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Research Article
Determination and Assessment of Glyphosate Exposure Among Farmers from Northern Part of Thailand

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Abstract

Background and Objective: The most popular herbicide used for crops in Northern Thailand is glyphosate which is a systemic, post-emergent, non-selective herbicide. The aim of present study was to modify a high performance liquid chromatographic-based method for analyzing of urine samples from farmers of Northern Thailand. **Materials and Methods:** The urine samples was cleaned by Solid Phase Extraction (SPE) after derivatization with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) and quantified by using high performance liquid chromatography with fluorescence detector (HPLC-FLD) to assessed glyphosate residue. **Results:** The modified HPLC-FLD method showed good accuracy and precision. The percent recovery levels obtained using control urine samples spiked at glyphosate concentrations of 2, 5 and 10 μg L^{-1} were 91.2, 79.7 and 103.9, respectively. The precision (RSD%) assessments reported as inter- and intra-batch variation were in rage of 3.9-11.5%. The limit of detection and limit of quantification were 0.5 and 2.0 μg L^{-1}, respectively. The modified HPLC-FLD method was applied to urine samples of farmers in Northern Thailand (Chiang Mai, Chiang Rai and Payao provinces) for analysis of glyphosate residue. The results showed glyphosate residue in 44.8% of urine samples collected with mean concentration 13.70±13.30 μg L^{-1} (11.89±26.08 μmol mol^{-1} creatinine) and the corresponding geometric mean was 9.50 μg L^{-1} (5.88 μmol mol^{-1} creatinine). **Conclusion:** The study successfully validated an HPLC-FLD for analyzing of glyphosate concentrations in human urine samples. The validated method was applied to measure glyphosate levels in urine samples collected from farmers of Northern Thailand region. The results indicated measurable glyphosate levels in 48% urine samples collected. In summary, the modified HPLC-FLD method may be used to assess the glyphosate body burden.

Key words: Glyphosate, assessment of glyphosate, glyphosate in urine, simple method for glyphosate, HPLC-FLD, FMOC-Cl derivatization, farmer

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Glyphosate [N-(phosphonomethyl) glycine] is an acidic herbicide[1] which is widely used in agricultural areas of Thailand. According to the classification of the World Health Organization (WHO), glyphosate has comparatively low toxicity to humans and animals so this herbicide has been extensively used. Several studies reported the glyphosate can be poisoned by various symptoms such as gastrointestinal symptoms[2], altered consciousness[3], hypotension, respiratory distress[4], metabolic acidosis and renal failure after expose to the compound[3]. The previous studies have suggested almost all glyphosate is excreted from the body within a week[5]. Even so, there is an ongoing debate about the safety of glyphosate, given its widespread use[6], people could be having frequent or even continuous, exposure to glyphosate in their food[7] and water[8,9]. A few studies have looked for glyphosate in humans[10]. The study of long-term toxicology of the low glyphosate residues has not been investigated in vertebrates and show effect to enzymes of the (Cyp450) family by inhibiting enzyme activity[11]. Glyphosate has been reported that indicated crucial for sex steroid hormone synthesis[12].

There are several methods have been developed for determining glyphosate in human urine i.e., gas chromatography coupled with mass spectrometry (GC-MS)[13,14], capillary[15] liquid chromatography coupled with mass spectrometry (LC-MS)[16,17], electrophoresis[15], UV detection (LC-UV) and fluorescence detection (LC–FLD)[8,18] after derivatization and automated amino acid analyzer. Thus, the liquid chromatography is suitable for glyphosate analysis more than gas chromatography because of the ion character of glyphosate and low volatility. The HPLC-FLD will be inexpensive and suitable for developing countries use in epidemiology and bio-monitoring.

Therefore, the purpose of this study is to modify the method for detecting glyphosate in urine samples and application used for determining of glyphosate farmer's urine from Northern part of Thailand by using high performance liquid chromatography with fluorescence detection (HPLC-FLD) after derivatization.

MATERIALS AND METHODS

Chemicals and organic solvents: Standard glyphosate (N-(Phosphonomethyl) glycine, 96%) and derivatization agent (9-fluorenylmethoxycarbonyl chloride, 97%) were purchased from Sigma-Aldrich, USA. Orthophosphoric acid (85% GR) and hydrochloric acid were obtained from Merck, USA. The organic solvent (acetonitrile and methanol) are HPLC grade were purchased from J.T. Baker, USA. The glyphosate standard stock solutions was prepared in methanol at a concentration level of 1000 μg mL^{-1} and stored in a refrigerator at 5°C. The stock standard solutions could be used for 3 months. Suitable concentrations of working standards were prepared from the stock solutions by dilution using a methanol immediately prior to sample preparation.

Study samples: The urine sample from farmer who worked in 4 provinces of Northern part of Thailand i.e., Chiang Mai, Chiang Rai, Payao and Nan. They were grew the vegetable and fruit in their areas. The morning urine samples were collected and aliquot in 15 mL PFP tube and then transported to Toxicology Laboratory, Environment and Health Research Unit, Center of Applied of Health Science, Research Institute for Health Science, Chiang Mai University. All samples were frozen at -20°C until analyzed.

Method validations: The present study was validated the parameters accuracy, precision, linearity and limits of detection (LOD) and quantification (LOQ). The recovery test was determined for accuracy of method at several concentration levels of 2, 10 and 50 ng mL^{-1}. The Relative Standard Deviation (RSD) i.e., repeatability and intermediate precision were reported as the method precision by carrying out the extraction and analysis of fortified samples within batch and difference days for analyzing. Linearity was determined by different known concentrations levels ranged between 1-100 ng mL^{-1} which were prepared by diluting the stock solution. The limit of detection (LOD, 0.5 ng mL^{-1}) and limit of quantification (LOQ, 2.0 ng mL^{-1}) were determined considering the LOD as 3 times the baseline noise and the LOQ as the concentration that produced a relation signal to baseline noise of 10, in a time close to the retention time of the analyst.

Application of the method for determination of glyphosate in urine samples: The method for detecting herbicide glyphosate was modified from previous study[19].

Urine samples preparation: The analytical method used to detect glyphosate in urine sample involves a derivatization step with 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) as derivatized agent. In brief, 3 mL of urine sample was transfer to glass test tube and incubated for 30 min with 0.5 mL of 50 g L^{-1} FMOC-Cl and 0.5 mL of 0.1 M borate buffer by stirring for derivatization. Then, added 30 μL of 2% H_3PO_4 and 4 mL distilled water and vertex mixed.

The samples were pre-concentrated by Solid Phase Extraction (SPE) for cleaning the sample before injected to HPLC. In brief, the 200 mg SPE cartridge C8/SAX was washed by 3 mL methanol 3 time and follow by 3 mL distilled water 3 times on VacElute SPS24 with flow rate 1 mL min^{-1} and then washed again with 3 mL distilled water. After the SPE dryness, the concentrated sample was eluted by 1 mL eluting solvent (HCl:ACN, 1:1v/v), then the eluted sample was filtered by 0.2 µm syringe filter. Twenty microliters of filtrate sample was injected to HPLC for glyphosate analyzing. The glyphosate concentration was calculated against standard curve and report in µg L^{-1}.

HPLC analysis: A concentrated stock solution of the analyzed glyphosate (1000 ppm) was prepared by dissolving 10 mg of the glyphosate in 10 mL of methanol. Stock solution were used to prepare calibration curve with different concentrations in methanol/water (50/50 v/v). For solid-phase extraction, recovery studies and calibration curves, pooled urine sample was spiked with the standard glyphosate and kept in freezer prior to analysis.

High performance liquid chromatography (HPLC: Agilant, 1100) equipped with fluorescence detector (FLD: Agilant, 1046A). The sample volume injected into the HPLC system was 20 µL. Analytical column Discovery® C18 SUPELCO (15 cm×4.6 mm×5 µm) column (Supelco Analytical, USA) was used for separation of the glyphosate. The isocratic mobile phase consisted of 2% H_3PO_4 in water: ACN, 70/30, v/v) as mobile phase at flow rate 0.8 mL min^{-1}. The total run time was 25 min. The fluorescence was set at excitation 242 nm, emission 388 nm and FLD signal was collected and found glyphosate peak at 5.73 min.

RESULTS AND DISCUSSION

HPLC optimization: The HPLC-FLD system proved to be a good option for the determination of glyphosate in urine samples, allowing analysis with good sensitivity. Typical chromatograms of a standard solution of the glyphosate at 20 ng mL^{-1}, (1) Pure standard, (2) Spiked urine sample, (3) Sample urine and (4) Blank urine are shown in Fig. 1.

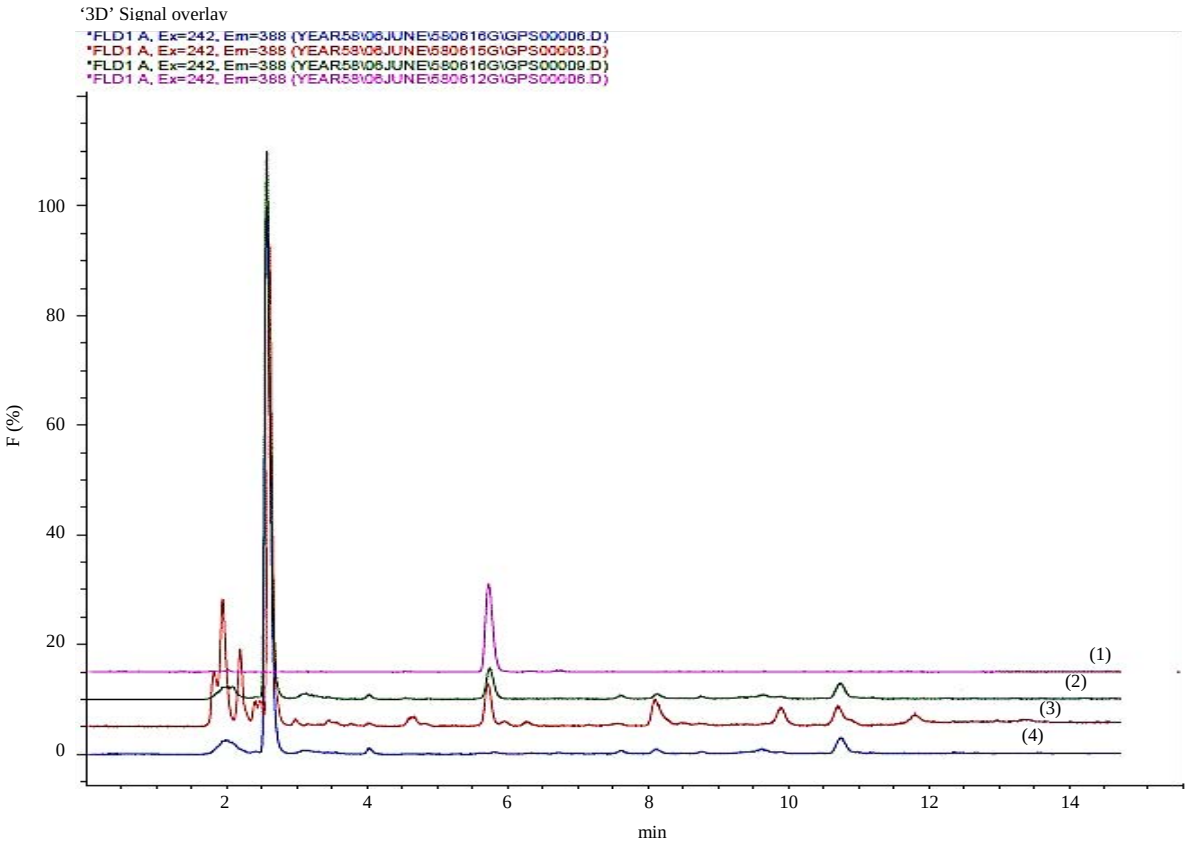

Fig. 1: Chromatogram of glyphosate in urine, 1: Pure stand, 2: Spiked urine, 3: Sample urine and 4: Blank urine

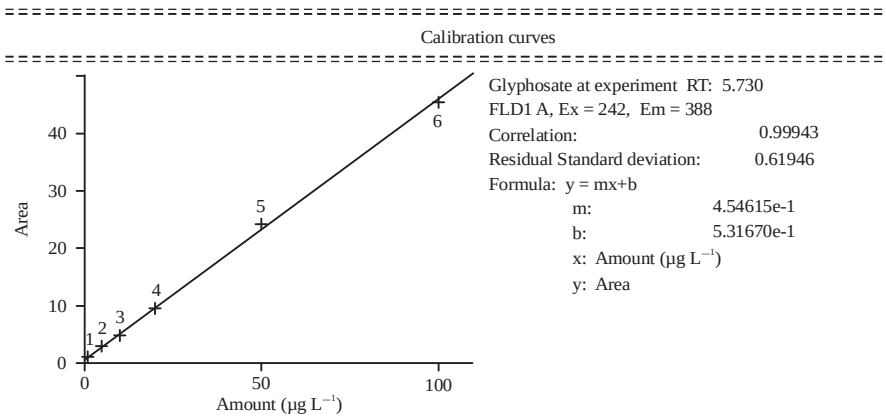

Fig. 2: Calibration curve of glyphosate at concentration 1, 5, 10, 20, 50 and 100 ng mL^{-1}

Table 1: Qualifications parameters of HPLC-FLD method

Parameters	Values
Recoveries (%), n = 3	
2 ng mL^{-1}	91.20
10 ng mL^{-1}	79.70
50 ng mL^{-1}	103.90
Precisions (RSD%)	
Intra-batch (n = 5) 10 ng mL^{-1}	8.86
Inter-batch (n = 20) 2 ng mL^{-1}	11.46
50 ng mL^{-1}	3.93
Linearity	0.9994
Limits of detection (LOD) (ng mL^{-1})	0.50
Limits of quantification (LOQ) (ng mL^{-1})	2.00

Table 2: Previous reported for analyzing glyphosate residue in difference matrix type, limit of detection (LOD) and limit of quantification (LOQ)

Studies	Detection	Matrix type	LOD (µg L^{-1})	LOQ (µg L^{-1})
Wang *et al.*[20]	HPLC-FLD	Sea water	0.60	2.0
		Pure water	0.24	0.8
Han *et al.*[21]	LC-MS-MS	Whole blood	50	100
Zouaoui *et al.*[3]	HPLC-MS-MS	Urine and plasma	20	50
Tzaskos *et al.*[24]	Spectroscopy	Water	93	309
Yoshioka *et al.*[22]	LC-MS-MS	Serum	30	90
Motojyuku *et al.*[23]	GC–MS	Serum	250	3000
Present study	HPLC-FLD	Urine	0.50	2

Accuracy, precision, linearity, limits of detection (LOD) and quantification (LOQ): Recoveries of spiked urine samples were determined. The recovery after extraction and cleaning up steps were carried out at 2, 10 and 50 ng mL^{-1} fortification levels for glyphosate in pooled spiked urine. The recovery data and relative standard deviation values obtained by this method are summarized in Table 1.

The precision of extraction method was performed from RSD% (inter- and intra-batches). Three levels spiked urine were extracted and analyzed for glyphosate concentration. The RSD% were 8.86% at 10 ng mL^{-1} (inter-batches), 11.46% at 2 ng mL^{-1} and 3.93% at 50 ng mL^{-1} (intra-batches) fortification concentrations.

The linearity was performed by using different known concentrations of glyphosate (1, 5, 10, 20, 50 and 100 ng mL^{-1}). The standard solutions were injected to HPLC-FLD and recorded the peak areas. A calibration curve has been plotted of concentration of the standards versus area observed and the linearity of method was evaluated by analyzing 5 levels. The peak areas obtained from different concentrations of glyphosate herbicides were used to calculate linear regression equations. These were Y = 0.4546X+0.5317 with correlation coefficients of 0.9994 A calibration curve was show in Fig. 2.

The limit of detection (LOD) of the method was 0.5 ng mL^{-1} at a level of approximately 3 times the back ground of control injection around the retention time of the peak of interest. The limit of quantification (LOQ) was 2 ng mL^{-1} as 10 times the baseline noise in the chromatogram.

The previous study reported chromatographic based methods for analyzing glyphosate in several kinds of samples such as water, soil and wheat. The present study showed a rapid and good method for analyzing glyphosate by using SPE after re-privatized with FMOC. The result showed good base line chromatogram when comparison with pure standard. The quantification parameters showed good sensitivity, linearity and precision. The LOD and LOQ were better than other previous report which using high technology and more expensive instrument. The previous studied were shown in Table 2[3,20-23].

Determination of glyphosate from farmer's urine samples: The developed method was applied in farmer's urine. The urine samples were collected from the period between June to December, 2014. They have been worked as farmers in agricultural areas. The ages of farmers were range from 18-65 years. The urine samples were collected from 210 male

Table 3: Detection and concentration of glyphosate residue in urine samples of farmers Northern Thailand

	Detection		Concentration (ng mL^{-1})		Concentration (μmol mol^{-1} creatinine)	
Provinces	No.	%	GM	Mean±SD	GM	Mean±SD
Chiang Mai (n = 102)	69	67.6	11.51	16.80±15.7[a]	6.67	11.80±15.2
Male (n = 76)	57	82.6	12.32	17.90±16.5	6.76	12.40±16.22
Female (n = 26)	12	17.4	11.51	11.60±9.5	6.27	8.90±8.2
Nan (n = 102)	55	53.9	8.62	11.10±9.2[b]	5.54	13.20±36.8
Male (n = 58)	36	65.5	8.38	11.30±10.2	5.01	12.10±36.6
Female (n = 44)	19	34.5	9.09	10.60±6.8	6.69	15.30±38.3
Payao (n = 93)	9	9.6	3.94	6.19±8.58[a,b]	3.26	4.10±3.23
Male (n = 76)	7	9.2	4.25	7.08±9.68	3.13	4.14±3.77
Female (n = 17)	2	11.8	3.02	3.04±0.56	3.78	3.96±1.70
Volunteer's (n = 297)	100	44.8	9.50	13.70±13.30	5.88	11.89±26.08
Male (n = 210)	100	47.6	9.96	14.76±14.54	5.75	11.74±25.06
Female (n = 87)	33	37.9	8.23	10.49±7.82	6.31	12.35±29.36

[a,b]Significant difference by Bonferroni at $p<0.05$ and GM: Geometric mean

and 87 female who identified themselves as farmer and working in their farms with rice, vegetables, fruit crops or multiple crops growing. The glyphosate in urine samples was shown in Table 3.

The results showed the highest detection was Chiang Mai province and the lowest was Payao province, while Chiang Mai and Nan province had significant higher than Payao province. The whole study showed 44.8% detected glyphosate residue in urine samples, male farmers had 5 detection higher than female farmers. The present study was shown higher GM of glyphosate residue in urine than other which studied in farmers and their families[25,26].

The residue of glyphosate in urine from farmers who work in farm themselves or from occupational had no many reported yet. The most reported of glyphosate residue were in serum and blood from suicide case in the hospital[21]. The present study showed results that had glyphosate residue in urine samples from farmer. The farmer may exposed to glyphosate in their areas or they used by they own self but without any symptoms because the acceptable daily intake is high for glyphosate.

CONCLUSION

Glyphosate is the most popular use among farmers in upper Northern Thailand. They used for controlling annual and perennial weeds before growing their crops. The modified method of present study for analyzing glyphosate can be used in real urine samples with good sensitivity and inexpensive equipment. The application for determining glyphosate in farmer's urine found some residue in samples. The methods can be used for epidemic studying because fast, simple method and high volume of samples.

SIGNIFICANT STATEMENT

The present study reported simple and sensitive methods for determining glyphosate residue in urine sample after derivatization with FMOC-Cl, cleaning up by SPE and quantification by HPLC-FLD. The application used modified method to assess glyphosate in farmer's urine among Northern Thailand found glyphosate residue in urine samples. The farmer may exposed to glyphosate in their areas or they used by they own self before crop production.

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