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Quantitative determination of glufosinate in biological samples by liquid chromatography with ultraviolet detection after *p*-nitrobenzoyl derivatization

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Abstract

We have established a new HPLC method for derivatizing and quantifying glufosinate (GLUF) in human serum and urine using *p*-nitrobenzoyl chloride (PNBC). The *p*-nitrobenzoyl derivative of GLUF (PNB-GLUF) was produced quantitatively over 10 min at room temperature. PNB-GLUF possesses the property of ultraviolet (UV) light absorption with a λ_{max} of 272.8 nm, and was isolated from biological specimens by reversed-phase chromatography using Inertsil Ph-3. In experiments at a UV wavelength of 273 nm, GLUF has a quantitative detection limit of 0.005 μg/ml, and when it was added to both serum and urine to yield concentrations of 0.1–1000 μg/ml, its recovery rate was quite satisfactory: at least 93.8% in all cases. Further, the measured amounts of GLUF in 23 serum samples from patients intoxicated by ingestion of GLUF compared favorably with those obtained by fluorescence derivatization–HPLC using 9-fluorenylmethyl chloroformate (R=0.998). This technique of analysis is, in addition, applicable for Glyphosat, which possesses a chemical structure resembling that of GLUF, and it will be of great use in the determination of these two compounds. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, LC; Glufosinate

1. Introduction

Glufosinate ammonium (GLUF), the active ingredient of the non-selective herbicide BASTA, is a phosphinic acid analogue of glutamic acid, and has been registered in many countries since 1984 [1]. In Japan, poisoning of humans through the ingestion of BASTA increased in the 1990s, and has become a common form of herbicide poisoning, like that by paraquat or glyphosate (GLYP) [2–4]. The toxic symptoms of GLUF acute poisoning include a fall in the level of consciousness, respiratory arrest and general convulsions, which appear after a latent period of 4–60 h. To save the patient's life, it is important to counter these symptoms by maintaining

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the patency of the airways and administering artificial respiration [2–6].

Koyama et al. [7] reported that it is possible in advance to predict the severity of delayed central nervous system symptoms from the time that has elapsed after ingestion of GLUF and the current blood concentration. However, in a large number of cases, the patients have ingested GLUF with the intention of suicide, and often kept secret of what have been taken. Since there are no characteristic clinical symptoms during the latent period, it is necessary to carry out both qualitative and quantitative determinations of GLUF in blood.

Many reports of river water and soil analysis for the determination of GLUF levels have appeared, but there have been no international reports on the measurement of its levels in human biological samples [8]. In Japan, one method used is *O*-methyl and *N*-acetyl derivatization [9] and detection by nitrogen–phosphorus detection–gas chromatography (NPD–GC) after extraction with anion-exchange resins. Other methods of determining GLUF levels in biological specimens include fluorescence derivatization–high-performance liquid chromatography (HPLC) using 9-fluorenylmethyl chloroformate (FMOC-Cl) [10], post-column fluorescence derivatization–HPLC using *o*-phthalaldehyde [11], and direct analysis by electrochemical detection with ion-exchange chromatography without performing derivatization [12]. However, in actual clinical practice, the multi-purpose and speedy HPLC–UV technique is probably the most desirable.

Nishida et al. [13] are alone in publishing a technique of pre-column UV derivatization–HPLC in which UV absorption derivatization is performed at 60 °C for 20 min using phenyl isothiocyanate after solid-phase extraction of GLUF from human biological samples.

In the present study, we studied a method in which, after deproteinization, GLUF in serum and in urine was subjected gently and briefly (at room temperature for 10 min) to UV absorption derivatization using *p*-nitrobenzoyl chloride (PNBC) and to reversed-phase HPLC to isolate and quantify it. In addition, we carried out GLUF determinations in 23 serum samples taken from patients with GLUF poisoning, using pre-column fluorescence derivatization–HPLC with FMOC-Cl [10], and report the correlation between the results of the two types of quantification.

2. Experimental

2.1. Chemicals and solutions

GLUF and GLYP were purchased from Wako (Osaka, Japan), and PNBC was purchased from GL Sciences (Tokyo, Japan). The other reagents and culture media used were for HPLC use, or of special grade.

2.2. Biological specimen collection

The standard human serum specimens used were purchased from Sigma (St. Louis, MO, USA).

The urine specimens used for GLUF recovery were from healthy adult volunteers (five men, five women), and were collected with the subjects' consent.

The serum specimens used for application were collected from 15 patients with GLUF poisoning between 2 and 6 h after they were thought to have ingested 10–100 ml of BASTA fluid (GLUF content 18.5%) with the intention of committing suicide between the years of 1998 and 2001. A total of 23 serum specimens that had been preserved by freezing at −40 °C were used for this study.

2.3. Sample preparation

To 500 μl of each of the serum and the urine, 500 μl of acetone was added, and after both were mixed, they were centrifuged. From the supernatant of the resulting serum mixture, 100 μl was taken as a sample for derivatization; and the supernatant of the urine specimen was diluted 1:10 with 0.1 *M* boric acid buffer solution (pH 8.5), and 100 μl of the resulting solution was taken as a sample for derivatization.

2.4. Derivatizing procedure

To 100 μl of each sample, 200 μl of 0.1 *M* boric acid buffer solution (pH 8.5) was added, followed by

100 μl of 1% PNBC acetonitrile solution, and the mixture was agitated. This was then left at room temperature (22 °C) for 10 min to allow derivatization to take place. A 10-μl volume was used for HPLC.

2.5. HPLC–UV conditions

A sample that was infused into a HPLC instrument (SCL10A VP; Shimadzu, Kyoto, Japan) adjusted to a flow-rate of 0.8 ml/min of the mobile phase of ammonium acetate (10 m*M*, pH 5.0)–acetonitrile (90:10) was separated on an Inertsil Ph-3 column (150×4.6 mm, 5 μm particle size; GL Sciences) at a constant temperature of 40 °C, and, using a diode array detector (SPD-M10A VP Shimadzu), detection was carried out at a detection wavelength of 273 nm, and quantitation was performed by the absolute calibration curve method using analysis software (Class VP; Shimadzu).

2.6. LC–MS conditions

A structural analysis of the *p*-nitrobenzoyl derivative of GLUF (PNB-GLUF) was performed under the conditions listed below.

For HPLC, an Alliance 2690 (Waters, Milford, MA, USA) was used.

For MS, a platform LCZ (Micromass UK, Manchester, UK) was used

Ionization method: atmospheric pressure chemical ionization (APCI) method; corona voltage 3 kV; source temperature 130 °C; probe temperature 450 °C; cone voltage 30 V (positive and negative). The type and temperature of the column, and the composition and flow-rate of the mobile phase were the same as the corresponding conditions used for HPLC–UV (stated above).

2.7. Recovery test

A 10-μl volume of standard GLUF solution was added to 1 ml of standard serum, and adjustments were made to obtain solutions of 100, 10, 1 and 0.1 μg/ml. To urine samples collected from healthy adult volunteers, GLUF was added to achieve final concentrations of 1000, 100 and 10 μg/ml. These concentrations were set with actual GLUF poisoning patients in mind [4,7,14]. After preparation and derivatization were performed, the absolute calibration curve method was used to determine the GLUF concentration, and the GLUF values for blank samples of serum and urine to which GLUF had not been added were subtracted, GLUF standard solution was derivatized, and the recovery rate was calculated relative to the measured control values (serum, $n=5$; urine, $n=10$).

2.8. Application: correlation to the HPLC method of GLUF using FMOC-Cl reagent

The correlation between the GLUF concentrations in the sera of patients who had actually ingested a GLUF-containing preparation and the measured values obtained with a fluorescence derivatization–HPLC method [10] with the use of FMOC-Cl, which is easy to quantify, was examined.

HPLC was performed by a previously described method [10]. Briefly, a Shimadzu SCL10AVP HPLC system with a RF10AXL fluorescence detector and an Inertsil ODS-2 separation column (150×4.6 mm, 5 μm particle size, GL Sciences) were used. The excitation wavelength of the fluorescence detector was set at 265 nm and the emission wavelength, at 315 nm. Serum GLUF was directly derivatized with FMOC-Cl (Sigma) for 10 min at 40 °C, and injected into the HPLC system.

2.9. Application: glyphosate analysis

We conducted a study to ascertain whether the above-described UV derivatization–HPLC analysis method could be employed for the phosphorus-containing amino acid-type herbicide glyphosate (GLYP), which has a similar chemical structure to GLUF and is in world-wide use [15]. The chemical structures of GLUF and GLYP are shown in Fig. 1. Standard solutions of GLUF and GLYP were added to blood serum to achieve concentrations of 100 μg/ml of each, and then HPLC was performed after derivatization with PNBC.

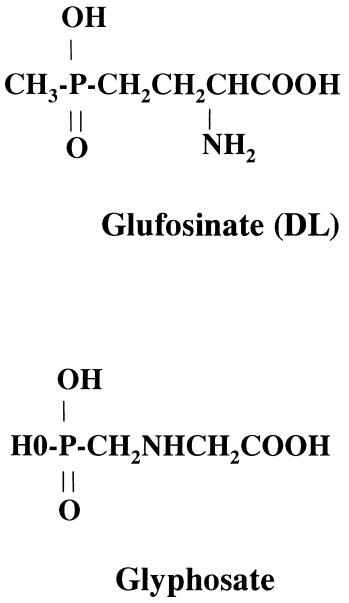

Fig. 1. Chemical structures of glufosinate and glyphosate.

3. Results

3.1. Derivatization of GLUF

The results of diode array detection using Inertsil Ph-3 and PNB-GLUF chromatography by means of LC–MS analysis (APCI method) are shown in Fig. 2. Peak 2 in Fig. 2 is that of the unreacted PNBC. In the UV spectrum, the λ_{max} of PNB-GLUF is 272.8 nm, and so in subsequent testing, a wavelength of 273 nm was used.

In MS of PNB-GLUF (APCI method), determinations were possible in both cationic and anionic modes. In addition, the product ion peaks m/z of 331 $[M+H]^+$ and 329 $[M-H]^-$ were obtained. The sensitivity of unreacted PNBC was low, and a peak was detected only in the negative ion mode.

After 100 μg/ml of a standard solution of GLUF was derivatized and left to stand at room temperature, the dynamics of the derivatization reaction was studied from the area obtained by analysis over time

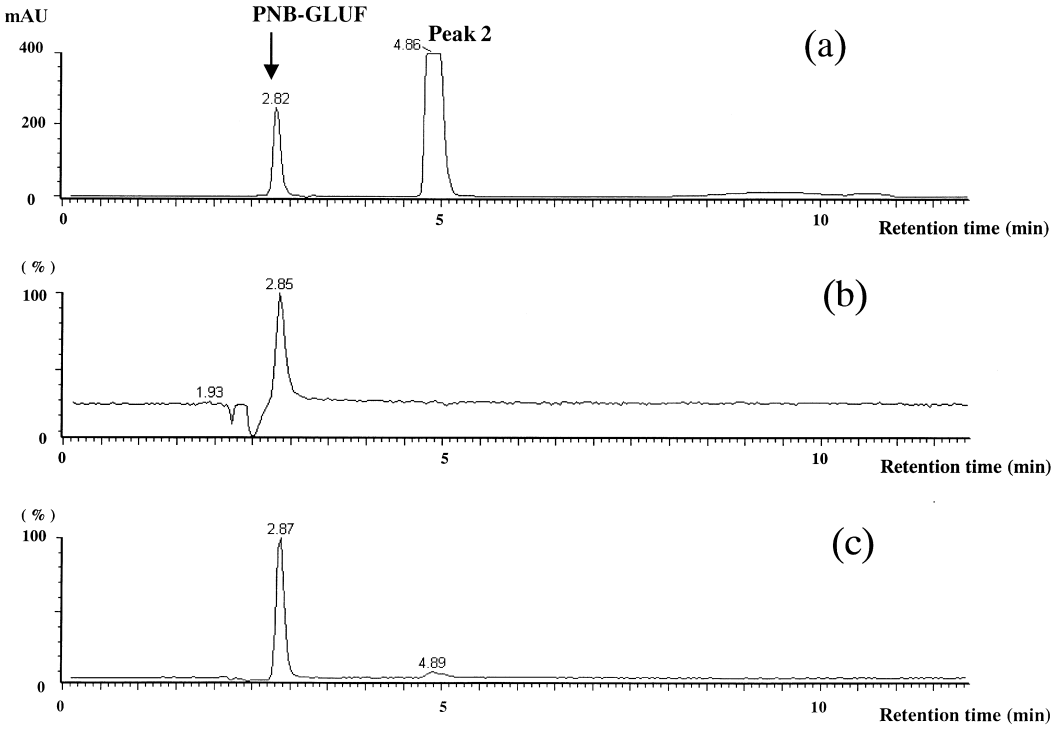

Fig. 2. Diode array (a) and APCI-mass (b: positive ion mode, c: negative ion mode) chromatograms of PNB derivatives of GLUF (125 ng).

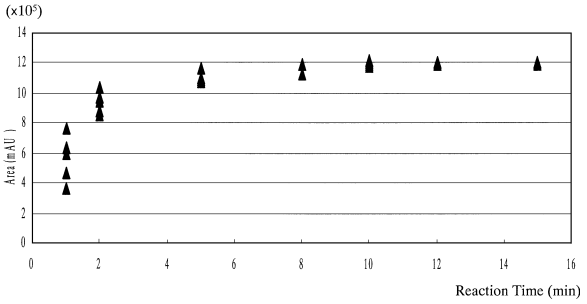

Fig. 3. Peak areas for GLUF as a function of the derivatization reaction at room temperature.

(Fig. 3). The derivatization reaction was at least 90% complete by the end of 5 min at room temperature, and had fully reached a plateau after 10 min. The relative standard deviation (RSD) was stable at 1.2% ($n=5$). The dynamics of the reaction were the same even when authentic specimen of the PNB-GLUF was tested; and therefore, derivatization conditions were set at 10 min at room temperature. Moreover, PNB-GLUF is stable for 24 h at room temperature.

Regarding the relationship between the derivatization reaction and the reagent pH, when 0.1 *M* boric acid buffer was adjusted to pH 8, pH 8.5 and pH 9 with 0.1 *M* hydrochloric acid, the areas obtained in the analysis showed no differences attributable to the differences in pH, and so it was decided thereafter to set the pH to 8.5.

In relation to the concentration of the PNBC acetonitrile solution, when 100 μg/ml GLUF standard solution was derivatized, the RSD of area at a concentration of 0.05% was 2% or less, but when GLUF was added before derivatization to normal human serum and specimens of urine obtained from 10 healthy individuals and diluted 1:10, in order to lower the RSD to 2% or less, it was necessary to have a concentration of 0.5%. Therefore, a concentration of 1% was used.

3.2. HPLC analysis

Separation was tested with an Inertsil ODS-2 column (150×4.6 mm, 5 μm particle size; GL Sciences), a widely used reversed-phase column, as well as with an Inertsil Ph-3 column. The results showed that the retention factor (k' value) of the GLUF peak was the same, 0.32, with each column, but the peak was broad in outline, and showed a tailing pattern. Later, a variety of C_{18} columns were tried, but the peaks were the same as those on the Inertsil Ph-3 column. This tailing phenomenon was not improved by changes either in the relative composition of the mobile phase or in the pH (pH 2.5, pH 3.0, pH 4.0, pH 5.0) resulting from the addition of 10 m*M* ammonium acetate. Consequently, the Inertsil Ph-3 column was used for the separation of PNB-GLUF.

For the calibration curve of PNB-GLUF standard solution obtained by UV detection at 273 nm, at GLUF concentrations from 0.01 to 100 μg/ml, $y=0.97x+0.72$; the correlation coefficient, $r=0.999$; lower limit of quantitation, 0.01 μg/ml; detection limit, 0.005 μg/ml with the signal-to-noise ratio set at 5.

3.3. Recovery of GLUF from biological specimens

The recovery rates of GLUF after its addition to standard human serum and to the urine of healthy subjects are presented in Table 1. In serum, after GLUF was added in amounts to yield concentrations of 0.1, 1.0, 10.0 and 100.0 μg/ml, the recovery rates were good: at least 93.8%. In the case of the urine specimens, the concentrations resulting from GLUF addition were 10.0, 100.0 and 1000.0 μg/ml, the rates also were good: at least 97.3%. From these results, it was seen that the measurements obtained with this method were reliable for serum concentrations of GLUF in the range 0.1–100.0 μg/ml, and for urine concentrations in the range 10.0–1000.0 μg/ml.

Table 1
Recovery of GLUF from biological specimens

Added[a]	Serum GLUF		Urinary GLUF	
	Recovery[b]	RSD (%)	Recovery[b]	RSD (%)
1000	–	–	101.6±3.8	3.7
100	98.6±4.3	4.4	98.7±4.5	4.6
10	96.6±6.5	6.7	97.3±6.3	6.5
1	95.3±7.1	7.5	–	–
0.1	93.8±7.7	8.2	–	–

[a] Amounts are expressed as μg/ml of specimen.
[b] Values are means±SD, $n=5$.

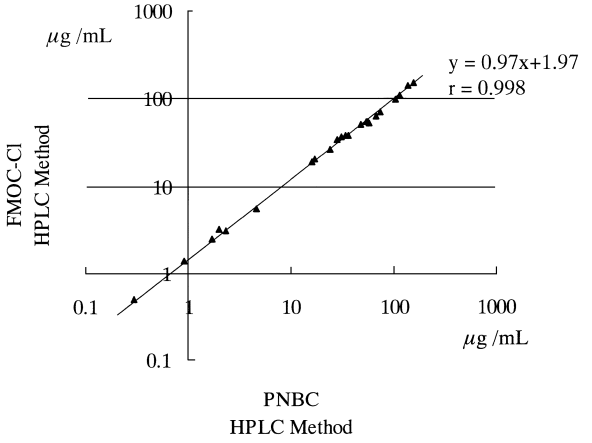

Fig. 4. Correlation between 23 serum GLUF concentrations obtained from 15 patients with acute GLUF poisoning according to PNBC and FMOC-Cl HPLC methods.

3.4. Applications

The measured values obtained by our method and by pre-column fluorescence derivatization–HPLC [10] using FMOC-Cl total of 23 specimens of serum obtained from 15 patients after acute self-inflicted BASTA poisoning are shown logarithmically in a scatter diagram in Fig. 4. The serum GLUF concentrations between 0.1 and 100 μg/ml gave a good correlation for both, with a regression line $y=0.97x+1.97$ ($r=0.998$).

Next, our method was applied to GLYP analysis. The chromatograms obtained are shown with the UV spectrum of GLYP (Fig. 5). The retention time of PNB-GLYP was 2.37 min, which is well differentiated from that of PNB-GLUF, and the λ_{max} values of 207.3 and 273.1 nm were obtained from the UV

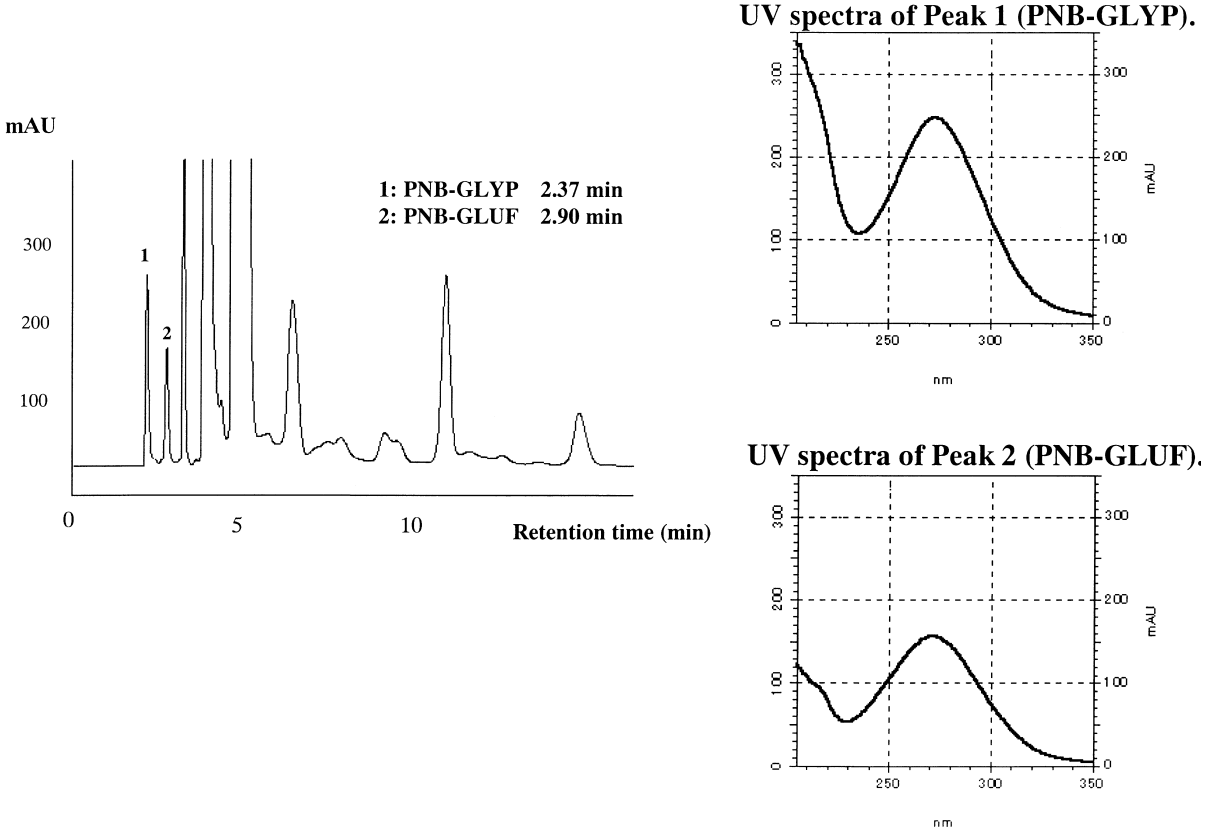

Fig. 5. Chromatograms of PNB derivatives of GLUF and GLYP added to the standard serum and its UV spectrum.

spectrum. In another study, performed after a further addition of GLYP, its ease of, and readiness for, quantification was seen.

4. Discussion

We have established a new method of pre-column UV derivatization–HPLC analysis for GLUF contained in biological samples. It is known that, in an alkaline solution, PNBC readily forms amide bonds with amines and amino acids, thus producing UV-absorbing derivatives. Since PNBC is also inexpensive and easily available, we used it for the derivatization of GLUF.

PNBC reacted quantitatively with GLUF in boric acid buffer at pH 8–9 at room temperature, and UV absorption derivatization took place even without any special means of heating. In addition, high yields were achieved at fast reaction rates when the derivatization of GLUF was carried out using PNBC at room temperature: 90% in 5 min, and 100% in 10 min. In the MS of PNB-GLUF, production peaks m/z of 331 $[M+H]^+$ and 329 $[M-H]^-$ were detected in the positive ion mode and the negative ion mode, respectively. Calculation from these molecular masses suggests that one molecule of PNBC links an amino group or a carboxyl group to an ester.

In this way, PNB-GLUF can be determined by LC–MS in either the cationic or the anionic mode, and future application for simple screening using the base ions will also be possible.

Furthermore, separation of PNB-GLUF was possible with reversed-phase liquid chromatography. However, when it was used as a serum reagent on the multipurpose ODS column, there was unsatisfactory separation from the peaks of interfering substances. As a result, we were able to separate the peaks from those of the interfering substances by using the Inertsil Ph-3 column, which undergoes a specific interaction with the phenyl groups. As a result, it became possible to dispense with the task of extracting GLUF from the serum.

In UV detection at 273 nm, the detection limit of GLUF is 0.005 μg/ml, and so measurement could be carried out on smaller amounts than was possible with pre-column UV derivatization–HPLC [13] using phenyl isothiocyanate, whose detection limit for GLUF is 1 μg/ml.

In cases of GLUF poisoning, the GLUF concentrations in serum can vary over a wide range between 0.1 and 1000 μg/ml [7]. GLUF is excreted rapidly in the urine after undergoing almost no binding to serum protein [16], and its concentration in the urine can rise above 1000 μg/ml [4]. Considering these concentrations, the rates of recovery in the serum and urine are good, at 93.8% or more, and for the 23 specimens obtained from the 15 GLUF poisoning victims, there was good correlation with the measured values obtained with fluorescence derivatization–HPLC [10].

Finally, we applied the present method of analysis for the simultaneous analysis of the herbicide GLYP, which has a similar structure to GLUF. It was possible to achieve good separation of GLYP that had been derivatized with PNBC, from interfering peaks in PNB-GLUF and serum. In addition, those peaks provided quantitative data. It is considered that this method will be useful as a rapid diagnostic technique in clinical practice in cases of poisoning by both chemicals.

5. Conclusions

A UV absorption derivatization reversed-phase liquid chromatography technique was tested for the rapid separation of GLUF in body fluid samples under mild conditions. On the calibration curve of this method, the detection limit was 0.005 μg/ml, and since the recovery rate of GLUF after its addition to blood serum and to urine was at least 93.8% at concentrations of between 0.1 and 1000.0 μg/ml, the method was considered reliable for quantitative determination. Also, the GLUF concentrations measured in the sera of patients who had ingested GLUF-containing herbicides showed good correlation with those obtained by fluorescence derivatization–HPLC. Furthermore, this analytical technique was used to carry out simultaneous analysis of GLYP, a phosphorus-containing amino acid-type herbicide chemically similar to GLUF. It is considered that this technique will be useful in the clinical sphere for the treatment of patients with

GLUF poisoning and for the diagnosis of herbicide intoxication.

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