Case Report


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Abstract

In Japan, poisonings by the glyphosate (GLYP)-containing herbicide Roundup and the gluphosinate (GLUF)-based herbicide BASTA® have been increasing since about 1987. We applied the gas chromatography-mass spectrometry (GC-MS) method of analysis, on which we have already reported in regard to the determination of the blood serum level of GLUF and its metabolite, for the determination of serum and urinary levels of GLYP and its metabolite aminomethyl phosphonic acid (AMPA). Derivatization using N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide was completed at a temperature of 80°C after 30 min, and the detection limit of GLYP was 10 pg using $m/z$ 454 and that of AMPA was 1 pg using $m/z$ 396. The full mass spectra of 100 pg GLYP and of 10 pg AMPA were obtained easily. In extractions for which the Isolute HAX cartridge was employed, the mean recovery rate of GLYP and AMPA added to serum to yield concentrations of 10-0.1 pg/mL (n = 5) was 91.6 ± 10.6% (or better), whereas that of GLYP and AMPA added to urine to yield concentrations of 100-1.0 pg/mL (n = 10) was 93.3 ± 6.6% (or better), both of which were good rates. Also, using this method of analysis, the presence of GLYP was identified in the full mass spectra obtained from the serum of a patient who may or may not have ingested Roundup.

Introduction

The phosphorus-containing amino acid-type herbicides, glyphosate [N-(phosphonomethyl)glycine] (GLYP) and gluphosinate [DL-homoalanine-4-yl(methyl)phosphinate] (GLUF), are broad-spectrum, non-selective herbicides with an increasing number of users, both in Japan and around the world (1).

Roundup herbicide, which was first available in the U.S. in 1974, contains 41% GLYP. The majority of the 56 cases of poisoning due to Roundup in Japan were marked by various symptoms and signs including pain in the pharynx and abdomen, digestive problems such as vomiting, falling blood pressure, and moderate impairment of consciousness. There is also a report of death after the ingestion of 100 mL or more of Roundup (2). Lee et al. (3) reported similar effects in 131 cases of ingestion of herbicide containing GLYP. In animal experiments on rats, on the other hand, the toxicity of GLYP was low, and the toxic symptoms resulting from Roundup ingestion were thought to be caused mainly by the surfactant that it contains (2,4). In addition, as Figure 1 shows, some GLYP molecules are metabolized into aminomethyl phosphonic acid (AMPA), the major metabolite (1).

In contrast, poisoning by BASTA, which was registered in 1984 as a herbicide and contains GLUF (5), is characterized by coma, apnea, and generalized convulsions that occur abruptly after a latent period lasting 4 to 60 h (6-10). The severity of the delayed central nervous system symptoms can be predicted from the time elapsed since GLUF ingestion and the serum concentration of GLUF (11). Therefore, immediate analysis of the serum GLUF level is of great importance because it offers an important index of whether or not management of the patient is necessary.
by artificial ventilation is necessary.

We reported the use of GC–MS analysis for determining the serum levels of GLUF and of the GLUF metabolite 3-methylphosphonicopropionic acid (MPPA) with the help of GLYP as the internal standard, in order to identify GLUF ingestion by a patient and to determine the blood concentration at the same time (12). However, approximately the same high number of cases of poisoning are caused in Japan by BASTA as by Roundup (13), and it was considered that a simultaneous analysis of both GLYP and GLUF was necessary. An examination of the literature revealed no previous report of a simultaneous analysis of GLYP and GLUF and their metabolites in biological specimens. The present study used DL-2-amino-3-phosphonopropionic acid (APPA) as the internal standard in an application of our GC–MS method for a simultaneous analysis of GLYP and its metabolite AMPA in the serum and urine of a patient with Roundup poisoning.

Case History

The patient was a 58-year-old woman who, in a suicide attempt, ingested 100 mL of herbicide that was kept in a store-room in her house. Vomiting started 30 min after ingestion and continued intermittently. After 8 h, she visited the hospital of her own accord for a medical examination. On arrival, she was lucid, and her pupil diameter, blood pressure, and pulse showed no abnormalities, but gastric lavage was performed as an initial treatment. Questioning of the patient’s family revealed that both Roundup and BASTA were stored in her house, but that the remaining amounts of the two herbicides did not indicate which she had drunk. On her arrival at the hospital (8 h after herbicide ingestion), we carried out an analysis of her blood serum to discover what she had ingested.

Analysis of her serum identified no GLUF and none of its metabolite MPPA, but GLYP and its metabolite AMPA were detected. On the basis of this result, artificial ventilation was not used for management of her condition, but she was given a saline infusion and kept under observation. On her second day of hospitalization, the patient recovered from her nausea and no abnormality was observed in her state of consciousness or her blood pressure. She was discharged on the seventh day of hospitalization. Also, a serum sample, collected 16 h after ingestion, and the urine, collected until the 7th day of hospitalization, were stored frozen at −40°C for later analysis.

Experimental

Chemicals and solutions

GLYP was purchased from Wako (Osaka, Japan), AMPA from Sigma Chemical Co. (St. Louis, MO), and APPA (as the internal standard) and N-methyl-N-(tert-butyldimethylsiloxy)trifluoroacetamide (MTBSTFA) from Aldrich Chemical Co. (Milwaukee, WI). All other compounds were of analytical reagent grade and obtained from Wako.

Biological specimen collection

The standard human serum specimens used for GLYP and AMPA recovery were purchased from Sigma Chemical Co. The urine specimens used for GLYP and AMPA recovery were from healthy adult volunteers (five men, five women), and were collected with the subjects’ consent.

GC–MS analysis

In the reports previously published on analytical methods for GLUF and MPPA (12), the internal standard used was GLYP. In the present study, APPA was used for this purpose, but no changes were made in specimen preparation, derivatization, or GC–MS conditions.

Briefly, a 100-mg Isolute HAX cartridge (International Solvent Technology Ltd., Mid Glamorgan, U.K.) was conditioned by washing with 1 mL of methanol followed by washing with 1 mL of 0.1 mol/L NaOH at a flow rate of 1 mL/min. A 100-μL volume of acetone was added to a 100-μL blood serum sample or to a 100-μL urine sample previously diluted 1:10 with distilled water and supplemented with 10 μL of the internal standard solution containing 1 μg of APPA. After being mixed for 2 min, the mixture was centrifuged at 1200 × g for 3 min. The aqueous phase was dissolved in 1 mL of distilled water, and applied to the cartridge. After washing with 1 mL of water, GLYP, AMPA, and APPA were eluted with 200 μL of 1 mol/L hydrochloric acid/methanol (4:1) at a flow rate of 200 μL/min. The solvent was evaporated to dryness at a reduced pressure and a temperature of 50°C.

To the dry residue were added 50 μL of MTBSTFA and 50 μL of dimethylformamide. The mixture was sonicated for 2 min at room temperature, then heated at 80°C for 30 min. After cooling to room temperature, samples of the solution containing the derivatives were used directly for GC–MS (Shimadzu GC17A

Figure 2. Kinetics curves for GLYP, GLUF, and their metabolites at 80°C. The reaction mixture consisted of AMPA (1 μg, *), MPPA (1 μg, ■), GLYP (1 μg, ▲), GLUF (1 μg, ○), and APPA (internal standard IS, 1 μg) in 100 μL of MTBSTFA-dimethylformamide (1:1). A 1-μL aliquot was injected into the GC–MS. For GC–MS conditions, see the Experimental section.
GC and QP5050A MS).

Chromatographic conditions for these analyses were as follows: a DB-5 fused-silica capillary column (15-m x 0.25-mm i.d., 0.25-μm film thickness, J&W Scientific, Folsom, CA); helium carrier gas at 1 mL/min; GC oven temperature program, 80°C (hold 2 min), 15°C/min to 300°C (hold 5 min); injector temperature, 300°C; split/splitless injector, splitless mode for 2 min; and injection volume, 1 μL. The MS was operated in the electron impact (EI) mode. EI ionization was employed at 70 eV with an electron multiplier set at 1200 V in either full scan operation mode for peak identification or SIM mode for quantitation purposes. The manifold temperature was 280°C.

Recovery test
Ten microliters each of GLYP solution and AMPA solution were added together to multiple 1-mL samples of standard serum to yield concentrations of 0.1 μg/mL, 1.0 μg/mL, and 10.0 μg/mL of GLYP and of AMPA. Next, the urinary concentrations of these two substances were also adjusted to 1.0 μg/mL, 10.0 μg/mL, and 100.0 μg/mL. Preparation and derivatization were performed, and quantitations of GLYP and AMPA were carried out by the internal standard method. Then the measured concentrations of GLYP and AMPA in blank serum and urine to which GLYP and AMPA had not been added were subtracted from the resulting values, and the recovery rates for the measured concentrations in the standard solutions of GLYP and AMPA were calculated (serum, n = 5; urine, n = 10).

Results and Discussion

GC–MS analysis
In the past, the determination of GLYP in biological specimens has been accomplished by HPLC (14–17) and by GC–MS (18). However, we were unable to find any published reports on the simultaneous measurement of the levels of GLYP and GLUF and their metabolites. In the present study, we have confirmed that our method of GC–MS analysis of serum GLUF and MPPA already reported (12) can be applied, by using APPA as the internal standard, to the simultaneous analysis of herbicides containing phosphorus-containing amino acids including GLYP and AMPA, together with their metabolites in the serum. The rates of the tert-butyldimethylsilyl (t-BDMS) derivatization reactions for GLYP, AMPA, GLUF, and MPPA are similar (Figure 2).

Table I. Recoveries of GLYP and AMPA from Biological Specimens

<table>
<thead>
<tr>
<th>Added*</th>
<th>GLYP Recovery* RSD (%)</th>
<th>AMPA Recovery* RSD (%)</th>
<th>GLYP Recovery* RSD (%)</th>
<th>AMPA Recovery* RSD (%)</th>
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<tr>
<td>100</td>
<td>96.6 ± 4.5</td>
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<td>98.3 ± 3.6</td>
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<td>10</td>
<td>94.3 ± 6.9</td>
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<td>96.7 ± 5.5</td>
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</table>

* Amounts are expressed as μg/mL of specimen.
* Values are mean ± SD, n = 5.
* Values are mean ± SD, n = 10.
The conditions for derivatization that were selected on the basis of the results of this, were 80°C for 30 min. Using 1 μg of APPA as the internal standard, 1 μg of each of GLYP, AMPA, GLUF, and MPPA was derivatized five times, and the relative standard deviation (RSD) resulting from GC–MS analysis was 2.3%.

The DB-5 capillary column was able to clearly separate the t-BDMS derivatives of GLYP, AMPA, GLUF, and MPPA, and of APPA as the internal standard (Figure 3). The fragment ions at [M-57]+ (loss of C(CH₃)₃), that is, m/z 454, 396, and 568, were the base peaks for the GLYP, AMPA, and APPA derivatives, respectively (Figure 4). Quantitative determination was performed in the selective ion mode using these base ions. The calibration curves for GLYP and AMPA analyses, in which APPA was used as the internal standard, were linear over the ranges 100 pg–100 ng (y = 21.1x - 0.06, r = 0.999) and 10 pg–10 ng (y = 0.82x + 0.03, r = 0.999). The respective detection limits of GLYP and AMPA for the signal-to-noise ratio of 5 were 10 pg and 1 pg. The full mass spectra of 100 pg GLYP and 10 pg AMPA were obtained easily.

The recoveries of GLYP and AMPA added to the serum and urine are shown in Table 1. When GLYP and AMPA were added to the serum to give concentrations of 10–0.1 μg/mL, the mean recovery was 91.6 ± 10.6% (or better), and when GLYP and AMPA were added to the urine to give concentration of 100–1 μg/mL, the mean recovery was 93.3 ± 6.6% (or better), demonstrating excellent results. Under these extraction conditions, GLUF and MPPA had approximately the same recoveries (12).

Analysis of patient blood serum and urine

A chromatogram of the patient’s serum obtained 8 h after she ingested the herbicide is presented in Figure 5. The t-BDMS derivatives of GLUF and MPPA give base ions at m/z 466 and 323, respectively (1,12). However, clear peaks were not seen with SIM mode chromatography using these base ions. In contrast, with SIM mode chromatography using the ions at m/z 454 and 396, separate peaks were seen for GLYP and AMPA at their respective retention times. In addition, it was confirmed with full scan mass fragments that these peaks were those for GLYP and AMPA, their respective concentrations being 22.6 μg/mL and 0.18 μg/mL.

Determinations of GLYP and AMPA yielded levels of 4.4 μg/mL and 0.03 μg/mL, respectively, 16 h after the poison ingestion. By means of this analysis, it was possible to deduce that the patient had ingested a herbicide containing GLYP, rather than one containing GLUF. If this analysis had not been performed, a delayed respiratory disturbance may have been predicted, and as a result, management of artificial ventilation not required in cases of poisoning with GLYP herbicide may have been carried out. In Japan, Roundup and BASTA came into widespread use about 1987, when the manufacture of paraquat preparations was discontinued (13). However, because poisonings by the two newer preparations require different treatments, a method of analysis that can simultaneously identify GLYP and GLUF is extremely useful for treatment planning.

Few studies have been done on the analysis of the GLYP content of human clinical specimens after poisoning by GLYP-containing herbicides. Hiraiwa et al. (16) used fluorescence derivatization HPLC (19) to determine serum levels of GLYP, and reported a case in which a patient who had ingested 80 mL of Roundup was found to have a serum GLYP concentration of 600 μg/mL 13 h later. This patient had diarrhea for two days, but improvement of the condition was seen, and on the fourth day, full recovery took place, and no sequelae remained. In the case that we treated, the blood level of GLYP 8 h after ingestion was 22.6 μg/mL, which was considered low for ingestion of 100 mL of Roundup. In animal experiments, the principal route of elimination of GLYP absorbed from the digestive tract was via the urine (13). Urinalysis in this case resulted in the detection of GLYP and AMPA until the fourth day of hospitalization, the total amounts being 3.7 g of GLYP and 25 mg of AMPA, which was calculated to correspond to the ingestion of 9 mL of Roundup. Before this patient arrived at the hospital, she had vomited many times, and the amount of GLYP actually absorbed was therefore probably less than would otherwise have been the case.

Finally, the ratio of the serum concentrations of GLYP and AMPA was 126:1 8 h after ingestion and 147:1 16 h after ingestion. The ratio between the total amounts of GLYP and AMPA eliminated in the urine was 148:1, indicating that probably only a small amount of the ingested GLYP was metabolized to AMPA.

The experience of this case in an emergency clinical setting shows that analysis of the phosphorus-containing amino-acid-type herbicides can both offer advantages for future methods of treatment and provide useful information on toxicokinetics.

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References


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