Journal of Chromatography B, 878 (2010) 3290–3296

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of a liquid chromatography–fluorescence–mass spectrometry method to measure glyphosate and aminomethylphosphonic acid in rat plasma

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ARTICLE INFO

Article history:
Received 13 July 2010
Accepted 16 October 2010
Available online 23 October 2010

Key words:
Glyphosate
AMPA
Rat plasma
HPLC–FLD–ESI-MS

ABSTRACT

A simple and fast method has been developed and validated to measure glyphosate (GLYP) and aminomethylphosphonic acid (AMPA) in rat plasma based on reversed-phase high performance liquid chromatography (RP-HPLC) coupled to fluorescence (FLD) and electrospray ionization mass spectrometry (ESI-MS) detection. After protein precipitation with acetonitrile, GLYP and AMPA were derivatized with 9-fluorenylmethylchloroformate (FMOC-Cl) and then separated on a C_{12} column (250 mm × 4.60 mm i.d.) using a gradient of an ammonium formate (20 mM, pH 8.5) and acetonitrile mobile phase. Selected ion monitoring (SIM) mode of the MS was used to obtain maximum sensitivity when quantifying GLYP and AMPA. The validation shows the method to be consistent and reliable, with an intra- and inter-day precision for GLYP and AMPA > 9% for both detectors. For both compounds the accuracy ranged from 2.1% to 7.8% for the intra-day readings, and from 4.1% to 8.6% for the inter-day values. The efficacy of GLYP extraction ranged from 87% to 93% and it was between 76% and 88% for AMPA. Moreover, the limits of quantification (LOQ) for GLYP and AMPA were 5 and 10 ng/mL, respectively with FLD, and 0.4 and 2 ng/mL with ESI-MS. The method was successfully applied to simultaneously measure both compounds in rat plasma samples several days after oral administration of glyphosate.

1. Introduction

Glyphosate [N-(phosphonomethyl)glycine] (GLYP) is a broad spectrum, non-selective, post-emergent herbicide that is used extensively in many countries. This compound is rapidly degraded into its aminomethylphosphonic acid (AMPA) metabolite [1], which accumulates and can be found in plants and animals [2,3]. Through recombinant DNA technology [4], it has been possible to produce glyphosate-tolerant transgenic crops with high levels of resistance [5], particularly varieties of soybean and maize. The increasing use of crops cultivars [6] with resistance to selected herbicides, and particularly to glyphosate, has generated certain concern in terms of environmental quality, food safety and consumer health. Indeed, despite the increasing use of this herbicide, its effects on non-target organisms have yet to be fully evaluated. As with other substances, potential effects of this herbicide should first be assessed in experimental animals, such as rats. Indeed, long-term exposure to glyphosate can provoke deficiencies in foetal ossification in pregnant rats [7] and other adverse effects such as liver toxicity [8]. The current assay was validated in rat plasma but could easily be adapted to plasma from other species, including human, to support the evaluation of potential effects of long-term exposure to GLYP in future surveillance programs.

Both GLYP and its main metabolite AMPA are polar and amphoteric compounds that are very soluble in water. These chemical properties hinder their analysis, as does the difficulty in detecting them, frequently exhausting the patience of many experienced analysts [9]. Moreover, their similarity to naturally occurring amino acids further augments the problem of measuring these residues in animal products. Thus, it is necessary to employ tedious and time-consuming cleaning procedures, frequently involving the use of ion-exchange solvents [10–14] and further derivatization, in order to facilitate their chromatographic separation by gas GC [15,16], GC–MS [17–21], LC [3,22–26] or LC–MS [27–34]. However, their low molecular mass and the effect of the phospholipid matrix also generate problems with LC–MS techniques, making it wiser

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doi:10.1016/j.jchromb.2010.10.013

to employ ionic chromatography and ICP-MS [35–37] or capillary electrophoresis coupled to ICP-MS [38]. Spectroscopy and other techniques have also been proposed as suitable methods to analyze these compounds [39–43]. Nevertheless, the determination of GLYP and AMPA in different matrices is still an important problem in contemporary applied analytical chemistry.

Among the derivatization options commonly proposed for LC methods, pre-column derivatization (the analytes are derivatized before passing through the chromatographic column) with 9-fluorenylmethylchloroformate (FMOC-Cl) is often preferred [31,44–47], even though post-column derivatization (when the analytes are derivatized after chromatography) has also been used [11,48]. A derivatization step is also required in gas chromatography, in this case using reagents such as N-methyl-N-(tert-butyldimethylsilyil) trifluoroacetamide [20] or N-methyl-trifluoroacetamide with tert-butyldimethylchlorosilane [15].

Despite the wide variety of techniques and methods proposed to evaluate GLYP and AMPA in different matrices, mainly environmental and food samples, only a few of these have been used for serum or plasma [3,17,33,43], reflecting the problems associated with this analysis. To develop a reliable method to analyze these compounds in rat plasma, we have considered all the aforementioned options, taking into account that HPLC methods are the most widely used for biological fluids. Thus, we employed liquid chromatography after FMOC-Cl pre-column derivatization and we used two different detectors, fluorescence and single quadrupole mass spectrometer. We had previously developed a RP-HPLC–FLD method to study the toxicokinetics of GLYP and AMPA in rat plasma with a LOQ of 25 ng/mL [3]. However, pushing the limit of detection of the assay was necessary, as that method was not sufficiently sensitive to measure GLYP and AMPA concentrations several days after single oral administration of glyphosate and it was impossible to detect the low plasma levels observed for both compounds, especially AMPA, following chronic exposure to the herbicide. For this reasons, we have attempted to improve the sensitivity (LOQ) of this technique by coupling a MS detector and optimizing the conditions. In addition, the earlier HPLC method has been improved by decreasing the analysis time as much as possible and enhancing the recovery of both compounds by treating the samples distinctly. The new HPLC–FLD–ESI-MS method has been fully validated, and the advantages and disadvantages of detecting GLYP and AMPA in rat plasma with both detectors have been assessed. The results obtained were also compared with earlier data to check the suitability of our new method. Finally, the validated method was successfully applied to analyze several plasma samples obtained from rats several days after single oral administration of glyphosate, demonstrating its capacity to be used in future toxicity studies needed for a major knowledge of GLYP and AMPA toxicity.

2. Materials and methods

2.1. Chemicals

GLYP [N-(phosphonomethyl)glycine: 95% pure, w/w], AMPA (amiomethylphosphonic acid: 99% pure, w/w) and 9-FMOC-Cl (fluorenylmethylchloroformate) were provided by SIGMA–Aldrich Chemie GmbH (Steinheim, Germany), acetonitrile (HPLC grade) was purchased from Labscan (Dublin, Ireland), and reagent grade boric acid, disodium tetraborate decahydrate, ammonium hydroxide and ammonium formate were all obtained from Scharlab (Barcelona, Spain). All other chemicals were obtained of the highest quality grade from commercial sources.

HPLC-grade water was obtained by purifying demineralised water in a Milli-Q system (Millipore, Bedford, MA, USA). A 0.1 M disodium tetraborate buffer solution [pH 9] was prepared in HPLC-grade water and a 10 mM solution of FMOC-Cl was prepared in acetonitrile.

Table 1
Results obtained from the flow injection analysis (FIA) tests of the MS-ESI parameters in positive mode for the selected mobile phase.

MSD parameter	Studied range	Optimal value
Capillary voltage (V)	2000–5500	3500
Drying gas (N_2) flow (L/min)	4–12	10
Drying gas (N_2) temperature (°C)	100–350	275
Fragmentor voltage (V)	10–370	70
Nebulizer gas (N_2) pressure (psi)	10–60	40

2.2. Standard solutions

Standard stock solutions were prepared by dissolving approximately 50 mg of powder in 100 mL of HPLC-grade water to a final concentration of approximately 500 mg/L. These solutions were further diluted with HPLC-grade water to concentrations ranging from 1000 to 0.4 or 2 μg/L for GLYP and AMPA, respectively. Blank plasma rat samples (0.2 mL) were spiked with different amounts of standards for calibration, between 5 and 1000 ng/mL GLYP or 10 and 1000 ng/mL AMPA for FLD, and from 0.4 to 1000 ng/mL GLYP or 2 to 1000 ng/mL AMPA for ESI-MS.

Each quality control (QC) sample was prepared using blank plasma rat samples (0.2 mL) spiked with the same standard stocks and working solutions of GLYP and AMPA used in the calibration studies. The concentration of the different QC samples were as follows: low QC level—1 ng/mL (GLYP) and 5 ng/mL (AMPA) for ESI-MS or 10 ng/mL (GLYP) and 25 ng/mL (AMPA) for FLD; medium QC level—300 ng/mL of each compound for both types of detection; and high QC level—1000 ng/mL of each compound for both types of detection.

All standard stocks and working solutions were stored in polypropylene containers and kept in the dark at +4 °C, and they were stable for over one month.

2.3. Chromatography system

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC–FLD–MS system was used that consisted of a vacuum degasser, a quaternary solvent pump, an autosampler with a column oven, a fluorescence detector (FLD) and a single quadrupole MS analyzer with an electrospray (ESI) interface, all controlled by a Chemstation software.

A Synergi 4 μm MAX-RP 80 (250 mm × 4.60 mm i.d.) was used as analytical column for LC and it was protected by a Synergi C_{12} security guard cartridge (4 mm × 3 mm i.d.), both obtained from Phenomenex (Torrance, CA, USA). After the optimization study, the mobile phase selected was a mixture of ammonium formate 20 mM [pH 8.5] in water (A) and acetonitrile (B), applied at a flow rate of 1 mL/min in a gradient mode as follows: (i) 0–5 min (A–B, 85:15, v/v); (ii) 5–10 min (A–B, 78:22, v/v); (iii) 10–13 min (A–B, 76:24, v/v); (iv) 13–18 min (A–B, 50:50, v/v); (v) 18–22 min (A–B, 0:100, v/v); and (vi) 22–30 min (A–B, 85:15, v/v), with a post-separation time of 5 min. The injection volume was set at 30 μL (draw speed 50 μL/min) and the temperature selected was 45 °C. The detection wavelengths were 240 nm (excitation) and 320 (emission) to quantify GLYP, and 250 nm (excitation) and 620 nm (emission) to quantify AMPA.

The ESI interface was operated in positive mode having performed flow injection analysis (FIA) tests of the MS parameters as reported in Table 1. Full scan LC–MS spectra were obtained by scanning from m/z 50 to 500. The most abundant ion of each compound was quantified in SIM mode.

2.4. Animals and treatments

The study was undertaken in accordance with the institutional ethics guidelines and it was authorized by the official ethical committee of the University Complutense de Madrid (Madrid, Spain).

In this study, 20 adult male Wistar rats (Charles River Inc., Margate, Kent, UK) weighing 180 ± 10 g were used. The animals were housed individually in polycarbonate cages with sawdust bedding and they were maintained in environmentally controlled rooms (22 ± 2 °C and 50 ± 10% relative humidity) on a 12 h light/dark cycle (light from 08.00 to 20.00 h). Food (A04 rodent diet, Panlab SL) and water were available *ad libitum*. The rats were divided into six groups: 5 animals as controls (Group 1), from which the blank rat plasma was obtained, and 15 animals were chosen for the treatment groups (Groups 2–6) that were orally administered glyphosate. The animals in groups 2–6 were deprived of food but they were allowed *ad libitum* access to water for 12 h before a single oral dose of glyphosate (100 mg/kg body weight) was administered by gavage in a volume of 0.5 mL corn oil/rat.

All animals were sacrificed by cervical dislocation (four animals at a time) and then exsanguinated 1, 2, 3, 4 and 5 days after oral administration of glyphosate. Blood samples were withdrawn and collected in heparinised tubes, and the plasma was separated by centrifugation and stored frozen in eppendorf vials until it was analyzed.

2.5. Sample analysis

Blank rat plasma spiked with standards (100 μL) or experimental rat plasma (100 μL) was transferred to an eppendorf tube, and 50 μL of acetonitrile was added. The mixture was vortexed for 1 min, it was placed in an ultrasound device for 10 min, and finally it was centrifuged at 10,000 rpm for 30 min at 12 °C to precipitate the proteins. The supernatant was collected (100 μL) and transferred to a different eppendorf tube to which 50 μL of acetonitrile was added, and the mixture was again treated with ultrasound and centrifuged under the same conditions as above. All the calculations reported here have been made taking into account the dilution factor of the plasma samples subjected to the protein precipitation protocol. The supernatant (100 μL) was derivatized with of 100 μL FMOC-Cl (10 mM) and 100 μL borate buffer (1.25 mM, pH 9), and the reaction was maintained at room temperature for 30 min. Finally 30 μL of this solution was injected into the HPLC–FLD–ESI-MS system.

2.6. Method validation

Validation was carried out following the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidelines [49,50], the International Union of Pure and Applied Chemistry (IUPAC) [51] technical report (2002) and the SANCO/10476/2003 document [52], determining recovery, selectivity, limits of quantification and detection, linearity, precision and accuracy.

The recovery of GLYP and AMPA was determined in 6 replicates at 3 concentrations (low, medium and high QC levels), comparing the peak areas of GLYP and AMPA from standard samples with those from: (i) extracted blank plasma samples from control rats, spiked with the same amounts of the compounds and then treated as described above (Blank plasma A); and (ii) plasma samples extracted from rats that were orally administered glyphosate, spiked with the same amounts of GLYP and AMPA and then treated as described above (Blank plasma B) in order to study the influence of the sample treatment; (iii) and to check the possible effect of the matrix on the ESI ionization, extracted blank plasma samples from control rats that were treated as described above and then spiked with the same amounts of GLYP and AMPA (Blank plasma C).

To check the selectivity of the method, extracts from blank and spiked plasma samples were assayed. The limit of quantification (LOQ) was determined by injecting a number of extracts from blank plasma samples ($n = 6$) and measuring the magnitude of the background response. We experimentally estimated the LOQ as ten times the signal-to-noise ratio (S/N).

Matrix-matched standard calibration curves were used to quantify the analyte residues in rat plasma. Blank plasma samples were spiked with variable amounts of GLYP and AMPA, in an analytical range between: 0.4 (ESI-MS), 5 (FLD) and 1000 ng/mL for GLYP; and 2 (ESI-MS), 10 (FLD) and 1000 ng/mL for AMPA. The samples were treated as indicated above, injected onto the LC–FLD–MS system, and the signal obtained for each concentration and detector was used to obtain the matrix-matched calibration curves. It was possible to obtain data for both detectors at the same time due to the on-line coupling and Chemstation software employed.

Intra-day precision and accuracy was determined concurrently with repeated sample analysis but using QC samples on the same day. In each run a calibration curve was established and six replicates of each low, medium and high QC samples were analyzed. Inter-day precision and accuracy were assessed by analyzing six sample replicates at three concentrations of GLYP and AMPA against a calibration curve on three consecutive days.

Precision was expressed as the percentage of the relative standard deviation (%RSD) at a given concentration for each QC samples. Accuracy was calculated through the relative error (%RE).

3. Results and discussion

3.1. Development of the method

Chromatographic conditions were optimized over several trails to achieve good resolution and increase the signal from analytes, as well as to minimize the run time. A gradient elution program was used that gave acceptable resolution between the derivatized AMPA and the peaks belonging to the excess reagent (see Section 2.3), which differed slightly from that reported previously [3]. Indeed, the percentage of acetonitrile was slightly higher in the second gradient step than that published previously, in order to decrease the analysis time as much as possible, without losing selectivity and sensitivity. By maintaining the flow rate at 1 mL/min, the total chromatography run time with this new gradient was 30 min, with a 5 min post-run time.

Tests were carried out to study the influence of the column temperature (between 25 and 60 °C in 5 °C intervals), which produced different retention times and peak symmetries. As expected, the retention times decreased slightly as the temperature increased and there was a loss of symmetry at low temperatures. Thus, the best results were achieved at 45 °C, as the peaks were narrow and there was good separation between the compounds and interfering peaks in the FLD chromatograms.

The possibility of enhancing the limits of quantification by injecting larger sample volumes was considered by testing the injection of control blank plasma spiked with 300 ng/mL of GLYP and AMPA in volumes from 20 to 50 μL. The results showed an increase in the S/N ratio for both compounds when up to 30 μL was injected, above which the S/N ratio did not improve. In fact, with this modification, the LOQ (FLD) for both compounds improved significantly, from 25 to 5 ng/mL for GLYP and from 25 to 10 ng/mL for AMPA.

Finally, the excitation and emission wavelengths were set at 240 and 320 nm for GLYP, respectively and at 250 and 620 nm for AMPA. These wavelengths were selected to avoid interference from plasma matrix compounds that might coelute with AMPA.

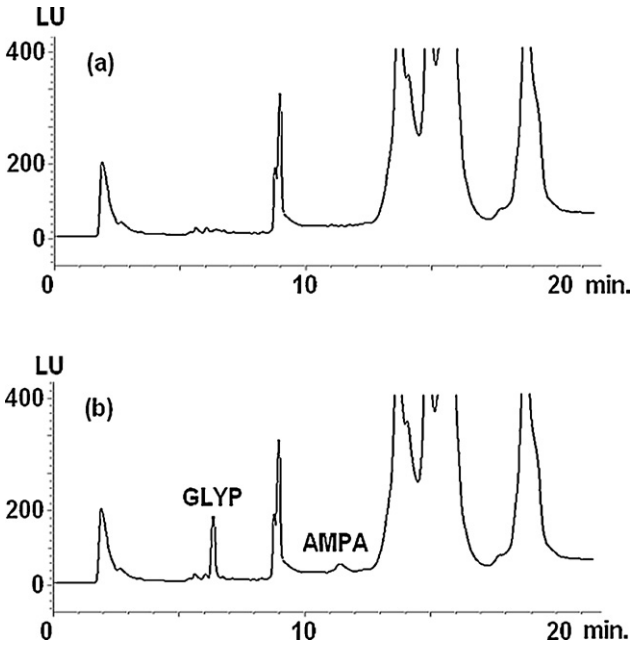

Fig. 1. FLD chromatograms (λ_{ex} 240 nm and λ_{em} 320 nm for GLYP, and λ_{ex} 250 nm and λ_{em} 620 nm for AMPA) of (a) a blank rat plasma sample and (b) a plasma sample taken 1 day after administering a single oral dose of glyphosate (100 mg/kg of body weight). The concentration of GLYP and AMPA were estimated as 919 and 101 ng/mL. The analytical column employed was a Synergi 4 μm MAX-RP 80 (250 × 4.60 mm i.d.) protected by a C_{12} security guard cartridge (4 × 3 mm i.d.). The mobile phase was a mixture of ammonium formate 20 mM [pH 8.5] in water (A) and acetonitrile (B), applied in the gradient mode described in Section 2.3 at a flow rate of 1 mL/min. The injection volume was set at 30 μL and the selected temperature was 45 °C. The chromatographic conditions are described in detail in Section 2.3.

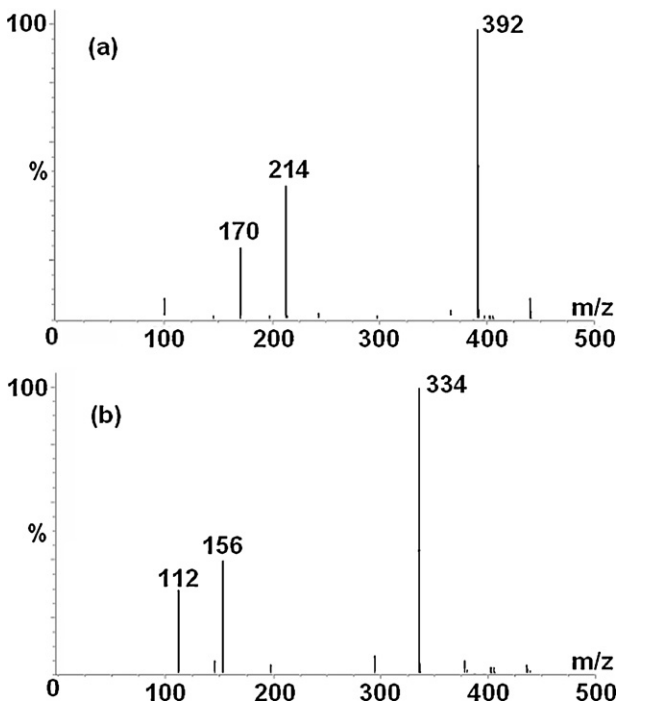

Fig. 2. Full scan ESI-MS spectra in positive mode of (a) GLYP–FMOC and (b) AMPA–FMOC derivatives.

3.2. Pre-column derivatization with FMOC-Cl

As mentioned in the Section 1, the aim of this work was to improve the sensitivity of an earlier method we developed. To achieve this, the tests carried out were based on the assumption that the FMOC-Cl derivatization employed previously was the most appropriate, and that the volume of acetonitrile was optimal to obtain the best LOQ and to avoid an unnecessary sample dilution, retaining the acetonitrile:sample ratio of 1:1.

An ultrasound step was introduced after sample centrifugation to try to improve the recovery without employing tedious and more time-consuming procedures. Distinct periods of ultrasound were tested (5–20 min), although times longer than 10 min did not improve the results. Tests were also performed on the sample centrifugation but the conditions selected previously (30 min centrifugation at 10,000 rpm and 12 °C) produced the cleanest chromatograms.

Finally, carrying out the same procedure twice consecutively, including the addition of 50 μL of acetonitrile, centrifugation and ultrasound, significantly increased the recovery of both compounds. Thus, performing two extractions with 50 μL of acetonitrile (instead of only one with 100 μL), and introducing an additional ultrasound step, improved the extraction yields.

Finally, the supernatant (100 μL) was derivatized with 100 μL of FMOC-Cl (10 mM) and 100 μL borate buffer (1.25 mM, pH 9) at room temperature for 30 min.

In a typical FLD chromatogram obtained using the conditions proposed from a plasma sample 1 day after rats were administered a single oral dose (100 mg/kg of body weight), both peaks were perfectly resolved from the matrix and derivatization reagents (Fig. 1). Indeed, the GLYP peak was symmetrical and it was much larger than the AMPA peak.

3.3. Mass spectrometry optimization

The first HPLC–MS experiments to select the optimal ESI-MS parameters and the appropriate ions were carried out by flow injection analysis (FIA) of the individual solutions of GLYP and AMPA derivatized with FMOC-Cl to monitor the MS intensity. Although these compounds are usually analyzed in negative ion mode [30,34], greater sensitivity was obtained here in the positive mode (as reported elsewhere: [27]) and hence, this positive ion mode was selected. The range studied for each parameter in positive mode is shown in Table 1 in which the conditions producing the greatest sensitivity for both compounds are also shown.

A major ion for each compound was evident in the mass spectra of the compounds (see Fig. 2), *m*/*z* 392 for GLYP–FMOC and 334 for AMPA–FMOC, corresponding to the protonated molecular ions $[M+H]^+$. Selected ion monitoring (SIM) mode was used to obtain the maximum sensitivity for quantitative analysis, and the following mass-to-charge (*m*/*z*) values were chosen for SIM analysis: 392 for quantification and 214, 170 confirmation of GLYP–FMOC and 334 for quantification and 156, 112 for confirmation of AMPA–FMOC.

To check how the matrix influenced the ionization, the peak areas of GLYP and AMPA in standard solutions were compared with those obtained in blank plasma C. The recovery of both compounds at the three concentrations assayed was close to 100% and they were not significantly different (Table 2). Hence, it was concluded that the matrix (rat plasma) did not affect the electrospray ionization of GLYP and AMPA.

3.4. Method validation

To assess the selectivity of the method, extracts from blank rat plasma were assayed, along with rat plasma spiked with GLYP and AMPA after a single oral dose (Fig. 1) and those spiked at the LOQ levels (Fig. 3). No matrix interference was evident in the FLD or ESI-MS chromatograms obtained. The LOQ for rat plasma was 0.4 ng/mL (ESI-MS) and 5 ng/mL (FLD) for GLYP, while the LOQ for AMPA were slightly higher at 2 ng/mL (ESI-MS) and 10 ng/mL (FLD). In both

Table 2
Extraction recoveries and matrix effect for both detectors of GLYP and AMPA from spiked rat plasma samples ($n = 6$).

Compound	Concentration (ng/mL)	Blank plasma A	Blank plasma B	Blank plasma C
		Mean (%) ± SD	Mean (%) ± SD	Mean (%) ± SD
GLYP	10[a]	93 ± 4.9	92 ± 3.1	93 ± 3.9
	1[b]	92 ± 4.3	90 ± 2.6	103 ± 2.6
	300[a]	89 ± 5.8	88 ± 4.9	88 ± 4.5
	300[b]	91 ± 5.6	91 ± 3.8	99 ± 3.8
	1000[a]	87 ± 4.9	90 ± 3.9	88 ± 4.1
	1000[b]	89 ± 4.2	88 ± 3.3	101 ± 3.3
AMPA	25[a]	88 ± 6.8	84 ± 6.2	85 ± 6.1
	5[b]	87 ± 6.0	85 ± 5.2	97 ± 5.2
	300[a]	76 ± 6.4	78 ± 3.9	78 ± 4.5
	300[b]	79 ± 5.3	77 ± 3.9	102 ± 3.9
	1000[a]	84 ± 4.2	82 ± 6.3	83 ± 5.1
	1000[b]	82 ± 3.1	83 ± 5.3	98 ± 5.3

[a] FLD.
[b] ESI-MS.

cases the LOQ were lower than those obtained previously using either detector [3] and as expected, they were better when ESI-MS was used. These LOQ values were higher than those obtained for GLYP and AMPA in other matrices (water, soils) and using more sensitive detectors (Ion trap, QQQ) with HPLC [27,28,30,32,34]. However, it is not possible to make a true comparison between these data as the matrix has a strong influence on the analysis of these compounds. Nevertheless, the LOQ obtained for GLYP was lower (ESI-MS) or equal (FLD) to the best LOQ found in the only previous study in which glyphosate was analyzed in serum (a similar matrix) by LC–QQQ [33]. Thus, the HPLC–FLD–ESI-MS technique developed produced results in serum samples that are equal to or better than the existing methods. Moreover, although with the proposed method the results for FLD were not as good as with ESI-MS, they were good enough in comparison with other methods, making FLD an economic alternative for experiments in which such high sensitivity is not required.

The extraction efficacy for GLYP ranged from 87% to 93% and it was between 76% and 88% for AMPA at the three concentrations tested. Hence, recovery has been improved with respect to our previous method, especially for AMPA. Indeed, there appeared to be no relevant differences in the recovery at the different concentrations, in the distinct rat plasma blanks, or using different detectors (Table 2). Taking into account the simple procedure to process the samples, these results were very good, particularly when compared with the often tedious and time-consuming procedures commonly employed with these compounds, such as SPE. Indeed, the recoveries obtained are in concordance with the recovery values obtained with SPE and other sample treatments [27,28,30,32,33,43], especially the recoveries of GLYP.

The graphs obtained were straight lines with an intercept not significantly different to zero ($p < 0.05$), confirming the linearity in the range studied. The absence of bias was checked using a t-test and studying the distribution of residuals. The determination coefficient values (R^2) were >0.99 (see Table 3) for all the linear ranges studied.

The intra-and inter-day precision (%RSD) for GLYP and AMPA was always below 9% for both detection methods (Table 3). The accuracy (%RE) for both compounds ranged from 2.1% to 7.8% for the intra-day readings, and from 4.1% to 9.1% for the inter-day values. It must be pointed out that the accuracy and precision values are slightly better for FLD than for ESI-MS, although both were sufficiently high. These results indicated that the present method is precise and accurate, and it could be said that although an internal standard is recommended in most validation guidelines, it was not necessary in this study due to the precision and accuracy of the method proposed.

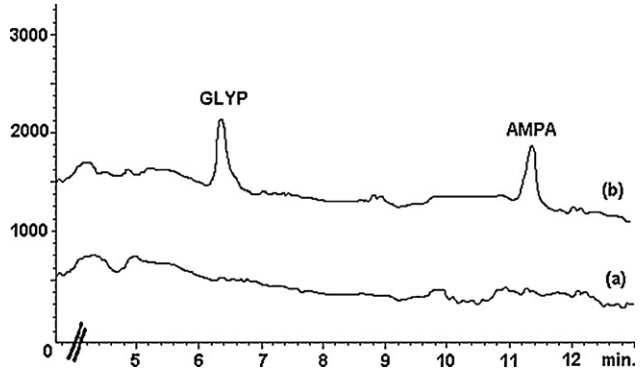

Fig. 3. Representative RP-HPLC–ESI-MS (SIM mode) chromatogram of (a) blank rat plasma and (b) blank rat plasma spiked with LOQ GLYP and AMPA (0.4 and 2 ng/mL, respectively). The analytical column used was a Synergi 4 μm MAX-RP 80 (250 × 4.60 mm i.d.) protected by a C12 security guard cartridge (4 × 3 mm i.d.). The mobile phase was a mixture of ammonium formate 20 mM [pH 8.5] in water (a) and acetonitrile (b), applied in a gradient as described in Section 2.3 and at a flow rate of 1 mL/min. The injection volume was set at 30 μL and the selected temperature was 45 °C. The following mass-to-charge (m/z) values were chosen for SIM quantification: 392 for quantification and 214, 170 for confirmation of GLYP–FMOC; and 334 for quantification and 156, 112 for confirmation of AMPA–FMOC. The chromatographic and MS conditions are described in detail in Sections 2.3 and 3.3.

3.5. Application of the method to measure GLYP and AMPA in rat plasma

The validated method was used to quantify GLYP and AMPA in rat plasma, demonstrating the possibility of monitoring GLYP and AMPA levels after oral administration of low concentrations (see ESI-MS results in Table 4). It should be noted with the MS method developed, GLYP and AMPA concentrations below 25 ng/mL could be quantified (the LOQ for the previous FLD method [3]). This sensitivity made it possible to monitor both compounds in this study, because the compounds existed below this concentration in several samples. As deduced from the data (Table 4), the concentrations of GLYP and AMPA in rat plasma samples decreased gradually from day 1 until day 5, the values at the end of this period being close to the LOQ levels for both compounds. These results indicate that AMPA was found at very low levels, making it necessary to use a MS detector, and that GLYP and its metabolite AMPA were eliminated from the plasma slowly. Thus, as could be seen, the method developed can be successfully applied for monitoring the low levels of GLYP and AMPA in plasma over a five day trial, highlighting the possibility of using this method in future surveillance studies or accidental poisoning in humans.

Table 3
Method validation parameters and mean calibration curves for GLYP and AMPA determination in rat plasma samples.

Validation parameter		GLYP		AMPA	
		FLD	ESI-MS	FLD	ESI-MS
Intra-day precision (%RSD)	Low	5.0	6.5	5.5	6.2
	Medium	4.1	5.2	5.2	6.6
	High	2.8	4.3	3.8	5.3
Inter-day precision (%RSD)	Low	7.1	8.5	7.0	7.9
	Medium	3.5	5.7	5.6	6.7
	High	4.9	6.1	5.2	6.5
Intra-day accuracy (%RE)	Low	−5.3	−6.2	6.8	7.8
	Medium	2.3	3.1	2.1	3.4
	High	−6.2	−7.3	−5.5	−6.4
Inter-day accuracy (%RE)	Low	6.5	8.1	−8.1	−8.6
	Medium	4.9	6.2	4.1	5.4
	High	−5.5	−6.8	−5.0	−5.7
LOQ (ng/mL)		5	0.4	10	2
Linear range (ng/mL)		5–1000	0.4–1000	10–1000	2–1000
Correlation coefficient (R^2)		0.999	0.998	0.994	0.999

Table 4
Time monitoring of GLYP and AMPA (ng/mL) in rat plasma after a single oral dose of 100 mg/kg of body weight employing ESI-MS detection.

Time	Animal group	GLYP	AMPA
1 day	2	919	101
		873	94
		848	99
2 day	3	448	60
		482	55
		497	57
3 day	4	136	23
		124	25
		129	22
4 day	5	39	10
		41	8
		46	9
5 day	6	8	3
		11	2
		9	4

4. Conclusions

For the first time, a fast and simple HPLC method with pre-column derivatization using FMOC-Cl has been developed and validated to measure low concentrations of glyphosate, as well as its main metabolite aminomethylphosphonic acid, in rat plasma samples using fluorescence and electrospray mass spectrometry detection. The validated data demonstrate that this method is consistent and reliable, with low %RSD values, little bias and good recovery. The limits of quantification for the analytes are sufficient to measure plasma concentrations after oral administration of glyphosate.

Although it has been demonstrated that the fluorescence detector could be an economic alternative to MS when determining these compounds in plasma, it was necessary to use MS to monitor the degradation of both compounds as the sensitivity required could only be reached using ESI-MS.

Acknowledgments

M.T. Martín and J. Bernal would like to thank the Spanish Ministry for their "Ramon y Cajal" and "Juan de la Cierva" contracts. This work was funded by the Spanish Ministerio de Educación y Ciencia (Project AGL2005-05320-C02-02).

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