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Rapid determination of glyphosate, glufosinate, bialaphos, and their major metabolites in serum by liquid chromatography–tandem mass spectrometry using hydrophilic interaction chromatography

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ABSTRACT

We developed a simple and rapid method for the simultaneous determination of phosphorus-containing amino acid herbicides (glyphosate, glufosinate, bialaphos) and their major metabolites, aminomethylphosphonic acid (AMPA) and 3-methylphosphinicopropionic acid (MPPA), in human serum. Serum samples were filtrated through an ultrafiltration membrane to remove proteins. The filtrate was then washed with chloroform, and injected into a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system. Chromatographic separation was achieved on a hydrophilic interaction chromatography (HILIC) column. Determination of the target herbicides and metabolites was successfully carried out without derivatization or solid phase extraction (SPE) cartridge clean-up. The recoveries of these compounds, added to human serum at 0.2 μg/mL, ranged from 94% to 108%, and the relative standard deviations (RSDs) were within 5.9%. The limits of detection (LODs) were 0.01 μg/mL for MPPA, 0.02 μg/mL for AMPA, 0.03 μg/mL for both glyphosate and glufosinate, and 0.07 μg/mL for bialaphos, respectively.

1. Introduction

Glyphosate [*N*-(phosphonomethyl)glycine], glufosinate [DL-homoalanine-4-yl (methyl) phosphinic acid], and bialaphos (L-2-amino-4-[(hydroxy) (methyl)-phosphinoyl] butyryl-L-alanyl-L-alanine) are phosphorus-containing amino acid–herbicides which are non-selective and carry a broad spectrum of use. These herbicides are widely used in many parts of the world and their underlying mechanism in which they take effect in plants is enzyme inhibition. Specifically, glyphosate affects the aromatic amino acid biosynthetic pathway by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), while glufosinate and bialaphos inhibit the enzyme, glutamine synthetase, which converts glutamate and ammonia to glutamine. This results in the accumulation of ammonia which ultimately inhibits photophosphorylation in photosynthesis [1,2].

These herbicides also give rise to a number of key metabolites, which assume a pivotal role during accidental or deliberate ingestion in humans. The major metabolites of glyphosate, glufosinate, and bialaphos are aminomethylphosphonic acid (AMPA), 3-methylphosphinicopropionic acid (MPPA), and L-glufosinate, respectively [1]. The chemical structures of these herbicides, along with their metabolites are provided in Fig. 1.

A large number of cases have accrued in which victims ingest these herbicides in an attempt at suicide [3–8]. Therefore, from the perspective of death investigation, a rapid and sensitive analytical method which is capable of identifying and confirming the presence of these herbicides is of monumental importance.

To date, a massive repertoire of analytical methods has been developed in order to detect these herbicides in body fluids. High-performance liquid chromatography (HPLC) with UV detection [9,10], HPLC with fluorescence detection [11,12], capillary electrophoresis (CE) [13], liquid chromatography/mass spectrometry (LC/MS) [14], and gas chromatography/mass spectrometry (GC/MS) [15] have all accumulated an excellent track record with regard to the successful determination of these herbicides. However, because these methods largely depend upon derivatization procedures using such reagents as *p*-toluenesulfonyl chloride [9,13], *p*-nitrobenzoyl chloride (PNBC) [10], 9-fluorenylmethyl Chloroformate (FMOC-Cl) [11], (+)-1-(9-fluorenyl)ethyl chloroformate [12], trimethyl orthoacetate [14], or *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTB-STFA) [15], the overall determination process is rendered as both tedious and time-consuming.

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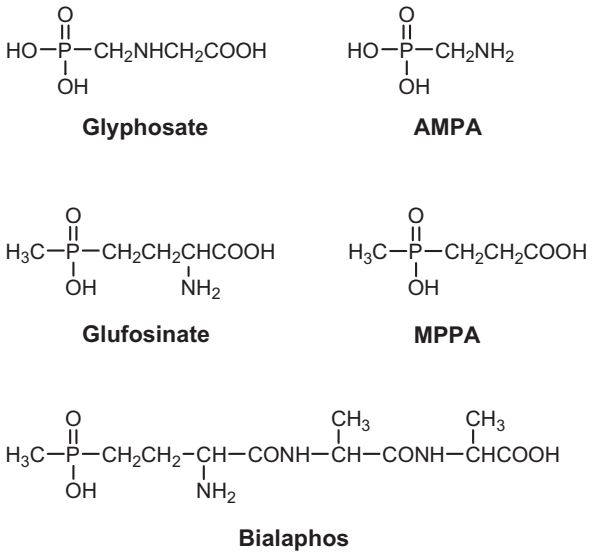

Fig. 1. Chemical structures of herbicides and their metabolites.

Meanwhile, alternative methods that don't resort to derivatization have been described, such as anion-exchange liquid chromatography with integrated pulsed amperometric detection (AEC-IPAD) [16] and ^{1}H NMR spectroscopy [17]. Unfortunately, these methods are not considered universal detectors and few laboratories are equipped with these costly instruments. While the LC–MS/MS method [18] has been reported to sufficiently detect glyphosate and glufosinate, this method requires ion-pair reagents, which suffers from poor robustness, requires a longer equilibration time, and has adverse impacts on column lifetime.

These phosphorus-containing amino acid–herbicides are amphoteric and polar. Therefore, without derivatization or ion-pair reagents, they result in poor peak shapes and insufficient peak separation in LC chromatograms under the use of conventional columns, such as reversed-phase (RP) or ion-exchange columns.

Recently, the analysis of glyphosate in agricultural products and water samples using hydrophilic interaction chromatography (HILIC) columns has been reported [19,20]. The HILIC column is suitable for hydrophilic and polar compounds which can not be retained by conventional RP chromatography; however, there have been no studies conducted on the analysis of phosphorus-containing amino acid–herbicides in body fluids without derivatization or ion-pair reagents.

The present study describes a rapid simultaneous analytical method for detecting glyphosate, glufosinate, bialaphos, and their major metabolites in human serum by LC–MS/MS using a HILIC column and without resorting to derivatization and solid phase extraction (SPE) cartridge clean-up.

2. Experiments

2.1. Chemicals and reagents

Standard glyphosate, glufosinate ammonium, bialaphos sodium salt, AMPA and MPPA were purchased from Wako Pure Chemical (Osaka, Japan). Chloroform, acetonitrile, and formic acid were of HPLC grade and also obtained from Wako Pure Chemical. Amicon Ultra (0.5 mL, 10 K MWCO) ultrafiltration cartridges were purchased from Millipore (Billerica, MA, USA). Water was purified using the Milli-Q water purification system (Millipore). All of the stock solutions (250 μg/mL) were prepared in water and stored in polypropylene (PP) bottles. Working solutions (50 μg/mL and 10 μg/mL) were prepared by mixing stock solutions with appropriate amounts of water to reach the target concentration.

2.2. Liquid chromatography

The analyses were uniformly performed using a Waters (Milford, MA, USA) Alliance LC–MS/MS system equipped with a 2695 separation module. Separations were conducted using an Obelisc N column (150 mm × 2.1 mm I.D., 5 μm, SIELC Technologies, Prospect Heights, IL, USA). The mobile phase was 0.1% formic acid–acetonitrile (80:20, v/v) at 0.2 ml/min and the injection volume was 2 μL. The column was kept at 40 °C in the column oven.

2.3. Mass spectrometry

Mass spectrometry was performed on a Waters Quattro micro API mass spectrometer using the multiple reaction monitoring (MRM) and negative electrospray ionization modes. Capillary voltage, extractor voltage, and multiplier voltage were set at 3.0 kV, 3.0 V, and 650 V, respectively. The source and desolvation temperatures were held at 125 °C and 350 °C, respectively. The desolvation gas and cone gas were set at flow rates of 600 L/h and 50 L/h, respectively. Optimization of the cone voltage (CV) and collision energy (CE) for each compound was achieved by infusion. Two precursor/product ion transitions were monitored for each target. The target ion transition with the highest intensity was used for quantitation, while the second target ion transition was used for confirmation. To confirm the presence of herbicides and metabolites in serum samples, the ratios of target ion peak area to second target ion were compared to a standard solution. The confirmation criteria of relative abundances and relative retention times for positive samples are set to 80–120% and 95–105% of the standards, respectively. Optimized parameters are summarized in Table 1.

2.4. Sample preparation

A serum sample was prepared from whole human blood by centrifuging for 10 min at 1200 × *g* (himac Centrifuge CF7D2, Hitachi Koki, Tokyo, Japan). A 500-μL aliquot of the serum sample was transferred to an ultrafiltration cartridge. After centrifugation for 15 min at 8000 × *g* (HIMAC Centrifuge SCT15B, Hitachi Koki), 300 μL of the filtrate was transferred to a microcentrifuge tube. Next, 200 μL of chloroform was added to the tube followed by vortex for 30 s. After centrifugation for 3 min at 8000 × *g*, 2 μL of the upper aqueous phase was injected into the LC–MS/MS system.

2.5. Method validation

A herbicide-free human serum pool was prepared by combining human sera from healthy volunteers in our laboratory. To make validation samples at fortification levels of 0.2 μg/mL and 5 μg/mL to serum, 10 μL of 10 μg/mL and 50 μL of 50 μg/mL working solutions were added to the 490 μL and 450 μL of herbicide-free serum samples, respectively. Following this step, samples were adjusted to volumes of 500 μL with serum using a volumetric flask. These samples were equilibrated for 1 h prior to extraction.

Intra- and inter-day reproducibility of the method was assessed by performing a series of replicate analyses. Intra-day repeatability was verified by analyzing six fortified samples in a single day. Inter-day repeatability was confirmed by analyzing samples on six separate days.

Table 1
Optimized MS/MS parameters of herbicides and their metabolites.

Compound	Molecular weight	Cone voltage (V)	Quantitation		Confirmation	
			Collision energy (eV)	MRM transition	Collision energy (eV)	MRM transition
Glyphosate	169	20	10	168 > 150	20	168 > 63
Glufosinate	181	25	20	180 > 85	15	180 > 95
Bialaphos	323	30	20	322 > 172	25	322 > 88
AMPA	111	30	10	110 > 81	15	110 > 79
MPPA	152	25	10	151 > 133	15	151 > 107

2.6. Case history

2.6.1. Case 1

A 50-year-old male was found dead in his parents' residence. On the table next to the deceased was a 500 mL bottle of Roundup®, which retained approximately 250 mL of the herbicide contents. Although there was no apparent note suggesting suicide, the victim suffered from chronic physical illness and was suspected to have committed suicide. The autopsy findings revealed bilateral congestive pulmonary edema. The gastric and esophageal mucosae were degenerated and compounded with a grayish discoloration. In the stomach was approximately 500 mL of grayish-green fluid, which emanated a chemical-odor suggesting pesticide ingestion.

2.6.2. Case 2

A 63-year-old female was found dead lying on the floor of her home with a plastic bag over her head. Because the bag was not sealed at the neck, suffocation was ruled out as the cause of death. As in case 1, there was a bottle of herbicide next to her, and she was suspected to have committed suicide by ingesting the herbicide which is assumed to be Roundup®. Autopsy finding were unremarkable with the exception of a mild congestion noted in the left lung. Post-mortem drug screenings conducted on urine using Triage DOA plus (Sysmex, Kobe, Japan) were negative.

3. Results and discussion

3.1. Optimization of LC conditions

Glyphosate, glufosinate, bialaphos, AMPA, and MPPA are extremely polar and amphoteric compounds. To achieve adequate separation and retention without ion-pair reagents, chromatographic separation was performed using a HILIC column. There are several types of HILIC columns commercially available. For example, bare silica, along with silica modified with amino, amide, or zwitterionic functional groups are used in the stationary phases. We evaluated four types of HILIC columns: Atlantis HILIC Silica (Waters), TSKgel Amide-80 (TOSOH, Tokyo, Japan), Unisol Amide (Agela technologies, Newark, DE, USA), and Obelisc N. In the mobile phase, 0.1% formic acid–acetonitrile and 10 mM ammonium formate/0.1% formic acid–acetonitrile were used for evaluation.

In evaluations using the Atlantis HILIC Silica column, in addition to broad peak shapes and peak tailing for glyphosate, poor peak separation was also documented. AMPA was difficult to retain on the Unisol Amide column, and the peak shape of glyphosate was broad. The TSKgel Amide-80 column also showed broad peak shapes for glyphosate and AMPA, and the sensitivity of these compounds were low. In contrast, the Obelisc N column resulted in sufficient peak separation and acceptable peak shapes for all analytes.

Ammonium formate is often added during the mobile phase to promote ammonium adduct formation and improve sensitivity. However, in this study the introduction of ammonium formate as the mobile phase adversely affected peak separation efficiency and peak shape, and furthermore caused a decrease in the sensitivity of the analytes. For these reasons, 0.1% formic acid–acetonitrile was used as the mobile phase.

Sufficient peak separation of these herbicides was achieved by isocratic elution without using a gradient elution. In order to shorten the overall time it takes to perform the analysis (including re-equilibration), isocratic elution was adopted in this proposed method.

3.2. Optimization of MS conditions

Optimization of ionizing parameters was carried out by infusion of standard solution for five compounds. Li et al. [20] analyzed glufosinate in positive ion mode, and glyphosate in negative ion mode in agricultural products and water samples with LC–MS/MS. Iwamuro et al. [21] reported that peak intensities of glyphosate, bialaphos, AMPA, and MPPA in positive ion mode were slightly higher than those in negative ion mode in analyzing soil and beverage samples with CE/MS. Our results show that the intensities of the five compounds in negative ion mode were slightly higher than those in positive ion mode with a mobile phase of 0.1% formic acid–acetonitrile (80:20). Optimization of CV and CE was carried out by changing the voltage from 10 V to 40 V, and 10 eV to 40 eV, respectively.

3.3. Sample pretreatment

Stalikas and Konidari [22] reported that phosphorus-containing amino acid–herbicides tend to be adsorbed on glass surfaces. To avoid unintentional adsorption, polypropylene (PP) bottles were used for storing the standard solutions.

In order to extract herbicides from serum samples, a deproteinization process is imperative. A number of chemical compounds

Table 2
Recoveries of the herbicides and their metabolites from human serum.

Compound	Intra-day recovery ($n = 6$)				Inter day recovery ($n = 6$)			
	Fortification level of 0.2 μg/mL		Fortification level of 5 μg/mL		Fortification level of 0.2 μg/mL		Fortification level of 5 μg/mL	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
Glyphosate	97.0	5.8	97.7	4.4	97.2	5.9	98.0	5.6
Glufosinate	103.2	5.0	102.8	4.6	98.0	5.1	99.9	3.7
Bialaphos	94.5	3.6	97.3	2.9	95.9	5.4	95.9	5.1
AMPA	101.2	3.1	102.6	4.2	96.1	3.3	103.9	4.5
MPPA	108.2	3.2	106.3	2.5	103.8	4.4	104.9	4.4

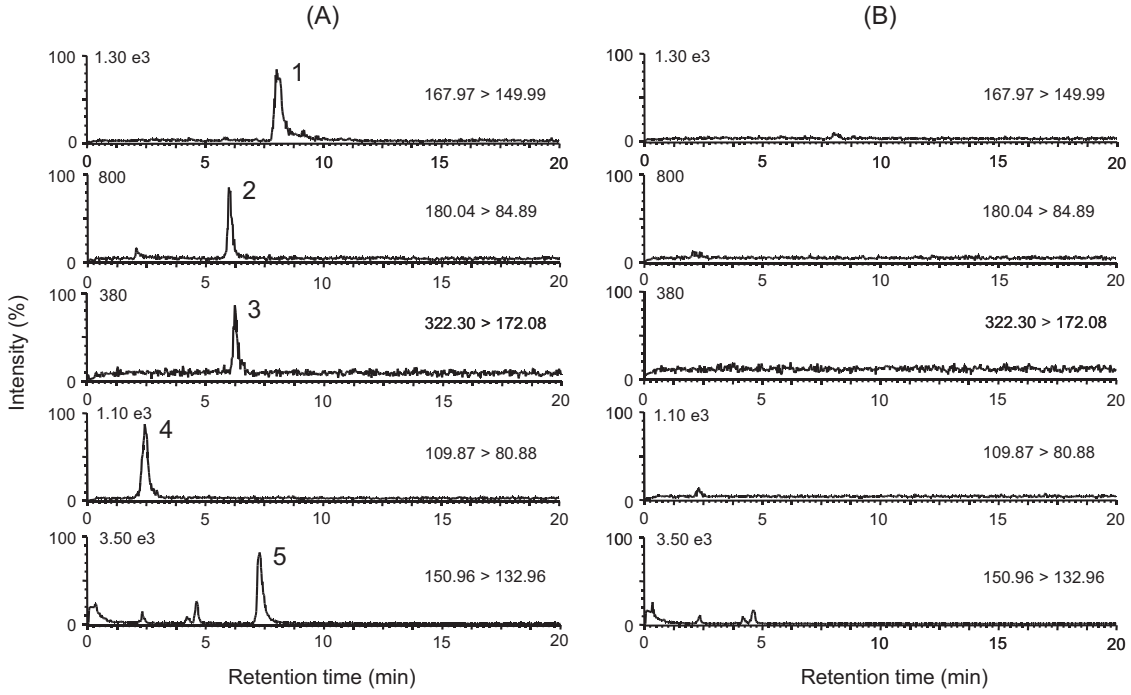

Fig. 2. Typical MRM chromatograms obtained from (A) human serum fortified with herbicides and their metabolites at a concentration of 0.2 μg/mL, and (B) blank serum. (1) Glyphosate; (2) glufosinate; (3) bialaphos; (4) AMPA and (5) MPPA.

are known to bind to proteins such as human serum albumin. Although deproteinization using an ultrafiltration membrane is an effective extraction method, releasing the protein for some compounds proves to be a difficult task.

Hori et al. [23] have previously reported that the mean ratio of free glufosinate to human serum is 0.99. To further investigate protein binding, concentrations of glyphosate were compared via two methods. A serum sample taken from actual casework and containing glyphosate was divided into two portions. One sample was deproteinized with the same volume of acetonitrile, and diluted with water. Meanwhile, the other sample was deproteinized only with the ultrafiltration membrane and diluted with water. Using this (acetonitrile deproteinization) method, total glyphosate – including its protein-bound form – was determined. It should be noted that only free glyphosate was determined under this ultrafiltration method.

The results obtained demonstrated that there was no significant difference (2.0%) between the concentrations in these two samples.

Moreover, in recovery studies, fortified serum samples were equilibrated for 1 h after spiking the standard solution prior to initiating the extraction procedure. Recoveries from the serum samples were more than 90% and it was shown that the effects of protein binding with these compounds were practically negligible. In order to increase the sensitivity, the deproteinization step using an organic solvent was skipped, and only ultrafiltration was adopted for extraction.

It should be noted that some interference remained in serum samples after ultrafiltration. To remove any traces of hydrophobic interference (i.e. lipids or fatty acids), we conducted a series of washing experiments using organic solvents and evaluated the washing efficiency of each organic solvent used. After ultrafiltration, each fortified serum sample was washed with chloroform, hexane, diethyl ether, and ethyl acetate. No significant differences were noted in the recoveries among the four solvents evaluated. Conversely, the densities of all of the solvents – with exception to chloroform – are lesser than that of water, and only chloroform has a greater density. Therefore, chloroform was adopted as a washing solvent, so as to position the serum samples along the upper layer of the mixture, thereby allowing for improved pipetting efficiency.

In LC/MS analysis – especially under electrospray ionization (ESI) mode – matrix effects resulting in ion suppression or ion enhancement with the coelution of the target analyte along with interference are often observed. To reduce these matrix effects, matrix-matched calibration curves are often used [15]; however, preparation of matrix-matched standards is time-consuming.

For the evaluation of the matrix effect under this method, recovery studies were performed using both solvent standards and matrix-matched standards. Serum samples from five volunteers with fortification levels of 1 μg/mL were used.

The recoveries of each compound ranged from 95% to 106% using solvent standards, and 91% to 109% using matrix-matched standards.

In the method proposed in this study, determination of herbicides using solvent calibration curves was possible without resorting to matrix-matched calibration. Additionally, the recovery study demonstrated that the absolute calibration method was sufficient without the addition of an internal standard (IS).

3.4. Validation of the method

Calibration curves were generated by injecting the five herbicides and metabolites in a standard mixture with concentrations ranging from 0.01 to 20 μg/mL. For glyphosate, AMPA, and MPPA, the calibration curves were linear in the range of 0.01–20 μg/mL with coefficients of determination (R^2) between 0.9994 and 0.9998. Similarly, for glufosinate and bialaphos, the calibration curves were also linear in the range of 0.02–20 μg/mL with R^2 between 0.9995 and 0.9996.

Table 2 shows the intra-day and inter-day recoveries from human serum. The percentage of recoveries was determined in sextuplicate at two concentration levels (0.2 μg/mL and 5 μg/mL) by comparing the peak area with the standard solution.

The intra-day recoveries ($n = 6$) ranged from 94.5% to 108.2%, with relative standard deviations (RSDs) ranging from 2.5% to 5.8%,

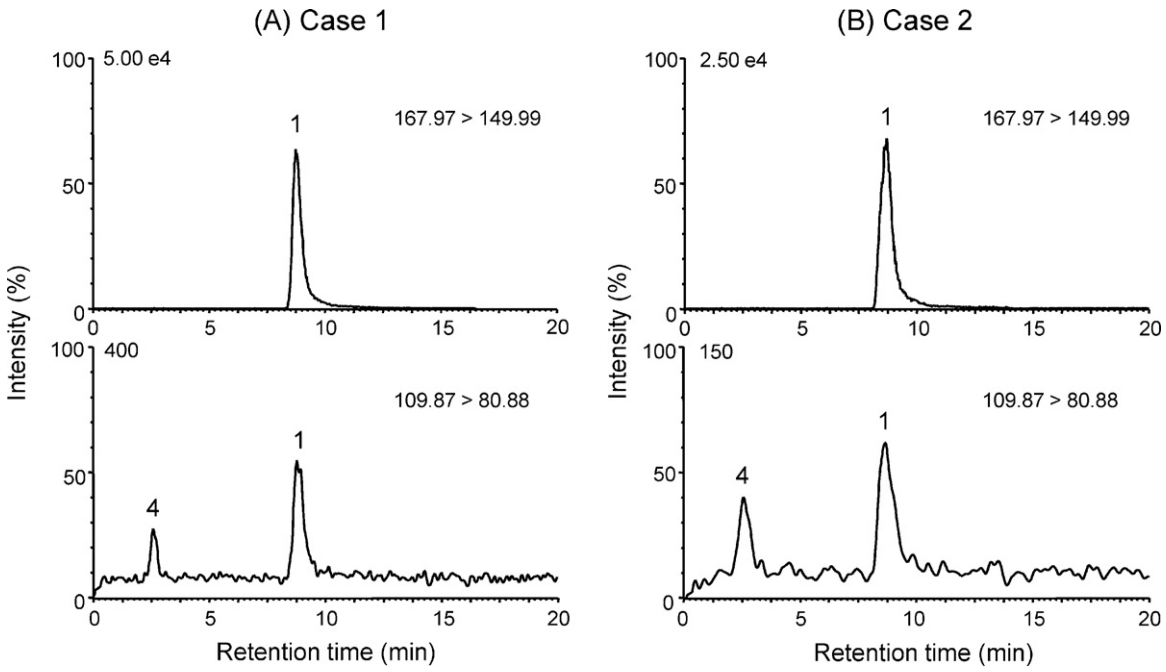

Fig. 3. MRM chromatograms obtained from human serum in herbicide poisoning cases. Chromatogram (A) is obtained from a sample diluted 250-fold, and (B) is obtained from a sample diluted 20-fold. (1) Glyphosate and (4) AMPA.

while the inter-day recoveries ($n = 6$) ranged from 95.9% to 104.9%, with RSDs ranging from 3.3% to 5.9%. Under the intra-day and inter-day recovery studies, the RSDs of the retention times ranged from 0.37% to 0.92% and 0.81% to 2.0%.

Satisfactory recoveries were obtained for all analytes, and both intra-day and inter-day accuracy and precision data showed promising repeatability.

Typical MRM chromatograms of human serum samples are shown in Fig. 2. These samples were fortified with five herbicides and their metabolites at a concentration of 0.2 μg/g. The limits of detection (LODs) and the limits of quantitation (LOQs) were defined as height values three or ten times the signal-to-noise ratio, respectively. The LODs were 0.01 μg/mL for MPPA, 0.02 μg/mL for AMPA, 0.03 μg/mL for glyphosate and glufosinate, 0.07 μg/mL for bialaphos, respectively. Additionally, the LOQs were 0.04 μg/mL for MPPA, 0.07 μg/mL for AMPA, 0.09 μg/mL for glyphosate, 0.10 μg/mL for glufosinate, and 0.22 μg/mL for bialaphos, respectively.

In the previously published method, the LODs in serum were 0.05, 0.03, 0.1, 0.2, and 0.05 μg/mL for glyphosate, glufosinate, bialaphos, AMPA, and MPPA, respectively, with a range of linearity of 0.2–5 μg/mL under the LC/MS derivatization method [14], and 0.25, 0.05, and 0.25 μg/mL for glyphosate, glufosinate, and AMPA, respectively with recoveries of 88–104% under the GC/MS derivatization method [15]. In comparison to these previously published methods, our method demonstrates sufficiently high recoveries and low LODs.

3.5. Application to forensic samples

In order to demonstrate its practicality, the method proposed in this study was applied to serum samples obtained from actual forensic casework. Serum samples were stored at −80 °C prior to analysis. For serum samples with herbicide concentrations beyond the highest point of the calibration curve, sample solutions were diluted with water to appropriate concentrations. Representative MRM chromatograms obtained from the victims' serum are provided in Fig. 3.

In case 1, glyphosate and AMPA were detected in the serum of the victim, with a concentration of 1477 and 1.5 μg/mL, respectively. In case 2, the concentrations of glyphosate and AMPA were 89 and 0.07 μg/mL, respectively. Glufosinate, bialaphos, and MPPA were not detected in either sample.

4. Conclusions

We developed a simple and rapid analytical method for the simultaneous determination of phosphorus-containing amino acid-herbicides, glyphosate, glufosinate, bialaphos, AMPA, and MPPA in human serum. After rigorous testing, our proposed method produced reliable and reproducible results with satisfactory detection limits.

These results were further augmented by a reduced burden of time consumption. As with other equivalent analytical techniques, the method proposed in this study requires a simple, standard preparation of serum samples. However, in comparison with other techniques, the herbicides and metabolites examined in the present study could be accurately determined within 30 min after the initial preparation of serum samples.

Our study not only supports this novel analytical method for proposed use in the forensic arena, but we also project that its application to various clinical spectrums can also culminate monumental benefits. One such medical field in particular where the method's uses could be exploited to their fullest extent would be emergency medicine. In situations where time is of the utmost essence, such as in apparent or suggested poisonings, a rapid method for detecting multiple herbicides would allow for rapid treatment intervention resulting in improved mortality rates.

While further research is required to validate the practicality of this method in actual clinical settings, the results documented in this study strongly suggest that its introduction into the existing arsenal of analytical techniques available to clinicians would render profound benefits.

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