QC samples were prepared using blank serum samples (0.2 ml) containing 0.6 μg (low QC level: 6 μl of 100 μg/ml; 3 μg/ml), 6 μg (medium QC level: 6 μl of 1 mg/ml; 30 μg/ml), and 18 μg (high QC level: 18 μl of 1 mg/ml; 90 μg/ml) μg of each compound. A total of 33 QC samples were prepared for each validation study (intra-, inter- and stability).

4. Extraction procedure

The calibration curves and the QC and clinical serum samples were extracted by employing a solid-phase extraction (SPE) technique utilizing a Sep-Pak® Plus PS-2 sorbent, obtained from Waters Corporation, along with a styrene-divinylbenzene sorbent.

Serum (0.2 ml) and acetonitrile (0.2 ml) were added to centrifuge tubes, and the mixture was vortexed (15 s). After centrifugation (5 min, 3000 rpm), 10 μl (100 μg/ml) of the IS solution was added to the 0.2-ml sample. The sample extracts were purified using Sep-Pak® Plus PS-2 (265 mg, 11 mm o.d. × 20 mm length) and loaded onto SPE cartridges conditioned with acetonitrile (4 ml) and distilled water (4 ml). The cartridges were dried (5 psi, 3 min) and then eluted with acetonitrile (2 ml). The eluates were reduced to dryness under nitrogen at 50 °C. The samples were reconstituted in 50 μl each of MTBSTFA with 1% TBDMCS and acetonitrile, and the mixture was vortexed (15 min). An aliquot (1 μl) of derivatized sample was injected into the GC–MS system.

4.1. GC–MS analysis

Gas chromatographic analyses were performed using an Agilent 6890 quadrupole gas chromatograph (Palo Alto, CA, USA) equipped with an Agilent 5975B mass spectrometer (MS). A 30 m × 0.25 mm HP-5MS fused-silica capillary column with a film thickness of 0.25 μm obtained from Agilent was used. Helium (purity ≥ 99.999%) was used as a carrier gas at a flow rate of 0.6 ml/min. A 1-μl extract was injected in a splitless mode at an injection temperature of 250 °C. The oven temperature was programmed to increase from an initial temperature of 100 °C (held for 3 min) to 300 °C (held for 3 min) at 20 °C/min. The temperatures of the quadrupole, ion source, and mass-selective detector interface were 150, 230, and 280 °C, respectively. The GC–MS system was operated in the selected-ion monitoring (SIM) mode with the electron multiplier tune value. The electron multiplier voltage for AMPA was set to 200 V relative to the tune value. The following ions were monitored (with quantitative ions in parentheses): AMPA (396.2), 144.1, 367.1; GLYP (454.2), 253.1, 352.2; GLUF (466.3), 364.2, 334.1; and IS (568.3), 466.3 and 408.2.

4.2. Matrix-matched curve

In order to prepare the matrix-matched calibration curves, blank serum samples were extracted using this method. The dried residue obtained after the completion of the procedure was derivatized after adding IS. In order to detect matrix effects, the target analytes in the serum were quantified against calibration solutions containing pure standard compounds and against calibration solutions prepared using cleaned extracts from the blank serum samples. The selection of IS and determination of concentration was performed according to a previous method [15].

4.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD is defined as a signal equivalent to five times the noise. The LOQ is defined as the concentration which has the precision of 20% and the accuracy of 80–120% [18].

4.3.1. Precision and accuracy

The intra- and inter-day %RSD were obtained using blank serum samples spiked with three QC samples. For the intra- and inter-day,

assays, each concentration was prepared and analyzed six times for 3 consecutive days. The accuracy was measured as the percentage deviation from the nominal concentration. The criteria for the acceptability of data included accuracy within ±15% of deviation from the nominal values and a precision within ±15% of RSD, except for the lowest LOQ for which it was not to exceed 20% of RSD [18].

4.3.2. Recovery

We evaluated the overall recovery of AMPA, GLYP, and GLUF from the blank serum by analyzing the six replicates of two sets of QC samples. The first set consisted of 0.2 ml of blank serum fortified with the three QC solutions and deproteinized with 0.2 ml of acetonitrile. To 0.2 ml of the deproteinized sample was added 1 μg of the IS. The second set consisted of 0.2 ml of blank serum after deprotenizing with the IS, and the three QC solutions were added to the eluate after the SPE. A comparison of the mean GLUF and GLYP integrated peak areas, (set A/set B) × 100%, provided the overall GLUF and GLYP recovery, expressed in percentage. The mean (S.D.) was calculated for the six replicates.

The efficiency of the SPE of AMPA, GLYP, and GLUF was also determined. A deproteinized serum solution was prepared in the same manner as the serum extracts, which were applied to the SPE columns. The first set ($n = 6$) was prepared by adding the three QC solutions along with 1 μg of the IS. The second set ($n = 6$) was prepared by adding 1 μg of the IS to the same deproteinized serum solution as used in the first set before the SPE, but the three QC solutions were added to the eluates after the SPE. To compute the SPE efficiency, the GLUF and GLYP area counts for the first set were divided by the area counts for the second set, and the difference was expressed in percentage.

4.3.3. Stability

No stability study in plasma has been previously conducted. Serum QC samples of AMPA, GLYP, and GLUF at three concentrations were used for stability experiments. The short-term stability of the serum samples was examined by analyzing the replicates of the three QC samples at room temperature for 24 h and 4 °C for 1 week. Each freeze–thaw cycle consisted of a minimum of 24 h freezing at −30 °C followed by a complete thaw at room temperature. Samples were analyzed after the third freeze–thaw cycle. The long-term stability of the four compounds in human serum was tested after storage at −30 °C for 4 weeks. The post-derivatized stability after extraction was determined for the three QC samples stored at room temperature (24 °C) for 24 h in autosampler vials. The concentration of each compound after the storage period was compared with the initial concentration that was determined from the samples that were freshly prepared and immediately processed.

4.4. Patient's samples

A 56-year-old Japanese male attempted to commit suicide by ingesting approximately 400 ml of Rundup® (41% glyphosate-isopropylammonium). He was transferred to our emergency department approximately 1.5 h after ingestion. On admission to our hospital, his Glasgow Coma Scale was 3-1-5 (Japan Coma Scale 200); systolic blood pressure, 100 mmHg; heart rate, 96 beats/min; respiratory rate, 18/min; and pupils, 3 mm, and a convulsion occurred. The biological examination was unremarkable. Serum samples were collected for conducting a toxicological analysis. Thereafter, he suffered a severe shock and received critical care under intubation. He was mainly treated by fluid infusion. He recovered after 15 days and was transferred to another hospital.

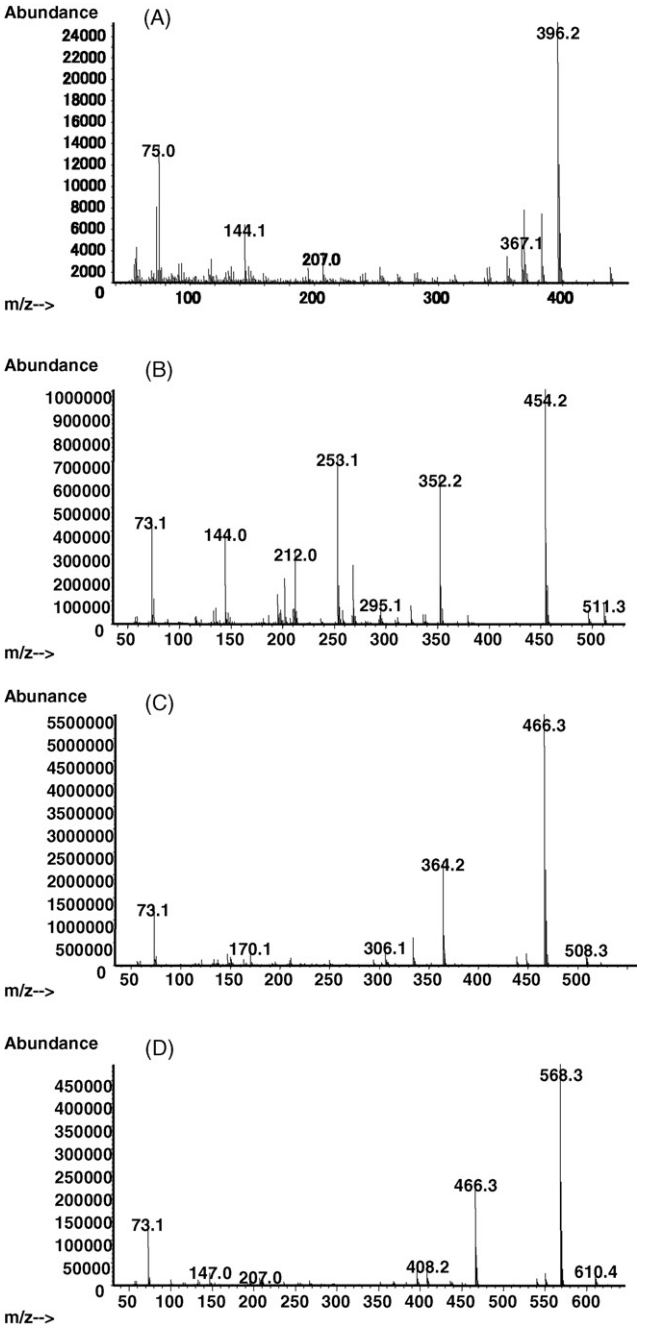

Fig. 2. Mass spectrum of (A) AMPA, (B) GLYP, (C) GLUF, and (D) IS.

5. Results and discussion

The previously reported sample derivatization methods are time-consuming [12–14], whereas this method is fast and suitable for GLYP and GLUF screening. The mass spectra of each compound are illustrated in Fig. 2. These mass spectra were the same as those reported elsewhere [13,14]. The typical chromatograms of the blank serum and the spiked one are shown in Fig. 3. AMPA, GLYP, GLUF, and IS were not detected in the blank serum. These chromatograms revealed that there was no interfering peak derived from the endogenous components at the elution times of the AMPA, GLYP, GLUF, and IS. The peaks corresponding to the derivatized AMPA, GLYP, GLUF, and IS were clearly observed at 11.07, 12.58, 13.00, and 13.34 min, respectively. Although this method could

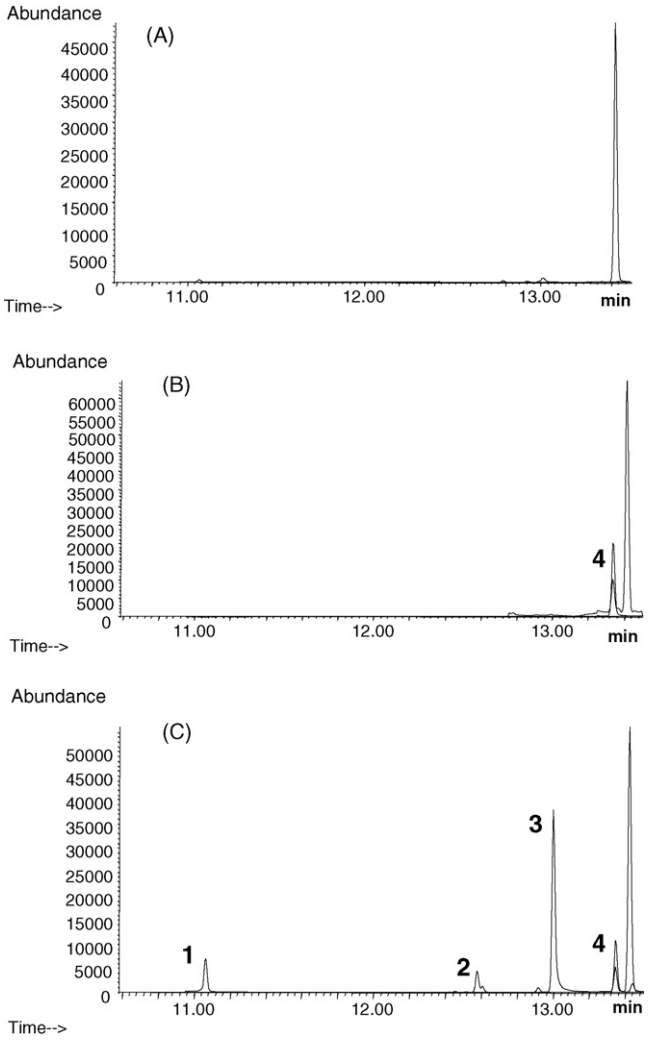

Fig. 3. GC–MS-SIM chromatograms obtained by Sep-Pak® Plus PS-2 column extraction of (A) blank human serum, (B) blank human serum with internal standard (IS), (C) blank human serum with low QC level (AMPA, 3 μg/ml; GLYP, 3 μg/ml; and GLUF, 3 μg/ml), and IS. 1: AMPA, 2: GLYP, 3: GLUF and 4: IS.

detect 3-MPPA, there existed an interfering peak derived from the endogenous components at the elution times of the 3-MPPA. Thus, 3-MPPA was not validated in this study.

The LOD of AMPA, GLYP, and GLUF was 0.25, 0.25, and 0.05 μg/ml, respectively. Based on the above criterion, the LOQ of AMPA, GLYP, and GLUF was determined to be 3 μg/ml when a 0.2 ml serum sample was used.

The validation data for the analytical method in terms of accuracy and precision are summarized in Table 1. Presented in Table 1 are data from the QC samples that were run in sextuple at each concentration on each of these 3 days.

The calibration curves of AMPA, GLYP, and GLUF were linear over the concentration ranges from 3 to 100 μg/ml. A regression linear analysis of the plot of the peak-area ratios (y) of the target to the IS versus the target concentrations was performed. The regression equations for AMPA, GLYP, and GLUF were $y = 0.013x - 0.0503$, $y = 0.0331x - 0.1263$, and $y = 0.0686x - 0.0113$, respectively. The average regression was always greater than 0.998. The experimental peak-area ratios were interpolated on the calibration curve, and the concentrations were back-calculated. The mean back-calculated concentrations approached the spiked concentrations by an RSD of <15%.

Table 1
Validation data of AMPA, GLYP, and GLUF from human serum.

	3 μg/ml (n = 6)	30 μg/ml (n = 6)	90 μg/ml (n = 6)
AMPA			
Intra-day accuracy (%)*	93.5 (7.0)	91.6 (6.9)	94.7 (5.8)
Inter-day accuracy (%)*,**	93.1 (6.9)	92.0 (6.5)	96.1 (5.8)
Precision RSD (%)	7.5	6.9	10.6
Intra-day RSD (%)[a]	8.4	7.2	7.7
Inter-day RSD (%)[b]	8.9	9.1	8.3
GLYP			
Intra-day accuracy (%)*	94.5 (8.5)	103.7 (8.1)	98.3 (4.5)
Inter-day accuracy (%)*,**	95.3 (8.8)	101.7 (7.6)	98.6 (4.5)
Precision RSD (%)	7.2	8.5	10.6
Intra-day RSD (%)[a]	9.4	9.1	11.5
Inter-day RSD (%)[b]	10.8	9.6	12.6
GLUF			
Intra-day accuracy (%)*	92.9 (6.7)	88.2 (5.7)	91.7 (5.3)
Inter-day accuracy (%)*,**	93.7 (7.6)	88.6 (4.5)	92.1 (5.8)
Precision RSD (%)	6.4	7.9	8.8
Intra-day RSD (%)[a]	6.2	7.6	7.2
Inter-day RSD (%)[b]	6.8	9.5	10.3

* Mean (S.D.).
** n = 18.
[a] Intra-day accuracy and precision results were obtained from sextuple samples for each concentration of the analyte analyzed on a single day.
[b] Inter-day accuracy and precision results were obtained by analyzing sextuple samples for each concentration of the analyte on three separate days.

The LOD for AMPA, GLYP, and GLUF as obtained by using our method was almost the same as that obtained using the previously reported method [13,14]. GLUF was analyzed in human serum, and it displayed a detection limit of 0.1 μg/ml [13,14]. These values appeared to be in the same order of magnitude as the values estimated by the methodology presented herein.

Further, Hori et al. [13,14] reported that the linearity range of GLYP and GLUF in the serum was 0.1–10 μg/ml. However, the GLUF concentrations in the serum a few hours after GLYP- and GLUF-poisoning were over several tens μg/ml at the least [4]. On the other hand, the linearity range for AMPA, GLYP, and GLUF obtained using our method was 10 times higher than that obtained using the previously reported method [13,14]. Probably, this difference in the linearity range was due to the derivatization efficiency of the used reagents. Thus, the present method is superior to the previously reported GC–MS method. In our method, while the sample concentrations of GLYP and GLUF were higher than the linearity range, the other deproteinized samples were extracted after dilution.

5.1. Assay precision and accuracy

%RSD of the intra-day assay (n = 6) for the serum with AMPA, GLYP, and GLUF was less than 11.5%, and good inter-day (n = 6) RSD values (less than 12.6%) were also obtained, as shown in Table 1.

5.2. Recovery

Table 2 presents the results of the recovery tests for the three QC levels. The overall recoveries in the serum samples ranged from 20.8% to 41.6% for AMPA, GLYP, and GLUF at the three QC levels. Although our extraction method after deproteinization used only 0.2 ml of the sample, given the overall poor recovery of AMPA, GLYP, and GLUF from the serum, we attempted to detect whether a greater loss was occurring during the extraction of the compound from the deproteinized samples or during the SPE process. Because the direct determination of recovery from serum was not feasible, we determined the SPE extraction efficiency. The mean SPE extraction efficiency for the three QC samples was revealed as 87.5–96.6%.

Table 2
Recovery study for GLYP, GLUF, and its metabolites in serum.

	QC concentration (μg/ml)		
	3	30	90
Mean (S.D.) overall recovery, % (n = 6)			
AMPA	20.8 (3.4)	22.1 (4.3)	22.3 (3.6)
GLYP	37.5 (2.7)	37.6 (3.4)	38.8 (3.5)
GLUF	38.8 (4.1)	39.3 (3.8)	41.6 (4.0)
Mean (S.D.) SPE* recovery, % (n = 6)			
AMPA	87.5 (3.6)	88.2 (3.8)	91.4 (4.1)
GLYP	93.5 (3.3)	94.2 (4.3)	95.3 (4.2)
GLUF	92.6 (4.1)	93.5 (5.1)	96.6 (4.5)

* Solid-phase extraction.

Thus, the greatest loss of GLUF and GLYP during sample processing occurred during deproteinizing. Similarly, IS was very poorly recovered from the serum sample; thus, the IS solution was added to deproteinized samples.

5.3. Stability

Table 3 presents the results for the short-term stability (24 h at room temperature and 4 °C for 1 week), 4 weeks stability at −30 °C, freeze–thaw stability (three cycles), and stability of the derivatized samples in the autosampler vials (24 h at 24 °C). The samples were considered stable if the deviation of the freshly prepared standard was less than 10%.

The three freeze–thaw cycles and 24-h room temperature storage for the three QC samples indicated that AMPA, GLYP, and GLUF are stable in human serum under these conditions. The serum QC samples are stable for at least 4 weeks if stored frozen at −30 °C, at 4 °C for 1 week, and subjected to three freeze–thaw cycles. The testing of the post-derivatized stability of the QC samples indicated that the three compounds are stable when stored at room temperature for up to 24 h.

5.4. Derivatization

The greatest advantage of the method described here is that the injection of the derivatized samples into a GC–MS was performed only by vortexing, without any heating. MTBSTFA with 1% TBDMCS reagent was directly derivatized to *tert*-butyldimethylsilyl (*t*-BDMS) compound at room temperature within a few minutes. The previously reported method took approximately 30 min at 80 °C for *t*-BDMS derivatization [12–14]. Therefore, the sample preparation time was remarkably decreased.

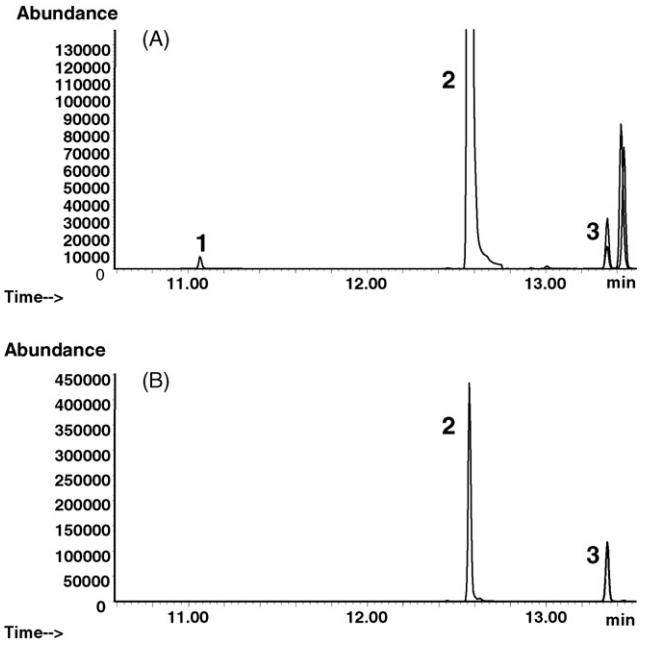

Fig. 4. GC–MS-SIM chromatograms obtained by Sep-Pak® Plus PS-2 column extraction of serum with IS from a GLYP-poisoned patient. (A) 0.2 ml serum sample extract. AMPA concentration was 15.1 μg/ml. (B) Serum sample was diluted 40 times before extraction. GLYP concentration was 6644.7 μg/ml. 1: AMPA, 2: GLYP, and 3: IS.

5.5. Application

The validated method was then applied to a sample analysis for patients with GLYP-poisoning. A chromatogram of a patient's serum sample is presented in Fig. 4, and the calculated concentrations of AMPA and GLYP were 15.1 and 6644.7 μg/ml, respectively.

In animal experiments, the majority of GLYP excretion occurred through the urinary system [19]. 99% of GLUF in human serum is not bound with protein [13]. Thus, GLUF and GLYP are excreted

Table 3
Stability of AMPA, GLYP, and GLUF in human serum and after derivatization.

	Nominal concentration (μg/ml)	Room temperature (24 h)	4 °C 1 week	Freeze–thaw (three cycles)	−30 °C 4 weeks	Room temperature (24 h) in vial
AMPA	3	2.8 ± 0.3	2.8 ± 0.4	3.0 ± 0.4	2.9 ± 0.1	2.9 ± 0.4
	%RSD	8.9	12.7	14.4	3.8	13.2
	30	27.5 ± 2.4	28.8 ± 1.3	28.8 ± 1.5	30.5 ± 2.7	28.2 ± 3.7
	%RSD	8.7	4.6	5.0	8.7	13.3
	90	85.2 ± 2.0	88.0 ± 2.0	88.4 ± 3.6	91.1 ± 3.9	88.8 ± 4.4
	%RSD	2.4	2.3	4.1	4.3	5.0
GLYP	3	2.0 ± 0.1	1.7 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	2.9 ± 0.4
	%RSD	6.7	10.7	10	10.2	13.8
	30	22.5 ± 2.9	20.1 ± 2.5	20.1 ± 1.6	21.2 ± 1.7	28.5 ± 3.9
	%RSD	12.9	12.3	8.1	8.0	13.7
	90	76.5 ± 5.5	78.7 ± 3.4	79.2 ± 2.5	75.6 ± 5.4	92.3 ± 4.2
	%RSD	7.2	4.3	3.1	7.1	4.6
GLUF	3	2.0 ± 0.2	2.7 ± 0.2	2.4 ± 0.2	2.7 ± 0.3	2.6 ± 0.3
	%RSD	7.9	7.4	9.8	10.5	12.4
	30	25.6 ± 0.8	25.0 ± 1.9	20.9 ± 2.3	26.1 ± 1.3	30.3 ± 2.4
	%RSD	2.9	7.6	10.8	5.1	7.9
	90	91.4 ± 3.1	80.0 ± 1.8	81.6 ± 3.4	84.8 ± 6.8	88.3 ± 6.6
	%RSD	3.3	2.2	4.1	8.1	7.5

n = 3 each.

largely via urine because of their high polarity. This methodology should be suitable to determine the GLUF and GLYP content in urine.

6. Conclusion

In conclusion, we have described a method for the determination of AMPA, GLYP, and GLUF in human serum. This method is specific, accurate, and precise and can be easily implemented into routine practice. As compared to the previously published GC–MS method, the application of MTBSTFA + 1% TBDMCS greatly reduces the sample preparation time, allowing for the reduction of the derivatization time from 30 min at 80 °C to a few minutes at room temperature; this makes it possible to apply this method to clinical samples in addition to the rapid toxicological screening of GLYP- and GLUF-poisoning.

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