HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY/UV DETECTION FOR DETERMINATION OF GLYPHOSATE IN SERUM AND GASTRIC CONTENT


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Abstract

In Thailand, poisoning by glyphosate (GLYP)-containing the herbicide, Roundup®, has been increasing. A high-performance liquid chromatographic (HPLC) method with UV detector has been developed for quantitating glyphosate. The serum samples (0.2 mL) were precipitated with acetonitrile (0.2 mL), and the supernatant was then injected directly into the chromatographic system using a fixed volume loop. The glyphosate was eluted on an adsorbosphere XL SAX 90A 10μ column (25 cm x 4.6 mm, I.D.). The mobile phase consisted of 0.8437 g of KH2PO4 in 960 mL of H2O and 40 mL of MeOH, and the pH was adjusted to 2.1 with 85% H3PO4. The peak area response, as measured by a UV detector, was quantitated by the external standard technique at 195 nm. The retention time of glyphosate was 7.3 min. The concentration was detected between 0.25-4.0 g/L. The limits of detection (LOD) and quantification (LOQ) were 0.27 and 0.44 g/L, respectively. The mean recovery was 92.1% and the coefficients of variation (CV) ranged from 6.7-12.9%. This analytical method is simple, and therefore appropriate for forensic purposes. Two fatal cases of glyphosate poisoning are presented, with a lethal serum concentration of 0.57 and 3.05 g/L, respectively. Chiang Mai Medical Journal 2008;47(4):155-162.

Keywords: glyphosate, high-performance liquid chromatography, poison, validation method

Glyphosate (GLYP) is a widely used broad-spectrum herbicide that kills unwanted plants. It acts through inhibition of the shikimate metabolic pathway in plants. This pathway is not present in mammals; therefore, glyphosate is classified as a low toxicity compound to mammals. However, glyphosate intoxication, including fetal cases, has been reported. An analysis of glyphosate in body fluid is crucial for the diagnosis of glyphosate intoxication. Several methods for determining glyphosate in different matrices (urine, serum) have been described. Glyphosate can be analyzed by
using thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) or nuclear magnetic resonance (NMR) techniques. The sensitivity is varied in different methods. In acute massive glyphosate ingestion, detection of glyphosate in gastric contents and serum is a part of the diagnosis. In this study, we verified the method for determining glyphosate in serum by using an HPLC with UV detector. This technique was less complicated than the HPLC previously reported, and the technique applicable for forensic cases in which a massive amount was ingested.

Materials and methods

Chemicals and reagents

The analytical reference standard glyphosate was kindly provided by the Monsanto Company, St. Louis, USA. Potassium dihydrogen phosphate was purchased from Fluka, Switzerland. Phosphoric acid was purchased from JT Baker, USA. Methanol and Acetonitrile HPLC grade were obtained from Fisher Chemical, UK. Drug-free human serum was obtained from the blood bank’s pooled serum at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand.

Equipment

A high-pressure system was used, consisting of a high-performance liquid chromatography 9012Q Pump Solvent Delivery System, ProStar 310 UV/Vis Detector, Rheodyne Injector equipped with a 20 μL loop, and star chromatography workstation (Varian™ Chromatography system, USA). The HPLC column was an adsorbosphere XL SAX 90A 10μ from Alltech, USA, and the guard cartridge adsorbosphere XL 90A SAX 5μ was also from Alltech.

Chromatographic conditions

The isocratic mobile phase consisted of 0.8437 gm of KH₂PO₄ dissolved in 960 mL of ultrapure water and 40 mL of MeOH, and the pH was adjusted to 2.1 with 85% phosphoric acid. A flow rate of 2.0 mL/min signals was monitored at 195 nm. The total run time was 20 min. Glyphosate was detected at 7.3 mins.

Preparation of calibration curve and quality control samples

A stock standard solution of glyphosate was prepared at the concentration of 10 g/L in ultrapure water. The standard solution was stable for more than one week at room temperature.

Calibration curves were prepared in water and drug-free human serum. Appropriate volumes of the stock solutions were added to each microtube (1.5 mL) containing 200 μL of water or drug-free human serum. Final concentrations of glyphosate in water and serum were adjusted to 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 g/L, and 0.25, 0.5, 1.0, 2.0 and 4.0 g/L, respectively.

Preparation of quality control samples was conducted by adding glyphosate into 200 μL of drug-free human serum, making final concentrations of 0.50, 1.0 and 3.0 g/L.

Samples preparation

Gastric content collected from victims was diluted with distilled water, then directly injected into the HPLC with standard concentrations of glyphosate in water (0.125-4.0 g/L).
A 200 μL of serum sample was added to 200 μL of acetonitrile for protein precipitation, then mixed and centrifuged at 8,000 rpm for 2 minutes. A 20 μL of supernatant was injected into the HPLC apparatus. The chromatogram of glyphosate is shown in Fig. 1. Quantification was carried out using a calibration curve of glyphosate. However, pure standards were also analyzed to determine recovery of the extraction process.

**Validation of the method**

The recovery of an assay was determined by comparing the analyst peak areas obtained from the quality control samples (n = 10) after precipitating protein to those obtained from the corresponding reference standard, which were prepared in water at the same concentration.

For linearity study, duplicate calibration curves were obtained in 1 day. Quantification was calculated from the peak area of glyphosate. The limit of detection (LOD) was defined as the lowest concentrations of the analyst, which produced a peak response corresponding to analysis. The average response (X̄) and standard deviation (SD) were calculated. The LOD was determined from X̄ + (3 SD).

The limit of quantitation (LOQ) was defined as the lowest concentration of the analyst, which can be measured with both an accuracy of + 10% of the true value and a coefficient of variation (CV) ≤ 20%. The LOQ was calculated from X̄ + (10 SD).

The within run and between run coefficients of variation were determined by replicate analysis of 0.50, 1.0 and 3.0 g/L of glyphosate in human serum, on either the same or separate days. The accuracy was determined by comparing the mean calculated concentration with the spiked target concentration of the quality control samples.

**Application of this technique to the postmortem biological samples**

The technique described has been used to measure glyphosate concentration in sera and gastric content samples from two fatal glyphosate poisoning cases.

**Results**

Under this condition, the retention time of glyphosate was 7.3 min. In all drug-free sera, there was no peak at this retention time. The chromatogram of spiked glyphosate in drug-free serum (1.0 g/L) is shown in Figure 1A. The percentage recovery of glyphosate at a concentration of 0.5, 1.0 and 3.0 g/L were 96.4, 91.5 and 88.3%, respectively. The calibration curves of glyphosate analyzed in water and serum, as shown in Figure 2, were linear in the ranges of 0.125-4.0 g/L (y = 0.0047x + 85.346, r = 0.998) and 0.25-4.0 g/L (y = 0.0106x + 52.082, r = 0.999), respectively. All validation criteria complied with the international standard, in which the intra-assay coefficient of variation less than 7.6%, inter-assay coefficient of variation was less than 12.9% and both intra-assay and inter-assay accuracies equal to or more than 80% (Table 1). The limit of detection (LOD) and quantitation (LOQ) was 0.27 and 0.44 g/L, respectively.

The technique described has been used to detect glyphosate in the sera and gastric contents of two massive fatal glyphosate poisoning cases. The chromatograms obtained from the serum and gastric content (dilution 1:50) of both cases are shown in Figure 1B, 1C, and Figure 3A and 3B. The quantitative results are demonstrated in Table 2.
Figure 1. Chromatogram of spiked glyphosate (1.0 g/L) in drug-free serum (200 μL) (1A), serum of first case report with a measured concentration of 3.05 g/L (1B) and serum of second case report with a measured concentration of 0.57 g/L (1C)
Figure 2. Calibration curves of glyphosate analyzed in water and serum.

Table 1. Precision and accuracy of the HPLC method for glyphosate in serum.

<table>
<thead>
<tr>
<th>Glyphosate (g/L)</th>
<th>Intra-assay (n=10)</th>
<th>Inter-assay (n=5)</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>%SE</td>
</tr>
<tr>
<td>0.5</td>
<td>0.40±0.06</td>
<td>15</td>
</tr>
<tr>
<td>1.0</td>
<td>0.90±0.12</td>
<td>8</td>
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<tr>
<td>3.0</td>
<td>2.85±0.40</td>
<td>5</td>
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Discussions and conclusion

Although glyphosate herbicide is claimed to be very low in acute toxicity and classified by the U.S. Environmental Protection Agency (EPA) as being in the least toxic category (class IV),\(^{16}\) massive oral ingestions and fatalities have been reported from time to time.\(^{17-19}\) The detection of glyphosate in the biological fluids of victims is necessary to diagnose glyphosate poisoning. Many techniques were reported in detecting glyphosate, but sophisticated equipment and techniques are needed. On the other hand, HPLC with a UV detector is a standard instrument in many forensic toxicological laboratories. However, few studies have reported the analysis of glyphosate in biological specimens using HPLC with UV detector.\(^{20,21}\) Tomita M,
Table 2. Quantitative data of the two massive fatal glyphosate ingestion cases

<table>
<thead>
<tr>
<th></th>
<th>Glyphosate (g/L)</th>
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<tbody>
<tr>
<td>Serum</td>
<td>Gastric content</td>
<td></td>
</tr>
<tr>
<td>First case</td>
<td>3.05</td>
<td>59.72</td>
</tr>
<tr>
<td>Second case</td>
<td>0.57</td>
<td>28.75</td>
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</table>

*et al.*²¹ used HPLC with UV detection for determining glyphosate in serum after extraction with an anion exchange resin column, and allowing it to react with p-toluene sulfonic chloride. The limit of glyphosate detection was 0.0003 g/L. The percentage recovery of glyphosate detected in serum was 75%, which shows that this method is highly sensitivity, and can detect a small amount of glyphosate in the serum of poisoned victims. However, it needs many steps of extraction. Compared to the method in this report, the analysis method was far more simplified. The specimen preparation before analysis was simple, with no derivatization. Nevertheless, the LOD was higher, but the percentage recovery better. This present method can be used to detect lyphosate in both serum and gastric contents in massive glyphosate poisoning cases.

In conclusion, the technique to measure the serum concentration of glyphosate...
has been validated. The calibration curve is linear. The inter- and intra-assay are within 13% and the accuracies are good. Only a small amount of specimen is needed. The analysis method is simple, using the protein precipitation technique for serum before directly injecting into HPLC. Nevertheless, the sensitivity was lower when compared to other methods. This technique is therefore suitable for cases of glyphosate overdose. It is applicable for fast quantitative analysis, especially in forensic toxicology that has constraints regarding sophisticated equipment.

Acknowledgements
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References
การตรวจหารำกำจัดวัชพืชไกลโฟลเซตในซีรั่มและสารในกระเพาะอาหารโดยวิธี HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/UV DETECTION


บทคัดย่อ ใบประเทศไทยปัจจุบันพบมีผู้เสียชีวิตจากการกินสารกำจัดวัชพืชไกลโฟลเซตซึ่งเป็นสารประกอบสำคัญในสารเคมีกำจัดวัชพืช "ราวดอก" ผู้วิจัยได้ทำการพัฒนาวิธีการตรวจหารำกำจัดวัชพืชไกลโฟลเซตโดยใช้เทคนิค high-performance liquid chromatography (HPLC) ร่วมกับเครื่องวัดแสงชนิด UV detector วิธีการเตรียมตัวอย่างทำได้โดยการตกตะกอนโปรตีนซีรั่ม 200 ไมโครลิตร แล้วตั้งทำละลาย acetonitrile 200 ไมโครลิตร นำส่วนตัวอย่างชีวิตซึ่ง HPLC โดยใช้ยี่ขันหน้า adsorbosphere XL SAX 90A 10 μ โดยเก็บชีวิต 200 ไมโครลิตร การแยกสารไกลโฟลเซตจากตัวอย่างโดยใช้สารละลายของสาร KH2PO4 0.8437 กรัมละลายในน้ำกลั่น 960 มิลลิลิตร ผสมกับ MeOH ปริมาตร 40 มิลลิลิตร ปรับให้เป็นกรด pH 2.1 ด้วย 85% ของกรดฟอสโฟลิค วัดด้วย UV detector ที่ความยาวคลื่น 195 นาโนเมตร ตรวจหารำไกลโฟลเซตที่ retention time 7.3 นาที สามารถตรวจคั่นตั้งแต่ 0.25-4.0 กรัม/ลิตร ความสามารถในการตรวจหารำ ต่ำสุด (LOD) เท่ากับ 0.27 กรัม/ลิตร และสามารถหาปริมาณได้ต่ำสุด (LOQ) เท่ากับ 0.44 กรัม/ลิตร รอบของการตรวจหารำไม่เกิน 2 ชั่วโมง สามารถตรวจหารำไกลโฟลเซตได้ตั้งแต่ 0.05 กรัม/ลิตร เชิงซีรั่มของผู้ป่วย 2551;47(4):155-162.

คำสำคัญ: ยาฆ่าหญ้า, ไกลโฟลเซต, HPLC, สารพิษ