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Simultaneous Determination and Quantitation of Paraquat, Diquat, Glufosinate and Glyphosate in Postmortem Blood and Urine by LC–MS-MS

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Abstract

A simple method, incorporating protein-precipitation/organic backwashing and liquid chromatography–tandem mass spectrometry (LC–MS-MS), has been successfully developed for the simultaneous analysis of four highly water-soluble and less volatile herbicides (paraquat, diquat, glufosinate and glyphosate) in ante- and postmortem blood, urine and gastric content samples. Respective isotopically labeled analogs of these analytes were adopted as internal standards. Acetonitrile and dichloromethane were used for protein precipitation and organic solvent backwashing, respectively, followed by injecting the upper aqueous phase into the LC–MS-MS system. Chromatographic separation was achieved using an Agilent Zorbax SB-Aq analytical column, with gradient elution of 15 mM heptafluorobutyric acid and acetonitrile. Mass spectrometric analysis was performed under electrospray ionization in positive-ion multiple reaction monitoring mode. The precursor ions and the two transition ions (*m/z*) adopted for each of these four analytes were paraquat (185; 169 and 115), diquat (183; 157 and 78), glufosinate (182; 136 and 119) and glyphosate (170; 88 and 60), respectively. Analyte-free blood and urine samples, fortified with the analytes of interest, were used for method development/validation and yielded acceptable recoveries of the analytes; interday and intraday precision and accuracy data; calibration linearity and limits of detection and quantitation. This method was successfully incorporated into an overall analytical scheme, designed for the analysis of a broad range of compounds present in postmortem samples, helpful to medical examiners' efforts to determine victims' causes of death.

Introduction

Paraquat (PQ), diquat (DQ), glufosinate (GLUF), and glyphosate (GLYP) are nonselective contact herbicides, widely used to control weeds for plant protection (1). In Taiwan, these herbicides are relatively inexpensive, readily available and often found in poisoning-related case samples. As shown in Figure 1, PQ and DQ are dipyridyl while GLUF and GLYP are organophosphorus compounds.

PQ is highly toxic and its mechanisms of action have been well established (2). PQ's mechanisms of action are associated with accumulation of reactive oxygen species and toxin-free radicals in various organs, especially in the lungs, to result in oxidation injures and eventually to irreversible pulmonary fibrosis and injures of vital organs. Death occurs within few days and is usually a consequence of multiorgan failure when a lethal amount of PQ is ingested. Compared to PQ, DQ poisoning is much less common but often

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causes severe toxic effect on the central nervous system. Early symptoms of poisoning resulting from DQ ingestion are similar to those caused by PQ, reflecting its corrosive effect on tissue (2). The toxic effects of GLUF and GLYP have not been as fully studied (3). GLUF-poisoning is characterized by various neurological symptoms such as disturbances of consciousness, convulsions and apnea, appearing several hours after ingestion. The mortality rate, with GLYP as a suicidal agent, is lower than the rates caused by PQ and other organophosphates.

PQ, DQ, GLUF and GLYP are often found in postmortem specimens collected from accidental, suicidal and homicidal poisoning cases in Taiwan. To assist medical examiners determining the causes of death in these cases, multiple analytical procedures are often needed to detect the presence of various compound categories in postmortem specimens. It is important to develop a simple method for simultaneous analysis of common herbicides.

PQ and DQ are traditionally analyzed by low sensitivity approaches—such as ultraviolet (UV) spectrometry (4, 5) and liquid chromatography (LC)/UV (6)—and gas chromatography-mass spectrometry (7). For effective analysis, GLUF and GLYP are often chemically derivatized to include suitable chromophore moieties to increase their molar absorptivity. Derivatizations can also be applied to improve volatility for fluorescence (8) and MS (9) detection.

Simultaneous analysis of these four herbicides by conventional liquid–liquid extraction and instrumental methods are often problematic because of these analytes' good solubility in water, low volatility, low molecular weight, thermal liability and different ion types in the gaseous phase. Improved sample preparation approaches (often costly and inconvenient), such as solid-phase extraction (SPE) (10–12), extraction (13) and protein precipitation (14) by acetonitrile and protein removal by ultrafiltration membrane (15), have been developed. More recently, LC–MS-MS methods have been applied to the analysis of water-soluble herbicides in water (9, 16, 17), vegetables (18), soil (9), urine (12, 13), plasma (13), serum (11, 14) and whole blood (10).

Having successfully developed highly accurate, selective and sensitive LC–MS-MS methods for the analysis of various drug categories in ante- and postmortem samples (19–23), we are applying the accumulated knowledge to the simultaneous analysis of PQ, DQ, GLUF and GLYP in blood, urine and gastric content samples. In this study, acetonitrile is used for protein precipitation and dichloromethane for organic backwashing. Isotopically labeled analogs (Figure 1) of these four herbicides were chosen as the internal standards (ISs) for quantitation.

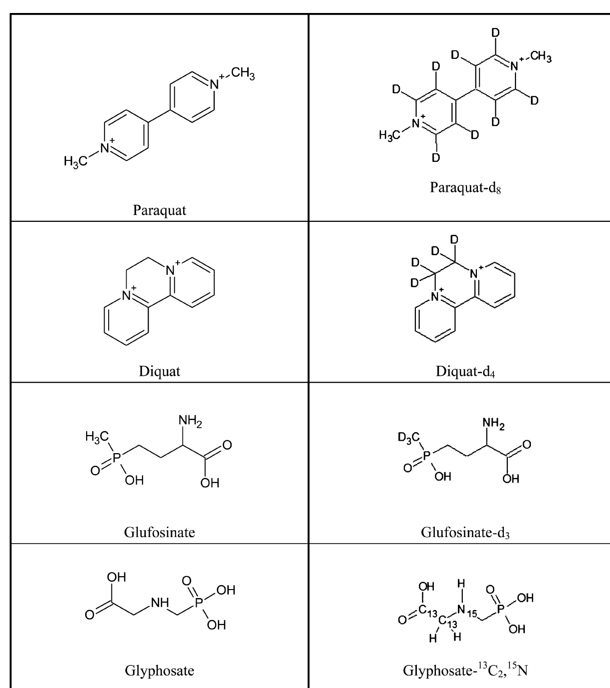

Figure 1. Chemical structures of PQ, DQ, GLUF, GLYP and their respective isotopic analogs adopted as the internal standard (IS) for the analytical protocol.

Materials and methods

Chemicals and reagents

Herbicide standards, PQ dichloride tetrahydrate, GLYP, DQ dibromide monohydrate and GLUF ammonium, were purchased from Chem Service, Inc. (West Chester, PA, USA) and Sigma (St. Louis, MO, USA). ISs, PQ-d_8 dichloride, DQ-d_4 dibromide, GLYP-$^{13}C_2$,^{15}N and GLUF-d_3 hydrochloride were obtained from Toronto Research Chemicals, Inc. (North York, ON, Canada) and Medical Isotopes, Inc. (Pelham, NH, USA). Chromatography grade acetonitrile, dichloromethane, 1,2-dichloroethane, ethyl acetate, methyl *tert*-butyl ether and *n*-hexane were purchased from J.T. Baker (Phillipsburg, NJ, USA). Heptafluorobutyric acid (HFBA) was purchased from Alfa Aesar (Ward Hill, MA, USA). Deionized water was produced by a PURELAB^{TM} Ultra water purification system from ELGA LabWater VWS Ltd. (Bucks, UK).

Herbicide-free blood and urine samples were obtained from forensic science specimens and healthy volunteers, respectively. These samples were checked by the same LC–MS-MS method to confirm the absence of the analytes.

Stock solutions, standard working solutions and calibration curves

A single herbicides stock solution (1 mg/mL) was prepared by weighing individual analytes and dissolving them into water to result in 100 mL of final volume: 0.1770 g of PQ dichloride tetrahydrate, 0.1967 g of DQ dibromide monohydrate, 0.1000 g of GLYP and 0.1094 g of GLUF ammonium salt. A 10-μg/mL standard working solution was prepared by using 0.1 mL of the stock solution in 10 mL of water. A stock solution of the ISs (100 μg/mL) was prepared using 1.366 mL of 1 mg/mL PQ-d_8 dichloride, 1.851 mL of 1 mg/mL DQ-d_4 dibromide, 1 mL of 1 mg/mL GLYP-$^{13}C_2$,^{15}N and 1.218 mL of 1 mg/mL glufosinat-d_3 hydrochloride in 10 mL water. A 10-μg/mL IS working solution was prepared by using 1 mL of the stock solution in 10 mL of water. For the preparation of calibration standards, appropriate volumes of 10-μg/mL working solutions (standards and ISs) were added to 0.1 mL blank blood (and urine) to achieve the following final concentrations: 1, 2.5, 5, 10, 20 μg/mL of the analytes and 5 μg/mL of ISs.

Sample preparation

An appropriate amount of the working IS solution (10 μg/mL) was added to 0.1 mL herbicide-free blood or urine, resulting in a concentration of 5 μg/mL for each IS. Additional water was added to each

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sample resulting in a final volume of 0.30 mL. For protein removal, 0.5 mL acetonitrile was added and the mixture was vortexed for 5 minutes and centrifuged at 4,000 rpm for 5 minutes. The upper solution was transferred to another tube, on which 1 mL of dichloromethane was added for backwashing. After vortexing for 5 minutes and centrifugation at 4,000 rpm for 5 minutes, 100 μL of the upper aqueous phase was transferred to an HPLC vial and 5 μL of sample was injected into the LC–MS-MS system.

Instrumentation for LC–MS-MS analysis

The LC–MS-MS system included an Agilent 1,200 Series LC and an Agilent 6410 Triple Quadrupole Mass Spectrometer (Agilent Technologies, Inc., Palo Alto, CA, USA). Chromatographic separations were performed at 40°C on an Agilent ZORBAX SB-Aq column (100 mm × 2.1 mm, 1.8 μm particle). The mobile phase included 15-mM HFBA (A) and acetonitrile (B) at a flow rate of 0.30 mL/min. The initial gradient composition (A/B 90:10, v/v) was held for 1.5 minutes; decreased to 0% A in 6 minutes and held for 2 minutes; then increased to 90% A in 1 minute and held for 1 minute.

The mass spectrometer was operated in positive-ion multiple reaction monitoring (MRM) mode. Optimized conditions were: nebulizer pressure, 40 psi; drying gas flow, 10 L/min at 350°C and HV capillary voltage, 4,000 V. The injection volume was 5 μL. Identification and quantification of each analytes were performed in positive mode using MRM of a quantifier and an additional qualifier ion. To meet the criterion for a positive identification, the ratio between the quantitative and the qualifying transition ions (derived from the precursor ion) must fall within ±20% of that established by the calibration standards.

Recovery

Recoveries were estimated at three concentration levels of these analytes. Recovery rates were calculated by comparing the peak areas derived from the following two sets of experiments. Samples in the first set included blank blood and urine samples spiked with the analytes (or their ISs) prior to the sample preparation steps. The second set of samples was the initial gradient LC–MS mobile phase containing equivalent concentrations of the analytes (or ISs). Each experiment was performed with three replicates.

Precision and accuracy

Precision and accuracy were evaluated over the linear dynamic range at five different concentrations (1–20 μg/mL). Intraday and interday precisions were assessed by five determinations per concentration conducted in one and five consecutive days, respectively.

Calibration and limits of detection and quantitation

Calibration curves of the four herbicides were prepared by the analysis of standard working solutions at the following concentrations: 1, 2.5, 5, 10 and 20 μg/mL. Analyte/IS peak area ratios were calculated by using Mass Hunter software (Agilent Technologies, Inc.). Data were fit to a least-square linear regression curve with a 1/x weighting factor and not forced through the origin. The limit of detection (LOD) was determined by analyzing a series of standard solutions with decreasing concentrations of the analytes and defined as the lowest concentration at which the acceptable criteria for the identification of an analyte were met, i.e., (i) the chromatographic peak shape was acceptable and (ii) quantitation/qualifier transition ion ratio was comparable to a calibration standard (within ±20%). The limit of quantitation (LOQ) was defined by both the MRM ion ratio tolerance (±20%) and the lowest concentration (of the analyte) at which the analyzed value was within ±20% of the expected value.

Evaluation of matrix effect

Matrix effect was assessed at three concentration levels, each with five replicate experiments. For this assessment, analyte peak areas derived from the following two sets of experiments were compared. Set 1: analytes were spiked into the solution resulting from the preparation of blank blood and urine samples; Set 2: equivalent concentrations of the analytes (and ISs) dissolved in the initial gradient LC–MS mobile phase. Matrix effect observed for each analyte was reported as a percentage value.

Evaluation of potential medium pH effect

Potential effect of medium pH was assessed using a set of six (different pH buffer) samples, containing 5 μg/mL of the four analytes, with pH 3, 4, 5, 6, 7 and 8. Analyte/IS peak area ratios of these four analytes observed in all samples (with different pH) were compared to assess potential pH effect, if any.

Results and discussions

With knowledge accumulated through years of experience in developing and applying LC–MS-MS methods for the analyses of various categories of drugs in ante- and postmortem samples (19–23), we were able to find the optimal chromatographic and mass spectrometric parameters suitable for the separation, identification and quantitation of the four analytes and their respective isotopically labeled ISs. These parameters were then used for the analysis of blank and standard blood, urine and gastric samples and to establish the validity of the entire analytical protocol. Analytical parameters validated included method specificity/potential interference, analyte recovery, matrix effect and quantitation parameters such as linearity, LOD/LOQ and precision/accuracy. Finally, data derived from the application of the method to the analysis of case samples were summarized.

Method development and validation

Chromatographic and mass spectrometric parameters

Chromatographic retention parameter, the precursor/product quantitation and qualifier ions and mass spectrometric parameters adopted for PQ, DQ, GLUF and GLYP and their ISs are summarized in Table I. Chromatographic parameters, using a ZORBAX SB-Aq column under gradient elution, were varied to achieve optimal separation and retention of the analytes and the ISs, with a total runtime of 10 minutes. Representative chromatograms of analyte-free blood and urine samples, which were spiked with 5 μg/mL of the four analytes and their respective isotopically labeled ISs, are shown in Figure 2 (blood) and Figure 3 (urine). Satisfactory separation and peak intensities were achieved under these conditions. No interfering signals were observed at the retention windows established for these analytes (and their ISs), when blank blood and urines samples were analyzed, indicating acceptable method specificity.

Recovery

A combination of the acetonitrile protein-removal approach (14) and the dichloromethane backwashing step was found effective to

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Table I. Retention time, transitions and MS–MS parameters for each analyte and IS

Compound	Retention time (min)	Precursor ion (*m/z*)	Fragment (V)	Quantitation ion (*m/z*)	Collision energy (V)	Qualifier ion (*m/z*)	Collision energy (V)
PQ	3.07	185	131	115	48	169	28
PQ-d_8	3.06	193	114	121	56	176	32
DQ	2.81	183	144	157	24	78	48
DQ-d_4	2.81	187	163	159	24	158	24
GLUF	1.12	182	88	136	8	119	16
GLUF-d_3	1.12	185	94	139	8	122	16
GLYP	0.87	170	65	88	4	60	12
GLYP-$^{13}C_2$,^{15}N	0.87	173	92	91	4	62	16

produce suitable product for LC–MS-MS analysis. Results of the recovery experiments reflected its effectiveness in recovering the analytes from the sample matrix. Data shown in Table II indicated acceptable recoveries, except for GLYP in blood matrix. Since an isotopically labeled analog of the analyte was used as the IS, low recovery will not affect quantitation; it may however affect achieving favorable levels of LOD and LOQ (see further discussion in the "Validation of quantitation protocols" section).

Matrix effect

In 1996, Buhrman and his co-workers (24) reported effects of sample pretreatment methods on the accuracy and precision of data derived from LC–MS-MS methods of analysis. They attributed this phenomenon to the suppression of the electrospray ionization (ESI) process, caused by the effects of substances simultaneously entering the mass spectrometer, during the chromatographic process (substances in the sample matrix may not always be completely removed by the sample preparation protocol). In particular, some molecules with low volatility could cause the ionization efficiency of the analyte to change unexpectedly, thus affecting the quantitation results (25).

Ion suppression occurs commonly, while enhancement has also been reported in rare cases (26). Further studies have indicated that matrix effect mainly originated from changes of the chemical process in the liquid phase. For instance, interfering substances' competition for charges or protons could change the drip dispersal, thus reducing the distribution of the analytes onto the dripping surface. As a result, the impact of the matrix effect on ESI is generally much greater than that on atmospheric pressure chemical ionization (27, 28).

Isotopically labeled analogs of the analytes are often used as the ISs (in a quantitation protocol) to eliminate or minimize the impact of matrix effects. With nearly the same physicochemical properties exhibited by the analytes, isotopically labeled ISs can compensate for matrix effects affecting the analytes in the sample pretreatment, chromatography and ionization processes (29–31). However, exceptions have also been reported (32). With this background in mind, potential matrix effects were evaluated using two different sets of data as illustrated below.

First, matrix effect data were calculated by comparing the signal (peak area) resulting from the following two sets of samples: (i) solution resulting from the preparation of blank blood and urine samples that were added known amounts of the analytes and (ii) samples containing the equivalent amounts of the analytes using the LC–MS-MS's initial mobile phase as the solvent. Matrix effects data are presented in percentage, a value that is significantly <100% would suggest ion suppression caused by interfering substances derived from the sample matrix. Data shown in Table III indicate the occurrence of matrix effects when urine samples were analyzed for GLUF and GLYP and when the blood samples were analyzed for GLYP.

A second set of matrix effect data was calculated using data derived from the same two sets of samples. However, the analyte/IS signal (peak area) ratios, instead of the analyte's signals, were used for the calculation. Thus, a value of 100% would indicate (i) both the analyte and the IS were free of matrix effect or (ii) the analyte and its IS exhibit the same extent of matrix effect. Data shown in Tables IV and V strongly suggest matrix effects observed in Table III (GLYP in urine and blood and GLUF in blood) were satisfactorily compensated for, when the analyte/IS signal ratios were used for the calculation. This is an indication that the analytes and their isotopically labeled analogs do not exhibit differential matrix effects.

It should also be noted that the ranges of percentage values observed for each analyte (in the same matrix) at three concentration levels, as shown in Tables IV and V, are significantly narrower than those shown in Table III. This is an indication that the IS method has also been effective in compensating for differential random errors observed among samples in the same analytical batch.

Validation of quantitation protocols

Data derived from the matrix effect study, as shown in Tables III–V, support the effectiveness of these isotopically labeled analogs in serving as the ISs. Shown below are data derived from the quantitation protocols incorporating the use of these ISs.

Summarized in Tables VI and VII are the intraday and interday precision and accuracy data derived from the analysis of the analytes in blood and urine matrices. Compatible data were obtained for the analysis of the analytes in blood and urine samples. As expected, the coefficient of variation (CV) values were significantly higher for samples with lower analyte concentrations (e.g., 1 µg/mL).

Method linearity, LOD and LOQ data are summarized in Table VIII. All calibration curves showed good linear response ($r^2 > 0.999$), over the 1–20 µg/mL range, for all analytes in both blood and urine matrices. These data indicate these analytes can be accurately determined at sub-µg/mL concentration level. The substantially lower recovery rate observed for GLYP from blood sample (Table II) did not appear to result in inferior LOD/LOQ data; however, it appeared to be the cause for the significantly higher CV data observed at the 1 µg/mL level (Table VI).

No specific study has been performed to assess the stability of the analytes in blood and urine samples. However, samples used for the interday study were all prepared at the first day of the study, and the last set of experiments was analyzed 5 days after the preparation of these samples. Analytical data shown in Table VI (blood)

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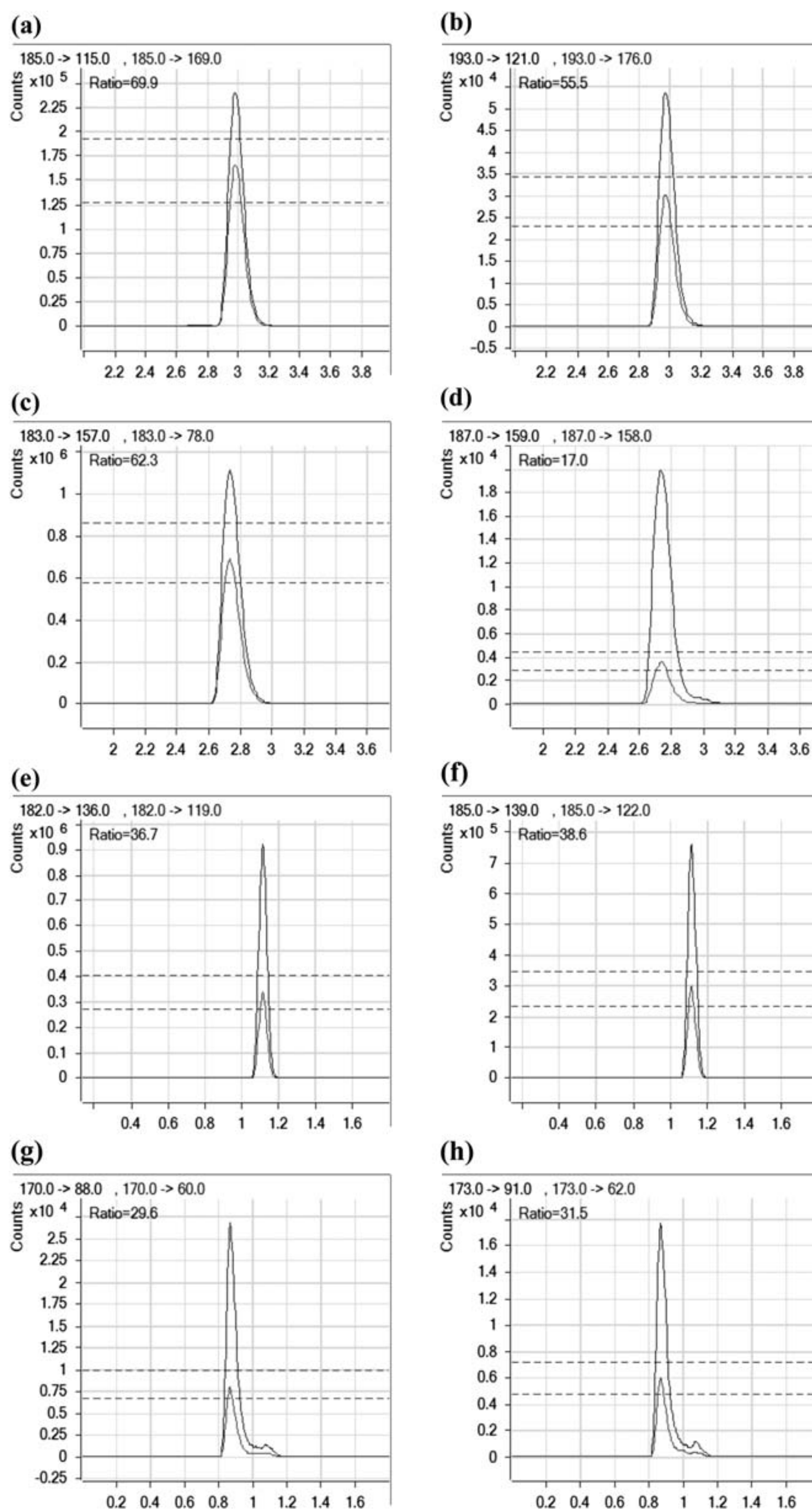

Figure 2. MRM chromatograms of analyte-free blood sample spiked with 5 µg/mL of standards and ISs: (a) PQ, (b) PQ-d_8, (c) DQ, (d) DQ-d_4, (e) GLUF, (f) GLUF-d_3, (g) GLYP, (h) GLYP-$^{13}C_2$,^{15}N.

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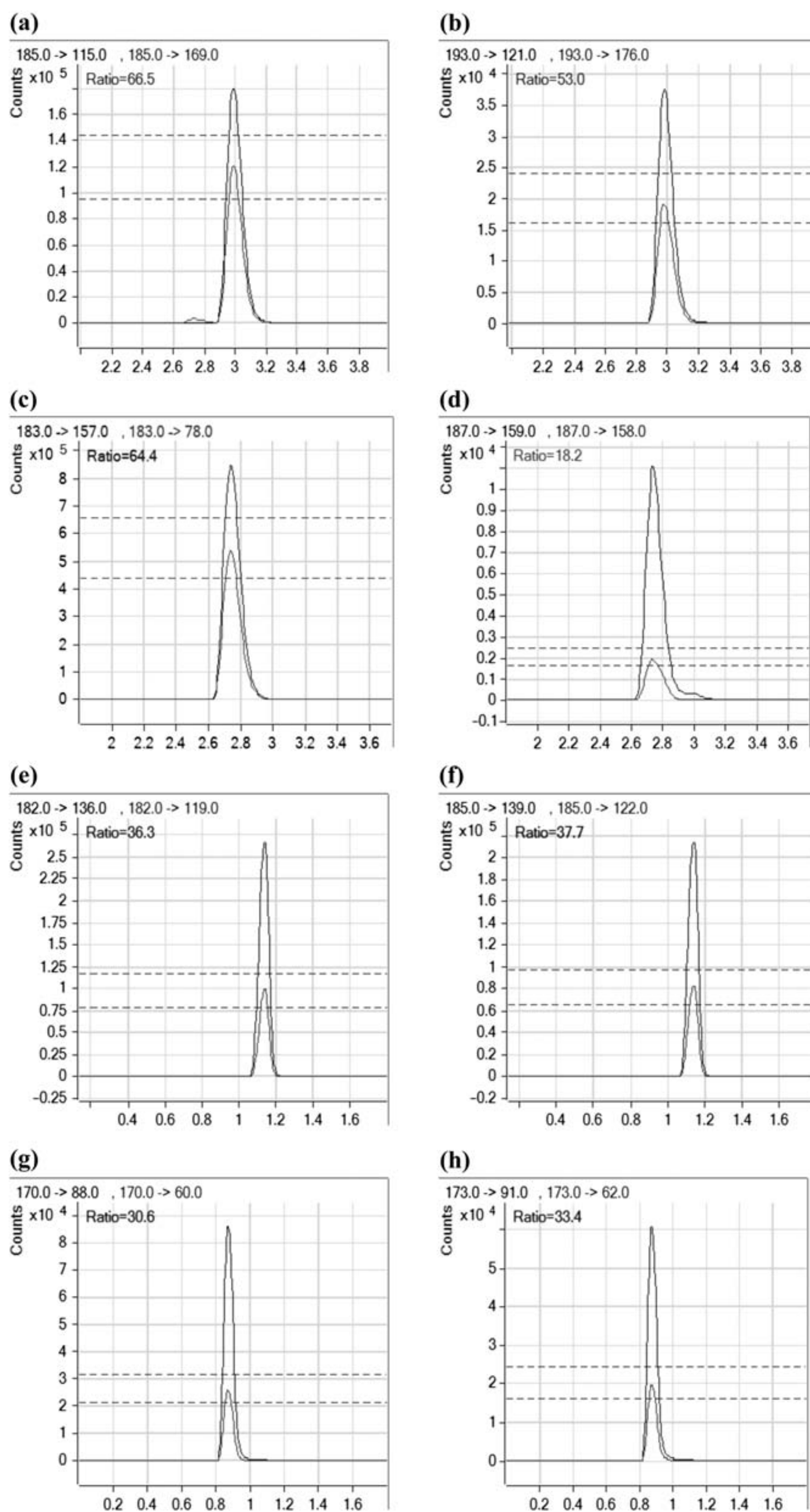

Figure 3. MRM chromatograms of analyte-free urine sample spiked with 5 µg/mL of standards and ISs: (a) PQ, (b) PQ-d_8, (c) DQ, (d) DQ-d_4, (e) GLUF, (f) GLUF-d_3, (g) GLYP, (h) GLYP-$^{13}C_2$,^{15}N.

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Table II. Recovery (%) of the analytes in blood and urine (mean ± SD; $n = 3$)

Compound	Concentration (µg/mL)	Recovery	
		Blood	Urine
PQ	2.5	91.61 ± 3.35	102.80 ± 5.56
	5	91.21 ± 3.68	94.98 ± 6.05
	10	89.55 ± 2.23	97.07 ± 3.03
DQ	2.5	84.85 ± 6.28	97.36 ± 1.80
	5	81.98 ± 1.70	97.91 ± 2.03
	10	86.61 ± 4.93	99.82 ± 1.20
GLUF	2.5	88.72 ± 6.22	72.84 ± 13.71
	5	75.17 ± 2.67	65.15 ± 5.46
	10	70.11 ± 5.65	62.15 ± 13.03
GLYP	2.5	46.11 ± 2.83	84.70 ± 2.69
	5	39.90 ± 5.43	76.16 ± 10.92
	10	24.36 ± 3.73	70.95 ± 5.32

Table III. Matrix effect (%) of the analytes as evaluated at three concentration levels (mean ± SD, $n = 5$)

Compound	Concentration (µg/mL)	Matrix effect[a]	
		Blood	Urine
PQ	2.5	121.04 ± 11.71	94.65 ± 5.13
	5	100.92 ± 2.83	78.67 ± 5.22
	10	94.11 ± 6.26	63.34 ± 2.61
DQ	2.5	104.10 ± 14.81	87.52 ± 2.02
	5	95.81 ± 14.03	75.68 ± 3.10
	10	94.86 ± 4.98	67.56 ± 2.05
GLUF	2.5	82.77 ± 2.26	26.90 ± 5.38
	5	85.33 ± 4.87	30.17 ± 5.27
	10	92.04 ± 3.80	28.45 ± 2.95
GLYP	2.5	33.74 ± 5.26	26.09 ± 7.27
	5	31.44 ± 1.81	29.06 ± 7.33
	10	52.12 ± 4.51	26.91 ± 2.72

[a]Matrix effect data were calculated by comparing the peak areas resulting from known amounts of analytes extracted from blank blood (or urine) samples to the peak areas resulting from the same amounts of the analytes dissolved in the initial mobile phase solvent (see text for fuller description).

Table IV. Peak area ratio of the analytes and their respective isotopic analogs: sample prepared in LC–MS-MS mobile phase and blank blood extract at three concentration levels ($n = 5$)

Analyte/isotopic analogs	Solvent[a]/matrix[b] (%)		
	2.5 µg/mL	5 µg/mL	10 µg/mL
PQ/PQ-d_8	2.236/2.348 (95.2)	4.957/4.853 (102.2)	10.569/10.290 (102.7)
DQ/DQ-d_4	9.489/10.562 (89.8)	19.632/21.519 (91.2)	39.946/41.144 (97.1)
GLUF/GLUF-d_3	0.513/0.494 (103.7)	1.046/1.043 (100.3)	2.140/2.158 (99.2)
GLYP/ GLYP-$^{13}C_2,^{15}N$	0.592/0.610 (97.0)	1.222/1.224 (99.8)	2.650/2.667 (99.4)

See text for fuller description for deriving data presented in this table.

[a]Analytes and their respective isotopically labeled analogs were dissolved in initial mobile phase used for LC–MS-MS analysis.

[b]Analytes and their respective isotopically labeled analogs were dissolved in the solution resulting from the preparation of blank blood or urine sample.

Table V. Peak area ratio of the analytes and their respective isotopic analogs: sample prepared in LC–MS-MS mobile phase and blank urine extract at three concentration levels ($n = 5$)

Analyte/isotopic analogs	Solvent[a]/matrix[b] (%)		
	2.5 µg/mL	5 µg/mL	10 µg/mL
PQ/PQ-d_8	2.165/2.200 (98.4)	4.865/4.561 (106.7)	10.526/10.264 (102.6)
DQ/DQ-d_4	9.684/11.512 (84.1)	20.554/23.588 (87.2)	41.703/50.103 (83.2)
GLUF/GLUF-d_3	0.499/0.493 (101.2)	1.050/1.084 (96.8)	2.249/2.322 (96.8)
GLYP/ GLYP-$^{13}C_2,^{15}N$	0.598/0.608 (98.3)	1.223/1.233 (99.2)	2.736/2.721 (100.6)

See text for fuller description for deriving data presented in this table.

[a]Analytes and their respective isotopically labeled analogs were dissolved in initial mobile phase used for LC–MS-MS analysis.

[b]Analytes and their respective isotopically labeled analogs were dissolved in the solution resulting from the preparation of blank blood or urine sample.

and Table VII (urine) indicate the analytes that remained stable during the time frame (5 days) experiments for the interday study were carried out.

Merits of the method

In many ways, this study is similar to an earlier one (11), also reported by Taiwan-based authors; however, our approach is simpler and less costly without using an SPE cartridge. More importantly, adopting respective isotopically labeled analogs of these four analytes as the ISs presumably would improve the accuracy of quantitation data. These ISs also increase the method's versatility, making it readily applicable to the analysis of various body fluids, such as blood, urine and gastric content, without having to precisely control the medium pH.

pH values of body fluids vary significantly. For example, the pH of human blood stays in a narrow range close to pH 7.40 (7.35–7.45), whereas urine pH value ranges from pH 4.6 to pH 8.0. Since an isotopically labeled analog and the analyte respond similarly during the analytical process, various body fluids can be readily analyzed without pH adjustment. As shown in Table IX, analyte/IS peak area ratios observed for these four analytes remain the same, regardless of the medium pH.

Application to the analysis of postmortem samples

The validated protocol has been applied to the analysis of these four highly water-soluble herbicides in urine, whole blood and gastric samples collected from postmortem cases. During the 2013–2014 periods, 22 cases were found to contain PQ or GLYP (or both); neither DQ nor GLUF was detected.

As shown in Table X, the mean values/ranges (in µg/mL) of PQ ($n = 15$) and GLYP ($n = 6$) found in blood samples were 291.5/2.9–1108.8 µg/mL and 991.0/3.4–3564.2 µg/mL, respectively. It should be noted that the mean blood concentration of PQ found in victims ($n = 4$) who had been treated in the hospitals was 11.1 µg/mL, significantly lower than the mean value (393.5 µg/mL) found in victims ($n = 11$) who had not been treated in the hospitals.

Suicide was the cause of death for all these 22 cases where PQ and/or GLYP were detected. In Taiwan, herbicides have been

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Table VI. Intraday and interday precision and accuracy data as presented in percentage ($n = 5$)—blood

Analyte	Concentrations (μg/mL)	Intraday			Interday		
		Mean ± SD	CV	Accuracy	Mean ± SD	CV	Accuracy
PQ	1	1.13 ± 0.03	2.2	112.7	1.12 ± 0.05	4.7	112.3
	2.5	2.51 ± 0.05	2.0	100.5	2.42 ± 0.05	2.2	96.7
	5	4.87 ± 0.09	1.9	97.3	4.72 ± 0.12	2.6	94.3
	10	9.76 ± 0.21	2.2	97.6	9.86 ± 0.16	1.6	98.5
	20	20.14 ± 0.39	1.9	100.7	20.59 ± 0.20	1.0	102.9
DQ	1	1.01 ± 0.06	5.5	101.4	0.96 ± 0.03	2.8	96.2
	2.5	2.49 ± 0.11	4.5	99.6	2.55 ± 0.06	2.3	101.5
	5	4.93 ± 0.18	3.7	98.6	5.067 ± 0.19	3.7	101.2
	10	10.19 ± 0.25	2.5	101.9	10.35 ± 0.19	1.9	103.5
	20	19.91 ± 0.98	4.9	99.6	19.67 ± 0.22	1.1	98.4
GLUF	1	1.07 ± 0.03	2.6	107.0	1.06 ± 0.03	2.4	106.1
	2.5	2.53 ± 0.03	1.3	101.1	2.48 ± 0.02	1.0	99.1
	5	4.90 ± 0.03	0.6	98.1	4.84 ± 0.06	1.1	96.8
	10	9.88 ± 0.18	1.8	98.8	9.84 ± 0.15	1.5	98.4
	20	20.06 ± 0.15	0.8	100.3	20.23 ± 0.21	1.0	101.1
GLYP	1	1.09 ± 0.13	12.0	109.4	1.08 ± 0.05	4.3	108.3
	2.5	2.54 ± 0.10	4.1	101.7	2.45 ± 0.12	4.7	98.0
	5	4.79 ± 0.11	2.4	95.9	4.73 ± 0.07	1.5	95.4
	10	9.69 ± 0.17	1.8	96.9	9.65 ± 0.16	1.6	96.5
	20	20.36 ± 0.52	2.6	101.8	20.56 ± 0.22	1.1	102.8

Table VII. Intraday and interday precision and accuracy data as presented in percentage ($n = 5$)—urine

Analyte	Concentrations (μg/mL)	Intraday			Interday		
		Mean ± SD	CV	Accuracy	Mean ± SD	CV	Accuracy
PQ	1	1.10 ± 0.01	1.3	109.8	1.11 ± 0.09	7.7	111.1
	2.5	2.44 ± 0.08	3.4	97.7	2.44 ± 0.07	2.7	97.7
	5	4.73 ± 0.06	1.2	94.6	4.79 ± 0.09	1.8	95.8
	10	9.80 ± 0.13	1.4	98.0	9.75 ± 0.18	1.8	97.5
	20	20.51 ± 0.10	0.5	102.5	20.29 ± 0.22	1.1	101.5
DQ	1	1.00 ± 0.07	6.9	100.3	1.00 ± 0.01	1.2	99.7
	2.5	2.59 ± 0.10	3.7	103.5	2.53 ± 0.07	2.9	101.0
	5	5.07 ± 0.32	6.2	101.3	5.00 ± 0.21	4.3	100.1
	10	9.99 ± 0.21	2.1	99.9	10.12 ± 0.21	2.1	101.2
	20	20.22 ± 1.02	5.1	101.1	20.08 ± 0.39	1.9	100.4
GLUF	1	1.06 ± 0.01	1.2	105.9	1.08 ± 0.04	3.3	107.7
	2.5	2.44 ± 0.05	1.9	97.4	2.45 ± 0.05	2.2	98.2
	5	4.84 ± 0.06	1.3	96.8	4.82 ± 0.05	1.0	96.4
	10	9.93 ± 0.10	1.0	99.3	9.81 ± 0.10	1.1	98.1
	20	20.18 ± 0.19	1.0	100.9	20.28 ± 0.20	1.0	101.4
GLYP	1	1.11 ± 0.02	1.8	111.0	1.13 ± 0.11	9.9	112.6
	2.5	2.47 ± 0.11	4.5	98.8	2.42 ± 0.10	4.1	96.8
	5	4.69 ± 0.08	1.8	93.9	4.78 ± 0.16	3.2	95.6
	10	9.63 ± 0.18	1.9	96.3	9.64 ± 0.32	3.3	96.5
	20	20.72 ± 0.19	0.9	103.6	20.34 ± 0.47	2.3	101.7

Table VIII. Linearity (1–20 μg/mL), LODs and LOQs data resulting from the analysis of the analytes in blood and urine matrices

Sample	Compound	Linearity (r^2)	LOD (μg/mL)	LOQ (μg/mL)
Blood	PQ	0.9993	0.1	0.2
	DQ	0.9996	0.05	0.1
	GLUF	0.9998	0.01	0.1
	GLYP	0.9995	0.1	0.1
Urine	PQ	0.9996	0.1	0.2
	DQ	0.9998	0.05	0.1
	GLUF	0.9996	0.1	0.2
	GLYP	0.9997	0.1	0.1

frequently found in suicide cases in the past (4, 5, 11); this phenomenon appears to remain an issue of social concern at this date.

Conclusion

With knowledge accumulated over years of practice in utilizing the LC–MS-MS instrumentation for the analysis of drugs, we have successfully developed an effective protocol for the analysis of four highly water-soluble herbicides in postmortem blood and urine samples. In summary, the protein-precipitation and organic

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Table IX. Analyte/ISs peak area ratios observed under various medium pH

Herbicides	pH value					
	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
PQ	7.02 ± 0.10	7.05 ± 0.12	7.04 ± 0.08	7.07 ± 0.19	7.00 ± 0.06	7.18 ± 0.02
DQ	17.27 ± 0.21	16.92 ± 0.54	16.74 ± 0.19	17.02 ± 0.05	17.68 ± 0.17	17.59 ± 0.33
GLYP	1.20 ± 0.16	0.89 ± 0.01	0.89 ± 0.01	0.89 ± 0.11	0.90 ± 0.01	0.89 ± 0.01
GLUF	0.94 ± 0.01	0.94 ± 0.01	0.95 ± 0.01	0.95 ± 0.01	0.95 ± 001	0.95 ± 0.01

Table X. Analytical findings of PQ and GLYP (μg/mL) in 22 postmortem case samples[a]

Case	Sample type	PQ	GLYP
1[b]	Blood	2.9	
2	Blood	18.5	3.4
3	Blood	616.5	
4[b]	Blood	4.9	
5	Blood	494.2	
	Gastric content	21617.2	
6[b]	Blood	30.3	
	Gastric content	3.0	
7	Blood	1108.8	
8	Blood	12.1	
9	Blood	379.3	
	Gastric content	645.1	
10[b]	Blood	6.1	
11	Blood	379.4	
	Gastric content	5918.0	
12	Vitreous humor	200.0	
13	Blood	476.7	
	Gastric content	1958.0	
14	Blood	663.7	
15	Blood	100.0	
16	Blood	79.8	
17[b]	Urine	0.7	0.6
18	Blood		282.3
	Gastric content		20492.9
	Urine		11549.2
19	Blood		3564.2
20	Blood		151.2
	Gastric content		5204.3
21	Blood		1598.1
22	Blood		346.6

Mean and range (blood): 291.5 and 2.9–1108.8 (PQ); 991.0 and 3.4–3564.2 (GLYP). Mean and range (gastric content): 6028.3 and 3.0–21617.2 (PQ); 12848.6 and 5204.3–20492.9 (GLYP).

[a]Neither DQ nor GLUF was detected in these case samples.

[b]Victims were sent to hospitals and died after unsuccessful treatment.

solvent-backwashing approach is effective for sample preparation, using a sample size as small as 0.1 mL—thanks to the specificity and sensitivity of the LC–MS-MS methodology. The short analytical time (10 minutes) is especially valuable for the incorporation of this method into an overall analytical scheme designed for the analysis of a broad range of compounds, possibly present in postmortem samples, to assist medical examiners determining victims' causes of death.

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