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Simultaneous detection and quantitation of highly water-soluble herbicides in serum using ion-pair liquid chromatography–tandem mass spectrometry

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ABSTRACT

We report the simultaneous screening of highly polar, water-soluble, and less-volatile herbicides, including glyphosate, glufosinate, paraquat, and diquat, in serum using liquid chromatography–mass spectrometry. The herbicides were separated by solid-phase extraction using a Strata-XC cartridge. A heptafluorobutyric acid solution was chosen as the mobile phase for ion-pair liquid chromatography. Mass spectrometry was used for analysis and was optimized for operation in the positive mode for all analytes. The serum specimens were screened for the presence of the herbicides at the following concentrations: 5 ng/mL for glyphosate, 2 ng/mL for glufosinate, 1 ng/mL for diquat, and 5 ng/mL for paraquat. This is the first report on the simultaneous detection of these compounds.

1. Introduction

Glyphosate (GLYP), glufosinate (GLUF), paraquat (PQ), and diquat (DQ) (Fig. 1) are widely used non-selective herbicides that kill a broad range of weeds in Taiwan; these herbicides account for 99% of the local herbicide market. In comparison with other pesticides, these compounds are readily available, relatively cheap, and often used illegally. There are several instances of food and drink being laced with these herbicides in murder and extortion cases. All these herbicides are highly and acutely toxic. Suicide victims who use these chemicals always experience great pain, and these compounds are also known to cause severe and acute long-term health problems. GLYP is less toxic than PQ but is still fatal in many suicide cases. In Taiwan, thousands of poisoning cases involving these herbicides are reported each year. The victims are generally unconscious when they arrive at the hospital. In such cases, it is very important to quickly detect the herbicide that has been ingested. It is more difficult to detect herbicides in clinical and forensic science specimens than in environmental samples. Every specimen may contain different types of herbicides. Although biological specimens are the major sources, food and water samples may also have to be investigated occasionally. The serum samples are the predominant matrix in clinical and forensics cases which would have the minimum sample size and should be clean up very carefully. Simultaneous detection of these herbicides can help in the provision of appropriate medical treatment and may also provide evidence for use in court at a later stage.

It is difficult to simultaneously detect herbicides that have low molecular weight (MW), low volatility, thermal lability, different ion types in the gaseous phase, and good solubility in water because these properties lead to problems in extraction. The traditional method for GLYP and GLUF detection involves increase in the molar absorptivity by conversion into chemical chromophore-containing derivatives or increase in the volatility of these compounds and subsequent detection by methods based on fluorescence [1–7] and mass spectrometry (MS) [8–10]. Well-known procedures include pre-column derivatization [1–5] using 9-fluorenyl methyl chloroformate (FMOC) and post-column derivatization using o-phthalaldehyde (OPA) followed by mercaptoethanol [6] or N,N-dimethyl-2-mercaptoethylamine [7]. The total phosphorus concentration of GLYP and GLUF is accomplished by reverse-polarity liquid chromatography-inductively coupled plasma/MS (RPLC-ICP/MS) [11] using tetrabutylammonium hydroxide (TBAOH) as the ion-pair reagent. Thus, GLYP and GLUF can be simultaneously detected by derivatization or using a basic ion-pair reagent. The quaternary ammonium compounds PQ and DQ are detected by ultraviolet (UV) analysis [12], gas chromatography (GC)/MS analysis [13], liquid chromatography (LC)/UV detection [14], and direct LC–MS analysis [15,16] using heptafluorobutyric acid (HFBA) as the ion-pair reagent for separation. However, the simultaneous detec-

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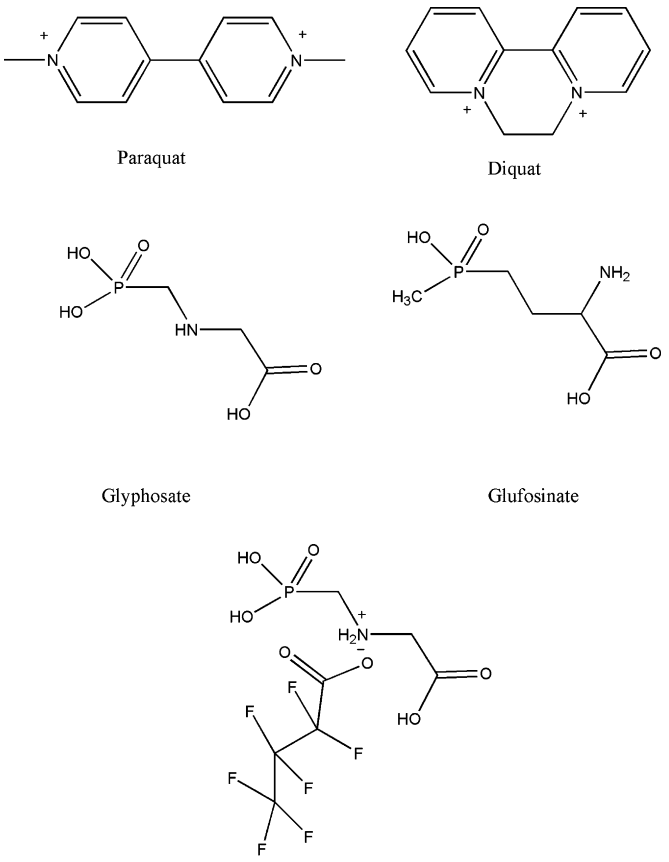

Fig. 1. Chemical structure of herbicides.

tion of GLYP, GLUF, PQ, and DQ has not been accomplished in previous studies.

Three challenges exist for the simultaneous detection of these two different kinds of herbicides. First, their high solubility in water causes extraction problems in the case of biological specimens. Second, these four compounds exist in different ionic forms: PQ and DQ bear two positive charges, while GLYP and GLUF are zwitterions. Finally, the charges of these ions change during their transition from liquid to gas phase. Therefore, there is a need for a method that allows the detection of these compounds with high sensitivity and selectivity.

There is no doubt that LC–tandem mass spectrometry (MS/MS) has great advantages in terms of selectivity and sensitivity, and these advantages can be effectively harnessed for the simultaneous detection of different kinds of compounds. The LC–MS/MS system was the main instrument used in this study. The charges of the molecules in the liquid phase depend on the pH value of the liquid. The ion source in MS/MS also helps in continuous generation of unique molecular ions. HFBA and TBAOH have been used as the ion-pair reagents for the separation of PQ from DQ and for differentiating GLUF from GLYP, respectively. However, we predicted that positive ions of these herbicide molecules can be continually generated in a mobile phase that had a lower pH value.

A commercial solid-phase extraction (SPE) cartridge can easily be used as a tool for specimen clean-up. Such a cartridge is convenient for automatic operation with an SPE workstation. Excellent recovery results have been reported when a cation exchange column was used as the SPE cartridge for PQ and DQ separation. The amphoteric character of GLYP and GLUF necessitates extensive specimen pretreatment, and liquid–liquid extraction and RP-SPE prior to derivatization are usually chosen for this purpose. HFBA was evaluated as the ion-pair reagent for the chromatography of these four compounds. SPE was carried out using a cation exchange column. Glyphosate-(1,2-^{13}C,^{15}N) and ethyl viologen (EV) were chosen as the internal standards for quantitation. The extraction procedure was optimized for analytes that bear positive charges in an acidic solution. The ion-pair formed between the analytes and HFBA could also be retained by the cation exchange column. The method for simultaneous detection was found to be the fastest and most flexible for different types of compounds, and it was sensitive enough to allow the detection and quantification of herbicides in clinical and forensic toxicology samples.

2. Experimental

2.1. Reagents and chemicals

Herbicide standards of paraquat dichloride, diquat dichloride, glyphosate, and glufosinate ammonium were purchased from AccuStandard (New Haven, CT, USA) and Riedel-de-Haën (Seelze, Germany). The internal standard ethyl viologen dibromate was obtained from Aldrich (Seelze, Germany), and glyphosate-(1,2-^{13}C,^{15}N) was from Isotec (Seelze, Germany). HPLC-grade acetonitrile (ACN) and methanol were purchased from JT Baker (Phillipsburg, NJ, USA). Ultra-pure water with a resistivity exceeding 18 MΩ was obtained by the purification of demineralized water in a Millipore Mini-Q System (Bedford, MA, USA). Reagent-grade HFBA, TBAOH, formic acid, ammonium formate, and ammonium hydroxide were purchased from Sigma (St. Louis, MO, USA).

A Strata-XC cation mixed-mode polymeric sorbent SPE cartridge (30 mg/1 mL) was purchased from Phenomenex (Torrance, CA, USA). Herbicide-free serum samples were obtained from the blood samples of clinical cases that had been prescreened by the same LC–MS method. These serum samples were collected in standardized non-heparinized rubber-stoppered vacuum tubes. After coagulation and centrifugation, the serum was collected.

2.2. Instrumentation and columns

The LC–MS system consisted of a PerkinElmer PE-200 (Woodbridge, Canada) separation module interfaced with an API 2000 triple quadrupole mass spectrometer (Applied Biosystems AB/MDS Sciex, Foster City, CA, USA). The LC column was a Luna Polar RP (150 mm × 2.1 mm ID) column purchased from Phenomenex. A Mettler-Toledo AX205 analytical balance (Columbus, OH, USA) was used for measuring the weights of the herbicides in their solid forms to an accuracy of 0.1 mg. The serum samples were pretreated in a Zymark SPE workstation and fast dried in a Turbovap (Hopkinton, MA, USA).

2.3. Stock solutions and standard working solutions

Herbicide stock solutions (100 μg/mL) were prepared by individually weighing and dissolving the following amounts of powder in 100 mL of water: 18.0 mg of paraquat dichloride tetrahydrate, 10.2 mg of glyphosate and its isotope, 20.3 mg of diquat dibromide monohydrate, and 11.1 mg of glufosinate ammonium salt. Standard working solutions for the LC–MS/MS analysis and fortification of samples were prepared by diluting the 100 μg/mL composite standards with demineralized water. All standard working solutions were stored in nonsilanized glass. A 10 μg/mL standard working solution was prepared by dissolving 1.0 mL of the stock solution in 10 mL of water. A 1.0 μg/mL standard working solution was prepared by diluting the 10 μg/mL standard working solution with

demineralized water. Thus, standard working solutions of two different concentrations, i.e., 1.0 and 10 μg/mL, were obtained. The concentration of the internal standards was 1.0 μg/mL. Calibration standards were prepared from the 10 μg/mL solution of each herbicide.

2.4. Sample pretreatment

2.4.1. Internal standards

Herbicide-free serum (0.5 mL) was added to a test tube, and internal standards of both glyphosate-(1,2-^{13}C,^{15}N) and EV at a concentration of 1.0 μg/mL and in a volume of 50 μL were added to obtain representative 1 mL serum samples containing 100 ng of both glyphosate-(1,2-^{13}C,^{15}N) and EV. Each test tube was supplemented with 250 μL of HFBA (20 mM, pH 2) and vortexed. The samples were made up with water to 1.0 mL for SPE.

2.4.2. SPE

A Strata-XC cation mixed-mode polymeric sorbent SPE cartridge was inserted into a Zymark SPE workstation. Two hundred microliters of methanol was added to the cartridge. The prepared sample was loaded onto the column. The column was washed with 500 μL of 20 mM HFBA and 500 μL water. The column was purged and dried with nitrogen for approximately 1 min. Eight hundred microliters of the eluent (containing 80 mL ethyl acetate, 20 mL methanol, and 1 mL ammonium hydroxide) was added to the column in the cartridge. The collected eluate was evaporated to dryness under nitrogen at 35 °C in a Turbovap. It was reconstituted with 500 μL HFBA (20 mM) solution and vortexed.

2.5. LC–MS adjustment

2.5.1. Optimization of the mobile phases

Three different kinds of mobile phases were prepared for determining the mobile phase with the best sensitivity to each herbicide ion: phase I (10 mM HFBA/ACN), phase II (15 mM HFBA/15 mM ammonium formate/ACN at pH 4.0), and phase III (20 mM ammonium acetate/TBAOH solution/ACN at pH 4.7) solutions.

Each standard was prepared in each mobile phase solution and was infused at a concentration of 100 ng/mL into the mass spectrometer by syringe pump and with the flow rate of 10 μL/min; subsequently, each compound's pseudomolecular ion was detected. The product ion scan (PIS) of the pseudomolecular ion used collision-induced dissociation (CID) to obtain a parent–daughter ion-pair. A prominent parent–daughter ion-pair was chosen for quantitation, while the others were used for qualification. All parameters, including the declustering potential (DP), focus potential, curtain gas, collision gas, temperature, etc., were adjusted to ensure that these ions were mass generated and reached the detector smoothly.

2.5.2. LC

All parameters of the LC–MS/MS system were set, and the samples were cleaned up. Five microliters of the blank, standard, and control samples was injected into a Synergy 150 mm × 2.1 mm Polar-RP column for separation in a PE Series 200 autosampler. The mobile phase was pumped at a flow rate of 0.2 mL/min with a gradient of 20 mM HFBA and ACN from 95:5 to 65:35 in 10 min using a PE series 200 micropump. Peek red tubing (1/16 in. × 0.13 mm ID) was used to transfer the liquid. The column was maintained at 40 °C in a Thermosphere TS-130 Phenomenex column oven.

Three solutions (phases I–III) were used to determine the best mobile phase for the simultaneous detection of the four herbicides. HFBA solutions (5–25 mM) were also prepared for optimizing the mobile phase. The mobile phase I (HFBA/ACN) was evaluated as eluent system. The herbides were carried out with the concentration of 150 ng/mL prepared by diluting ampule standards and eluent system. The average peak area (n = 3) of each quantitation ion was used to optimize the mobile phase.

2.5.3. Mass optimization

TurboIonSpray (TIS) was chosen as the ion source at an operating temperature of 450 °C and in the positive mode for all acquisitions. Conditions for mass spectrometric detection in the TIS mode were as follows: curtain gas pressure, 35 psi; electrode voltage, 5500 V; gas 1 pressure, 50 psi; gas 2 pressure, 65 psi; DP, 40 V for PQ and DQ; DP, 60 V for GLYP and GLUF; focusing potential, 370 V; entrance potential, −10 V; collision cell entrance potential, 25 V; CID using nitrogen with a collision gas pressure of 0.25 mTorr; collision energy (CE), 25 V for PQ and DQ; CE, 35 V for GLYP and GLUF; collision cell exit potential (CXP), 14 V for PQ and DQ; CXP, 10 V for GLYP and GLUF; CEM, 2050 V; and dwell time for each transition, 100 ms.

2.5.4. Multiple reaction monitoring (MRM) technique

The MRM technique was chosen for quantification and confirmation in this study. Two pairs of MRM ions of each analyte, two prominent MRM ions, one precusor and two daughter ions were selected to obtain 4 IP (identical point) in the LC–MS/MS analysis [17]. This MRM technique can bring the conformity to the minimum 3 IP accumulation level. For MRM of LC–MS, the most prominent MRM ion-pair was chosen for quantification, while the other was used for confirmation. In this study, the MRM ion ratio of the calibration standards, i.e., the ratio of the two MRM ions, had ion ratio tolerances ±25% relative to that of the calibrator or a quality control (QC) sample of similar concentration.

2.6. Calibration standards and validation

The calibration curves of the four herbicides were prepared by standard working solutions and serum at concentrations of 0.2, 0.5, 0.8, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100, 150, and 200 ng/mL. The concentration of the two internal standards in serum was 100 ng/mL, and these samples were used for evaluating the linearity, limit of quantitation (LOQ), and limit of detection (LOD). QC was carried out with the 20 and 150 ng/mL samples that were prepared by diluting different sources, ampule standards, and free serum for the evaluation of accuracy, ion suppression, and stability. For all analytes, a linear weighted least-squares model was used to prepare the calibration curves. The LOD was defined as the lowest concentration at which the MRM ion ratio was ±25% relative to that of the low concentration QC sample (20 ng/mL). The LOQ in the MRM mode for MS/MS was matched by both the MRM ion ratio tolerance mentioned above and the lowest point of the calibration curve that was calculated from the least-squares equation, and it had a bias of ±20% of the nominal values.

2.7. Matrix effects

Two solvent calibrations and two matrix calibrations samples were prepared at the same low (20 ng/mL) and high (150 ng/mL) concentrations for evaluating the matrix effects. Both solvent and matrix calibrations were performed by the same SPE procedure. The matrix effect was estimated by comparing the peak areas from the samples of matrix calibrations to those from the corresponding samples of solvent calibrations, and these were also reported as percentage values.

2.8. Stability test

Three QC samples of low (20 ng/mL) and high (150 ng/mL) concentrations were used to evaluate the freeze/thaw stability which were analyzed using both apparatus prior to (control samples, $n = 3$ each) and after three freeze/thaw cycles (stability samples, $n = 3$ each). For each freeze/thaw cycle, the stability samples were thawed and kept at room temperature for 4 h and frozen at −20 °C for 24 h. The control values were calculated from the daily calibration curves. The stability was tested against an acceptance interval of 90–110% for the ratio of the means (stability samples *vs.* control samples) and against an acceptance interval of 80–120% from the control samples' mean in order to obtain the 90% confidence interval of stability samples [18]. The analyte was stored for 1 month at −20 °C and tested for long-term stability by repeated freeze/thaw cycles.

3. Results and discussion

3.1. Mass optimization

Different kinds of MS techniques are available for analyzing herbicides. Use of a triple–quadrupole module is advantageous because the technique is sensitive and these compounds are stable. In these methods, the pseudomolecular ion and the more stable fragment ions are generated for analysis. The precursor (pseudomolecular) ions of the herbicides must be established. When both HFBA solution and HFBA–ammonium formate were used as the mobile phase for analyzing PQ and DQ [8,9,19]. The molecular weight (Mw) 186 and 184 of PQ and DQ, respectively, were found to have precursor ions $(M-H)^+$ with 185 and 183. The product ions of PQ and DQ were the same as those determined by a previous method [19] in this study. When a TBAOH solution of higher pH value was used, the predominant precursor ion was 93 (m/z 186/2) in the case of PQ; 92 (m/z 184/2), DQ; and 107 (m/z 214/2), EV. The prominent product ions had m/z of 171 and 155 for PQ, 157 and 84 for DQ, and 185 and 157 for EV.

It is convenient way to simultaneous detection of herbicides by injection of them without prior derivatization, with the same acquisition mode, significant precursors and product ions for sensitivity and specificity. GLYP (Mw 169) and GLUF (Mw 181) were only found to have relatively significant precursor ions (m/z) 170 and 182 using positive acquisition in the HFBA solution. The m/z of the product ions were 88 $(M+H-H_3PO_3)^+$ and 60 $(M+H-H_3PO_3-CO)^+$ for GLYP, 90 and 62 for a GLYP isotope, and 136 $(M+H-HCOOH)^+$ and 119 $(M+H-HCOOH-OH)^+$ for GLUF (Fig. 2). When these compounds were added to the commonly used HFBA–ammonium formate solution and the TBAOH solution, the significant findings in the negative mode was better than those in the positive mode. The precursor ions were 168 for GLYP and 180 for GLUF. The product ions were 150, 122, 81, and 63 for GLYP and 136, 119, and 56 for GLUF. The mass fragmentation of each herbicide is shown in Table 1.

The HFBA/ACN, HFBA–ammonium formate/ACN, and TBAOH/ACN mobile phase systems have all been used in the positive and negative modes acquisition. The final selected eluent was evaluated by those conditions, including its compatibility with extraction, separation by LC, and detection by the mass method.

3.2. Optimization of LC

The acid dissociation constants for GLYP have the following values: $pk_{a1} < 2$ (0.8, first phosphonic), $pk_{a2} < 2.6$ (2.3, carboxylate), $pk_{a3} < 5.6$, and $pk_{a4} < 10.6$ [11,20]. For GLUF, the values are $pk_{a1} < 0.8$ (phosphonic), $pk_{a2} < 2.9$ (carboxylate), and $pk_{a3} < 9.8$ (amine). The molecule preferentially bears a negative charge in the ammonium acetate buffer system (pH 4) during LC–MS analysis or in the pH range 5–9 for most biological specimens. The detection of GLYP and GLUF was carried out by prior derivatization and ammonium acetate buffer as mobile phase in LC–MS analysis [8,9] but never has been performed when HFBA solutionwas employed as the mobile phase. The acid dissociation constant for HFBA had a pk_a value of 0.4. The first acid dissociation of GLYP's and GLUF's phosphonic group was suppressed by ion-pair formation between the herbicide and HFBA. The positive charge of the herbicide could be predicted when HFBA was used as the mobile phase.

The three mobile phase systems containing HFBA/ACN, HFBA–ammonium formate/ACN, and TBAOH/ACN were evaluated for their use in the simultaneous detection of herbicides. As shown in Table 2, PQ and DQ as well as GLYP and GLUF were not separated by chromatography when mobile phase II was used. Further, PQ and DQ were not separated by mobile phase III (TBAOH/ACN). Moreover, higher LOQs were observed when both mobile phases II and III were used. Successful simultaneous separation of all compounds only occurred when HFBA/ACN was used as the mobile phase. The HFBA solution contributed to ion-pair formation with PQ and DQ as well as with GLYP and GLUF. The mobile phase I (HFBA/ACN) was found to have the completely separation of these herbicides and evaluated as eluent system.

A Synergy Polar-RP column that can be used with a mobile phase composed of 100% water and can handle solutions with a pH tolerance range from 1 to 10 was used for the separation. The four herbicides were only completely separated by the HFBA/ACN system. GLYP and GLUF could not be separated in the HFBA/buffer/ACN system. In the basic TBAOH system, PQ and DQ could not be detected in the previous MRM mode because the MRM ions were changed to 93/171 and 92/157. PQ and DQ also lost their ion-pair ability in both the HFBA–ammonium formate–ACN and TBAOH systems, resulting in incomplete separation. The experimental results showed that the ion-pair appeared in these herbicides only when HFBA was used. This indicates that ion-pairs of GLYP and HFBA were formed (Fig. 1) and that the HFBA mobile phase was suitable for simultaneous detection of these herbicides.

In this study, HFBA (concentrations ranging from 5 to 25 mM) with ACN was used as the mobile phase for optimizing the simultaneous detection of the herbicides, and the best chromatographic performance in terms of ion separation and intensity was observed in a 20 mM HFBA solution/ACN (Fig. 3).

3.3. Extraction

The herbicides, proteins, amino acids, and minerals were all highly soluble in water, and this led to problems in extraction when the solubility of herbicides in biological specimens was under investigation. Many different extraction procedures can be used with conventional detection methods. Liquid–liquid extraction can be applied to most chemical compounds; however, this method suffers from the drawbacks of low recovery and long time period required. A cation exchanger for biological specimens [19], silica Sep-Pak [21] for water samples, and on-line C-8 SPE [22] for drinking water have been successfully used for the extraction of PQ and DQ without pre- or post-column treatment.

PQ and DQ, which are quaternary ammonium herbicides, were successfully extracted by the cation exchange column since molecules with two positive charges definitely cannot be exchanged. GLYP and GLUF are zwitterions and carry a positive charge in solutions of low pH. The Strata-XC mixed-mode cation

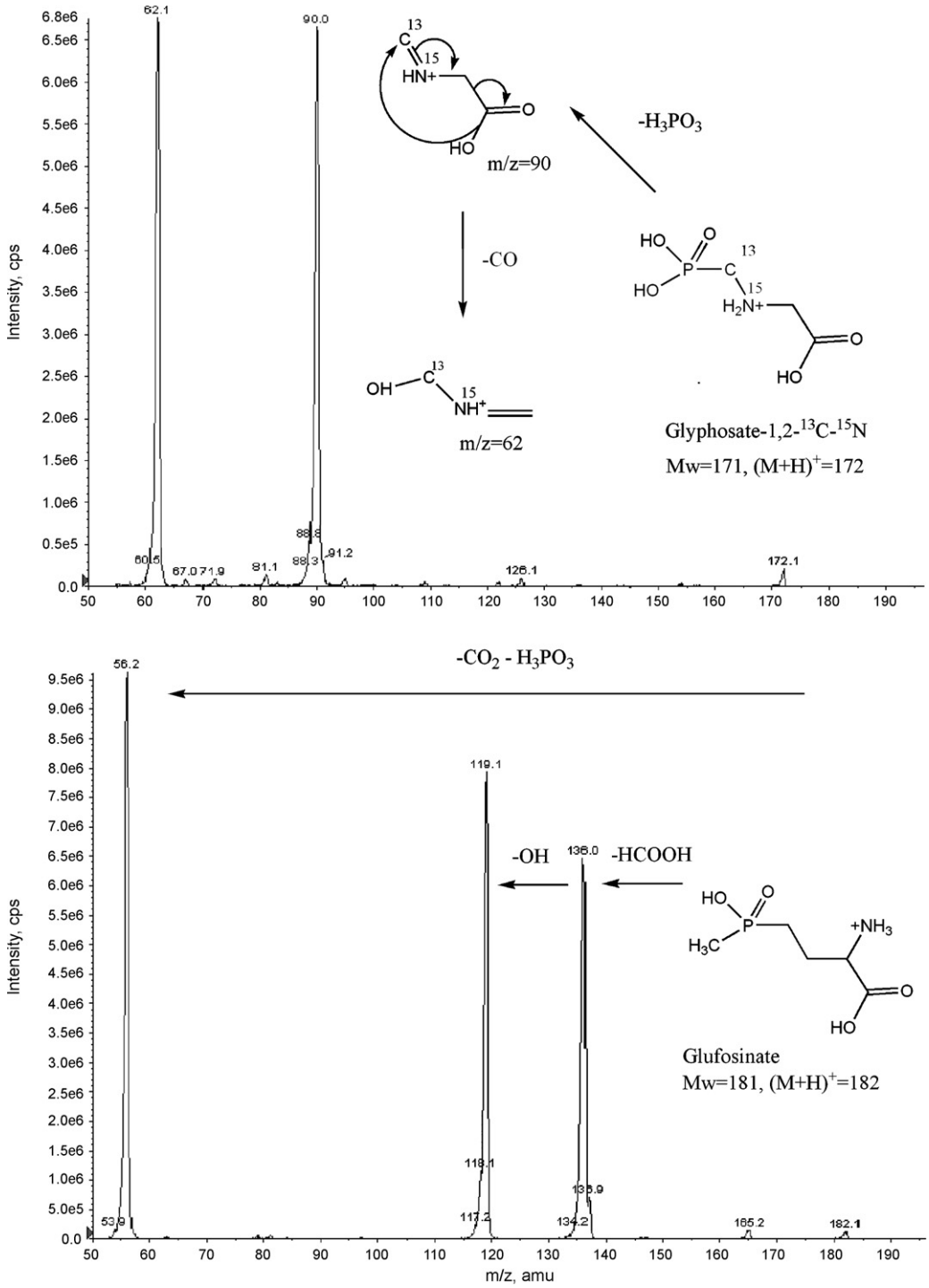

Fig. 2. Product ion scan spectrum of GLYP and GLUF.

exchange cartridge was evaluated as the SPE cartridge for these four herbicides. Since the pk_a of the strong cation exchange group was <1, the stationary phase was fully ionized (negatively charged) under aqueous conditions. The positive molecular ions of the herbicides could strongly bind with the sorbent. The HFBA solution was used as the ion-pair reagent and increased the acidity of the solution. Once an ion-pair containing HFBA and a molecular ion was formed and passed through the cartridge, the hydrophobic, polar (hydrogen bonding), and strong cation exchange forces helped in retaining the herbicide molecules. With the exception of the ionic bonds between the molecules and the stationary phase, the ion-pair with HFBA increased the intramolecular hydrogen bonding and hydrophobic interaction ability of the entire molecule, which could then be adsorbed onto part of the polymeric phase. The sample was washed with mineral acid and eluted with an ammonium hydroxide/methanol solution of higher pH 10. As shown in Table 2, the recoveries of the four analytes were within the acceptable range of 80–120%. Thus, this extraction procedure was considered to be sufficiently sensitive for these herbicides.

Table 1
ESI-MS/MS of herbicides.

Herbicides	Molecule weight	Acquisition mode	Precusor ion (*m/z*)	Product ion (*m/z*) (relative intensity, %)
Glyphosate	169	Positive[a]	170 (5)	88 (100), 60 (95)
		Negative[b]	168 (25)	150 (40), 122 (40), 81 (100), 63 (92)
Glyphsate-(1,2-^{13}C,^{15}N)	171	Positive[a]	172 (5)	90 (99), 62 (100)
		Negative[b]	170 (25)	152 (38), 124 (42), 81 (100), 62 (90)
Glufosinate	181	Positive[a]	182 (3)	136 (70), 119 (85) 56 (100)
		Negative[b]	180 (30)	162 (42), 134 (55) 81 (100)
Paraquat	186	Positive[a,c]	185 (100)	170 (65), 158 (45), 144 (35)
		Positive[b]	93 (22)	171.0 (100), 155 (47)
Diquat	184	Positive[a,c]	183 (70)	168 (100), 157 (70), 143 (10)
		Positive[b]	92 (100)	157 (22), 84 (49)
Ethyl viologen	214	Positive[a,c]	213 (100)	198 (10), 186 (25)
		Positive[b]	107 (41)	185 (100), 157 (52)

[a] HFBA solution.
[b] TBAOH solution at a higher pH value.
[c] HFBA–ammonium formate buffer solution (10 mM, pH 4) were used as mobile phase.

Table 2
Recovery and LOQ study were performed by using three different mobile phases, phase I: 10 mM HFBA/ACN, phase II: 15 mM HFBA/15 mM ammonium formate/ACN at pH 4.0, and phase III: 20 mM ammonium acetate/TBAOH solution/ACN at pH 4.7. $n = 3$ for recovery test.

Analytes		QC	Mobile phase		
			I	II	III
Glyphosate	*R* (mean ± CV, %)	150 ng/mL	92 ± 5.3	88 ± 6.3[a]	94 ± 4.5
		20 ng/mL	95 ± 6.8	92 ± 7.8[a]	88 ± 10
	LOQ (ng/mL)		5	15	2
Glufosinate	*R* (mean ± CV, %)	150 ng/mL	90 ± 7.5	97 ± 8.3[a]	102 ± 8.8
		20 ng/mL	98 ± 6.3	94 ± 6.4[a]	105 ± 7.4
	LOQ (ng/mL)		2	10	1
Diquat	*R* (mean ± CV, %)	150 ng/mL	96 ± 5.2	89 ± 3.2[a]	92 ± 3.6[a]
		20 ng/mL	92 ± 5.5	93 ± 8.9[a]	NA
	LOQ (ng/mL)		2	5	20
Paraquat	*R* (mean ± CV, %)	150 ng/mL	97 ± 8.9	102 ± 3.9[a]	91 ± 4.4[a]
		20 ng/mL	94 ± 5.5	105 ± 7.3[a]	NA
	LOQ (ng/mL)		5	5	30

[a] Represent the separations were not complete.

3.4. Results of LC–MS

Three different solvent systems were tested for their application in ion-pair LC. A much lower LOQ was obtained when GLYP and GLUF were separated in the TBAOH system than in the HFBA system. In the HFBA/buffer system, the ions were suppressed, resulting in a higher LOQ. HFBA can easily form ion-pairs with highly soluble compounds, and this feature is useful in ion chromatography.

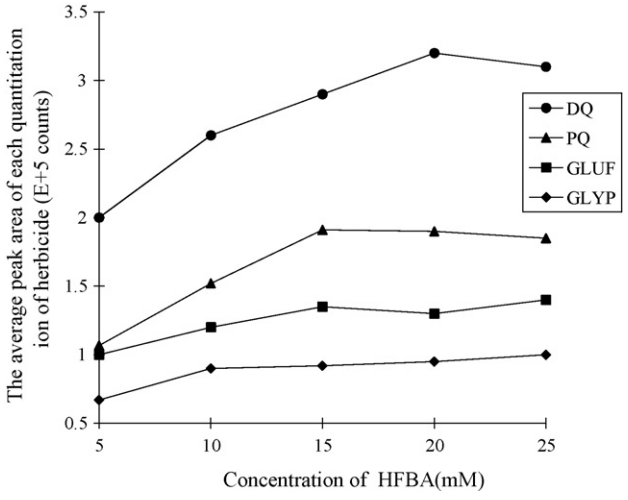

Fig. 3. Optimization of mobile phase in LC–MS/MS analysis.

The results of the chromatography of the four herbicides and internal standards are shown in Fig. 4. The retention times of GLYP, GLYP-(1,2-^{13}C,^{15}N), GLUF, DQ, PQ, and EV were 1.62, 1.62, 2.26, 5.56, 5.78, and 6.90 min, respectively. Complete separation of GLYP and GLUF was indicated by ion-pair formation of these two compounds with HFBA. The results showed that the pH value of the HFBA solution was approximately 2, and the total chromatographic time, including that required for column equilibration, did not exceed 10 min.

These herbicides were recovered from the cation exchanger column at similar levels using different mobile phases. The cation exchanger has the advantage of being flexible in application. Using a strong cation exchanger, acidic, neutral, and basic compounds can be forced to bear a positive charge in the ion-pair on the cation exchanger, and extraction can be achieved with high recovery.

3.5. Method validation

The method presented here, i.e., cation exchange extraction followed by analysis with HFBA ion chromatog-

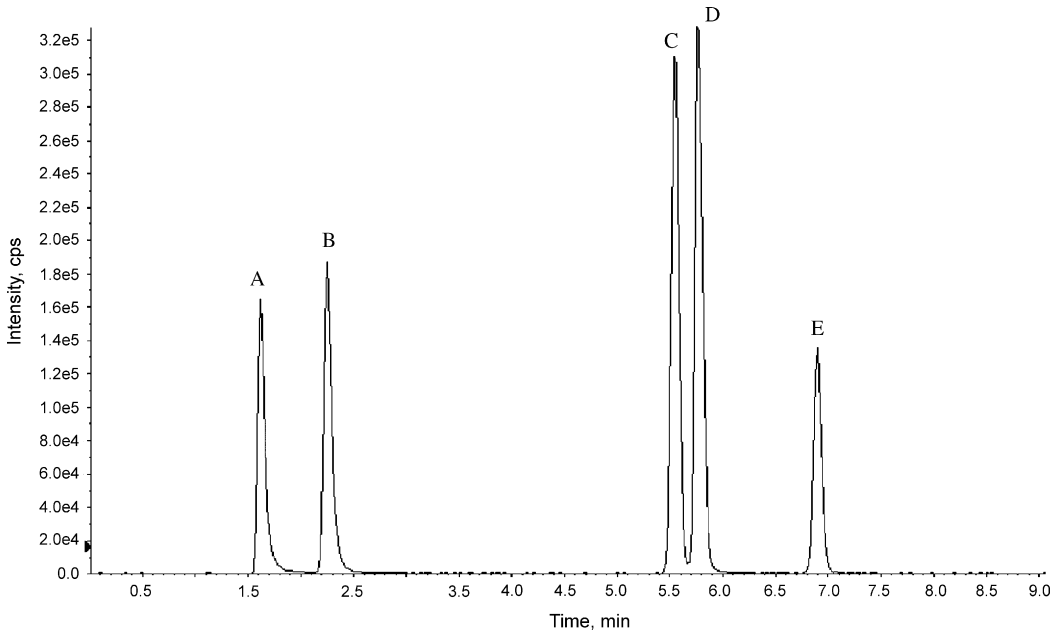

Fig. 4. MRM of the ion chromatography of herbicides: (A) TIC of GLYP and GLYP-(1,2-^{13}C,^{15}N), (B) TIC of GLUF, (C) TIC of DQ, (D) TIC of PQ, and (E) TIC of EV.

Table 3
Validation report, $n = 3$ for the studied analytes.

Analytes	Mean ± CV (%)				Linearity r	LOQ (ng/mL)
	tR (min)	QC recovery	Matrix effect Low	High		
Glyphosate	1.62 ± 0.4	96 ± 2.6	94 ± 5.5	91 ± 8.1	0.9998	5
Glufosinate	2.26 ± 0.5	95 ± 6.2	105 ± 8.3	97 ± 5.7	0.9992	2
Diquat	5.55 ± 0.6	104 ± 7.3	98 ± 6.6	93 ± 4.2	0.9985	1
Paraquat	5.78 ± 0.6	102 ± 3.6	96 ± 2.6	90 ± 2.1	0.9999	5

raphy was validated. The validation report is shown in Table 3. The coefficient of variation (CV) value of the retention time for each herbicide was below 1% and was stable during separation. The LOQ values for PQ and DQ were comparable to those obtained using previous method [15,16]. On the other hand, the LOQ values of GLYP and GLUF were 5 and 2 ng/mL, respectively. In the clinic, survival is likely if the PQ concentration in the plasma does not exceed 2.0, 0.6, 0.3, 0.16, and 0.1 mg/L at 4, 6, 10, 16, and 24 h, respectively, after ingestion [23]. The LOQ values of these herbicides in the plasma samples were sufficiently low to meet the requirements of clinical medicine and forensic toxicology. The calibration concentration range was from the LOQ to 200 ng/mL. The coefficient of determination ranged from 0.9985 to 0.9999.

As shown in Table 3, there were no relevant matrix effects for these herbicides at low (20 ng/mL) and high (150 ng/mL) concentrations. The highest matrix effects were observed for PQ, and this was considered to be acceptable due to good reproducibility. The analytes were also stable at low and high concentrations for a period of more than 24 h. In the freeze/thaw and long-term stability experiment, the ratio of the means (stability samples *vs.* control samples) were within 90–110%, whereas the 90% confidence intervals for stability samples were within 80–120% of the respective control means, thus fulfilling the acceptance criteria for the four herbicides at both concentrations. The body maintains the pH of the arterial blood in a narrow range close to pH 7.40 (7.35–7.45). Disturbances in acid–base homeostasis can be life-threatening. The recovery of herbicides in serum of low pH values was analyzed to evaluate matrix effects in acidosis samples. The four herbicides were spiked in the serum sample with the pH adjusted to 6.8, 7.0, 7.2, and 7.4 using an acetic acid solution. The recoveries of these serum samples at the different pH values ranged from 90.5 to 104.9% (Table 4).

3.6. Application to real cases

Practical application of the new technique described in this paper was demonstrated in case I in which drinks and a threatening letter were sent to the laboratory. The drinks were cleaned up by liquid–liquid extraction, and the extracts were screened by GC/MS. Only the anion surfactant, polyethoxylated tallow amine (POEA), which allows herbicides to penetrate the waxy surfaces of plants, was detected. The ICP/MS data revealed that the drink had a total phosphorus concentration that was hundreds of times higher than the normal. GLYP was finally detected by the proposed method, and the concentration was in the range 52–320 μg/mL. In case II, an old couple was found dead at home. Their serum extracts were directly injected into the GC/MS system. No toxic substances were detected. The ICP/MS data were also found to be normal. PQ was

Table 4
Recovery of specimens at various pH, $n = 3$ for recovery test.

Herbicides	pH value			
	6.8[a]	7.0[a]	7.2[a]	7.4[a]
Glyphosate	96.0, 3.6	98.3, 3.2	93.1, 2.6	95.3, 3.1
Glufosinate	95.8, 3.6	95.3, 3.3	96.8, 2.0	98.4, 3.5
Diquat	96.0, 3.6	98.3, 2.4	93.7, 3.6	93.4, 4.1
Paraquat	102, 2.9	97.1, 3.2	97.8, 4.9	98.9, 3.4

[a] Recovery, CV (%).

finally detected by the method presented here, and the concentrations in the old couples' serum samples were 2.5 and 2.8 μg/mL, respectively.

4. Conclusions

We developed a specific, sensitive, and accurate method for the simultaneous detection and quantitation of highly polar herbicides in human serum by using ion-pair LC coupled with MS/MS. The LOQ for all tested herbicides was <10 ng/mL in a serum specimen volume of 0.5 mL. At least two ion-pairs were chosen for confirmation by MRM; the prominent one was used for quantitation, while the ratio of the two ion-pairs was used for qualification. The analytes were well separated with high recoveries when the appropriate SPE technique was used, and internal standards allowed the rapid screening of herbicides using small quantities of specimens. The proposed method, in which an SPE column and the LC–MS/MS system were used, has the potential to be extended to all compounds that bear positive or negative charges. This potential will be investigated in further studies.

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