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Short Communication

High-performance liquid chromatographic determination of glyphosate and (aminomethyl)phosphonic acid in human serum after conversion into *p*-toluenesulphonyl derivatives

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ABSTRACT

We have developed a simple, highly sensitive and fast assay method for determining glyphosate and its major metabolite, (aminomethyl)phosphonic acid (AMPA), in serum by high-performance liquid chromatography with ultraviolet detection. Both compounds were successfully extracted with an anion-exchange resin column and allowed to react with *p*-toluenesulphonyl chloride. The detection limits were 0.3 μg/ml for glyphosate and 0.2 μg/ml for AMPA. Recoveries of glyphosate and AMPA spiked to serum were *ca.* 75% and *ca.* 88%, respectively. We are convinced that this procedure, in practice, allows medical examiners to analyse both compounds in the serum of poisoned patients within a short time.

INTRODUCTION

Glyphosate, N-(phosphonomethyl)glycine, has been used extensively as the active ingredient of the commercial herbicide Roundup®, which has a broad spectrum and is a non-selective weedkiller. (Aminomethyl)phosphonic acid (AMPA) has been shown to be the major metabolite in plants, water and soil. In spite of the comparatively low toxicity of glyphosate, this formulation has been used as a suicide agent in Japan and other countries. Glyphosate has been analysed by gas chromatographic [1–3] and high-performance liquid chromatographic (HPLC) [4–9] methods. Most of these methods, however, have been applied to the determination of both glyphosate and AMPA in vegetables, fruits,

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water and soil, and determination in biological specimens has been extremely tedious [10–12]: very time-consuming, or not adequately quantitative, or not readily acceptable (*e.g.* NMR).

We previously developed a simple HPLC method for the determination of glyphosate and AMPA as the *p*-toluenesulphonyl derivatives [13], and have now applied this method extensively to the determination of glyphosate and AMPA in human serum extracted with an anion-exchange resin column.

EXPERIMENTAL

Materials

Glyphosate was generously donated by Monsanto Japan (Tokyo, Japan), and AMPA was obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile was purchased from Nacalai Tesque (Kyoto, Japan). *p*-Toluenesulphonyl chloride was purchased from Tokyo Kasei (Tokyo, Japan). An anion-exchange resin (AG-1X8, 100–200 mesh, chloride form) was obtained from Bio-Rad (Richmond, CA, U.S.A.). Before use, the *ca.* 150 ml of resin were converted in a suitable column from chloride form into the acetate form, by washing successively with 3.0 l of 1 *M* NaOH, 1.5 l of distilled water, 1.2 l of 1 *M* acetic acid and 1.5 l of distilled water. The prepared resin was stored at 4°C until used. All other chemicals were of analytical reagent grade.

Stock solutions

Glyphosate and AMPA were dissolved in doubly distilled water at concentrations of 1 mg/ml and stored at 4°C in plastic tubes. These solutions were used to prepare standard solutions and spiked sera containing various concentrations of both compounds.

Sample preparation

A 0.5-ml volume of serum was diluted 1:8 with distilled water, and then mixed with 1 ml of 10% trichloroacetic acid solution. After centrifugation (900 *g* for 5 min), the supernatant was washed twice with 8 ml of diethyl ether, and then diluted with distilled water to 20 ml. This supernatant was applied to the prepared anion-exchange resin column (90 mm × 16 mm *I.D.*), washed with 30 ml of distilled water and then eluted with 30 ml of 1.0 *M* or 20 ml of 0.1, 0.2, 0.5 and 1.0 *M* HCl. The eluate was collected, evaporated and then reconstituted in 0.5 ml of distilled water.

Derivatization

Derivatization was performed as described previously [13]. Briefly, 0.5 ml of sample solution containing glyphosate and AMPA was mixed with 0.25 ml of 0.4 *M* phosphate buffer (pH 11.0). The mixture was allowed to react with 0.1 ml of *p*-toluenesulphonyl chloride (10 mg/ml in acetonitrile) at 50°C for 5 min in a

heating bath. An aliquot of the reaction mixture was injected into the HPLC column.

HPLC apparatus and conditions

A Model 6A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a UV spectrophotometric detector set at 240 nm was used. The HPLC separations were performed on a Cosmosil 5 C_{18} packed column (250 mm × 4.6 mm I.D., Nacalai Tesque) with a guard column (Nacalai Tesque). The mobile phase was 0.2 *M* phosphate buffer (pH 2.3)–acetonitrile (85:15, v/v). The column temperature was ambient, and the flow-rate was 1.5 ml/min. Peak areas were integrated with a C-R6A Chromatopac (Shimadzu).

RESULTS AND DISCUSSION

Although we had previously developed an HPLC method for determining glyphosate and its major metabolite AMPA simultaneously, its practical application to serum samples required the removal of interference that affected the retention time on the chromatogram and the recoveries. As both glyphosate and AMPA are anionic molecules, we examined an anion-exchange resin column. As a result, glyphosate and AMPA in deproteinized serum samples were successfully eluted with 1.0 *M* HCl and their *p*-toluenesulphonyl derivatives showed peaks at 9.8 min and 7.4 min, respectively. However, the 1.0 *M* HCl eluate still contained a small amount of interfering substance. Therefore, we examined a stepwise elution method, from 0.1 to 1.0 *M* HCl. As a result, we found that AMPA and glyphosate were separately and specifically eluted with 0.2 *M* and 1.0 *M* HCl, respectively (Fig. 1). Since an alkaline pH gave the best yields in the derivatization [13], it was important to evaporate the acid eluate completely. The detection limits obtained were 0.3 μg/ml in serum for glyphosate and 0.2 μg/ml in serum for AMPA, following a 20-μl injection, at a signal-to-noise ratio of 2. In addition, both derivatives could be extracted by ethyl acetate under acidic conditions. Thus, when necessary, it was possible to determine much lower levels of both compounds, although this could not be verified. The precision and overall average recoveries of both glyphosate and AMPA from spiked serum (2 and 20 μg/ml) are listed in Table I. Although the precision and recoveries were acceptable, the recoveries of glyphosate were lower than those of AMPA, which indicates that glyphosate is more readily linked to resin or serum components. In two earlier studies, Sprankle *et al.* [14] and Glass [4] showed that glyphosate readily adsorbed in various clay fractions and organic matter from soils.

On the other hand, in practice, the stepwise elution method is not necessary and elution with 1.0 *M* HCl is sufficient to remove interference. Glyphosate and AMPA in the serum of patients admitted to a hospital following ingestion of Roundup can be simultaneously determined after elution with 1.0 *M* HCl, since the concentration of glyphosate in the serum is estimated to be as high as 100

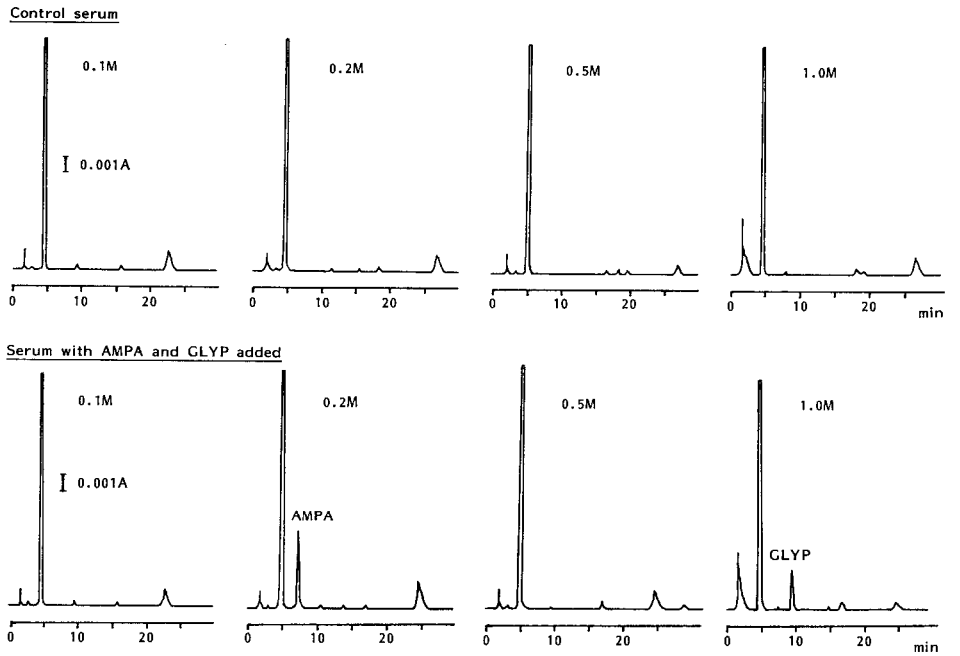

Fig. 1. Chromatograms of stepwise eluates from 0.1 to 1.0 *M* HCl. (Aminomethyl)phosphonic acid (AMPA) and glyphosate (GLYP) were separately and specifically eluted with 0.2 and 1.0 *M* HCl, respectively.

TABLE I

RECOVERY FROM SPIKED SERUM SAMPLES AND THE RELATIVE STANDARD DEVIATION (R.S.D.) OF REPLICATE ASSSAYS (n = 5)

Compound	Added concentration (μg/ml)	Recovery (%)	R.S.D. (%)
Glyphosate	2	78.5	1.1
	20	73.9	2.0
AMPA	2	90.1	5.5
	20	86.9	3.9

μg/ml [10,12]. Thus, it requires *ca.* 1 h for a serum analysis by medical examiners who must apply an emergency procedure.

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