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Determination of glyphosate residues in Egyptian soil samples

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ABSTRACT

A sensitive linker-assisted enzyme-linked immunosorbent assay (L'ELISA) was developed for the analysis of glyphosate in Egyptian soil samples. Polyclonal glyphosate antibodies were produced from rabbits immunized with glyphosate protein conjugate. The conjugate was prepared by activating the carboxylic groups of proteins; thyroglobulin or bovine serum albumin with 1-ethyl-3- (3-diaminopropyl) carbodiimide hydrochloride and N-hydroxysulfosuccinimide followed by directly coupled to the amino group of glyphosate. The L'ELISA used succinic anhydride to derivatize glyphosate, which mimics the epitopic attachment of glyphosate to thyroglobulin. L'ELISA recognized the derivatized glyphosate with a limit of detection (LOD) of 0.8 ng g^{-1} and sensitivity (IC_{50} value) of 0.018 μg g^{-1}. The recovery values of the spiked soil samples with different concentrations of glyphosate were in the range of 87.4–97.2%. Good correlation was achieved between L'ELISA and conventional high-pressure liquid chromatography (HPLC) with fluorescence detection. This study demonstrated the utility and convenience of the sensitive, simple, practical and cost-effective L'ELISA method for glyphosate analysis in soil samples. Also, it is ideal for rapid screening of a large number of environmental samples.

Introduction

Glyphosate, N-(phosphonomethyl) glycine, is a systemic, post-emergence, nonselective and broad-spectrum herbicide in Egypt. The active molecule was developed in the 1970s and marketed as a product called Roundup in 1973 by Monsanto Co. It can effectively control 76 of the world's 78 worst weeds. It is currently one of the most important herbicides worldwide. Its unique properties provide for a wide range of uses in agriculture but also in non-agricultural areas [1,2]. However, its widespread application has created problems regarding environmental contamination; therefore, its detection in environmental samples has become increasingly important [3].

Glyphosate analysis in environmental matrices is problematic because it is a small molecule and has structural similarity to many naturally occurring plant materials such as amino acids and secondary plant compounds. It is highly soluble in water thereby making its extraction with solvents difficult. Therefore, glyphosate isolation and quantitation poses a challenge to the analytical chemist due to the necessity of removing matrix effects before analysis. Plant and soil matrices contain co-contaminants that render analysis more costly and time-consuming [4].

Chromatography is the most used method for the determination of glyphosate, including GC [5], GC/MS [6], HPLC with UV [7], diode array [8], fluorescence [9] detection and LC/MS [10,11]. Due to its physicochemical characteristics of glyphosate fit better with LC analysis, although the lack of adequate chemical groups (e.g. chromophores, UV absorption, fluorogenics) hamper their measurement by conventional detectors, thus different methods for the analysis of glyphosate have been developed. For these reasons, both pre-column and post-column derivatization procedures have been employed. Pre column procedures are based mainly on derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl) to form fluorescent derivatives (improve detection) and/or to reduce the polar character of the analytes facilitating the chromatographic retention. In post-column procedures, the most common reaction is with *o*-phthalaldehyde (OPA) and mercaptoethanol or with OPA and *N*,*N*-dimethyl-2-mercaptoethylamine. Normally, HPLC has been used in combination with fluorescence detection after derivatization [12]. Recently, high selectivity and sensitivity are usually achieved using an LC–MS/MS method, based on pre column derivatization with FMOC-Cl and this has gradually come to play an important role in the analysis of glyphosate [13]. Unfortunately, these methods usually considered expensive, complex instrumentation and are out of economical reach for many laboratories, limiting their application in the field of residue analysis [14]. Meanwhile, the existing wide variety of methods also indicates the difficulties with glyphosate analysis.

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ELISA is easy to carry out for most laboratories and suitable for a wide range of matrices, fast, robust, selective, accurate and precise [11]. **As** well as it is a valuable tool in residue analysis and complements conventional analytical methods [15]. Only a few references to a glyphosate immunoassay could be found in the literature which used antibodies produced in their laboratories [4,16,17]. The linker-assisted competitive enzyme-linked immunosorbent assay (L'ELISA) is an environmental method using a derivatized analyte to enhance its affinity to the antibody. Also the results of glyphosate ELISA in a sub-part-per-billion reporting limit, which is needed for routine, rapid, low-cost monitoring of the glyphosate [18,19].

The main objective of this study was to develop and characterize an indirect competitive immunoassay based on polyclonal antibody for glyphosate. Then the resulting assay was validated with spiked-recovery studies from the fortified untreated matrix. The recovery values were also compared to the conventional chromatographic method (HPLC). Finally, the developed assay of glyphosate was applied to the analysis of Egyptian soil samples.

Materials and methods

Chemicals and instruments

The analytical standard of glyphosate (purity 99.7%), formulated glyphosate (Round up 48% WSC), 1-ethyl-3-(3-diaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), thyroglobulin (TG), bovine serum albumin (BSA), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), skim milk powder, goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate and 3,3′,5,5′-tetramethylbenzidine (TMB), succinic anhydride, 9-fluorenylmethyl chloroformate (FMOC-Cl), analytical grade acetonitrile and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co (Germany). Reagent-grade sodium tetraborate, hydrochloric acid (HCl), potassium hydroxide (KOH) and LC-grade water were purchased from standard chemical companies.

96-well flat bottom polystyrene microtiter plates (Nunc, no. 442404 Denmark) were purchased from Sigma-Aldrich Co, ELISA microplate reader (STAT FAX - 2100), ultraviolet visible spectrophotometer (T-80 + UV/VIS spectrometer PG Instrument Ltd) and high pressure liquid chromatography (HPLC) Agilent Technologies HP-1200, USA were used in this study.

Buffers and solutions

Conjugation buffers (0.01 M potassium phosphate buffer, pH 5.0 and 0.2 M potassium phosphate buffer, pH 8.5), dialysis buffer (10 mM phosphate buffer saline (PBS), pH 7.4), coating buffer (50 mM carbonate buffer, pH 9.6), washing buffer PBST (10 mM PBS, pH 7.4 containing 0.05% Tween-20), blocking solution (5% skim milk in PBS), antibody dilution and glyphosate concentrations (50 mM Tris-HCl, pH 9.0 with 100 mM NaCl (TBS)) and substrate buffer (100 mM phosphate citrate buffer, pH 5.4). TMB substrate solution (400 µL of 0.6% TMB-DMSO and 100 µL of 1% H_2O_2 in 25 mL of substrate buffer) and stopping solution (2 M H_2SO_4) were used. A solution of 40 mM sodium tetraborate, pH 8.5 in HPLC-grade water was used for extraction and solution containing 12 mg mL^{-1} of FMOC-Cl in acetonitrile was used for the derivatization step prior to the HPLC analysis.

Glyphosate derivatization

I. *For L'ELISA*: Add 150 µL of each glyphosate concentration or sample to 10 µL of sodium bicarbonate solution (0.5 M $NaHCO_3$), followed by 10 µL of 2% succinic anhydride in DMSO (w/v). The reaction mixture was mixed and incubated at RT for 30 min [18].
II. *For HPLC:* Two mL of the extracted sample (as will mention in 2.7.2.) was acidified with 40 µL of 6 M HCl to pH 1.0 to eliminate possible interactions of glyphosate with matrix components according to the procedure described by Ibáñez et al., [20]. To maximize the dissociation of possible analyte–cation complexes, the pH 1.0 was maintained for 1 h, then neutralized with 40 µL of 6 M KOH. After that, 120 µL of 40 mM borate buffer, pH 8.5 and 120 µL of 9-FMOC-Cl stock solution were added and allowing the reaction to take place overnight at RT. Thereafter, the reaction was stopped by acidifying the solution to approximately pH 1.5 by adding 200 µL of 1 M HCl.

Preparation of hapten-protein conjugates

The hapten (glyphosate) contains an amino group was coupled to carrier proteins; TG or BSA via active ester method using EDC and Sulfo-NHS according to Staros et al., [21]. 100 mM of EDC and 5 mM of sulfo-NHS were added to 40 mg of TG or BSA pre-dissolved in 4 mL of 10 mM phosphate buffer, pH 5.0. The reaction mixture was stirred for 10–15 min followed by the addition of 4 mL of 100 mM of glyphosate in 0.2 M phosphate buffer, pH 8.5. The mixture was stirred for 3 h at RT and then overnight at 4 °C to complete the conjugation. The conjugate glyphosate-TG or glyphosate-BSA was dialyzed against 10 mM phosphate buffer, pH 7.4 for 24 h at 4 °C. During dialysis the buffer was changed four times. The dialyzed material was finally lyophilized and stored at −20 °C. The conjugate was applied as an immunogen (glyphosate-TG) or a coating antigen (glyphosate-BSA). Conjugate formation was confirmed spectrophotometrically by the UV scan for glyphosate, proteins (TG or BSA) and their conjugates in the range of 190–300 nm. UV–Vis spectral data were used to ensure the successful coupling between the hapten and carrier proteins.

Antibody production

Three male New Zealand white rabbits weighing 2.4 ± 0.1 kg obtained from Poultry Department, Faculty of Agriculture, Alexandria University, Egypt were immunized with the immunogen to prepare polyclonal antibody. The experimental protocols were approved by the Ethical Committee for the use and care of laboratory animals. Routinely, 10 mg of the conjugate dissolved in 0.5 mL of sterile saline solution (0.9%) emulsified with CFA (1:1 vol ratio) was injected subcutaneously at multiple sites on the back of each rabbit. This was followed by four secondary boosters of the same dose of immunogen emulsified with ICFA at 2 weeks intervals and bled 7–10 days after each boosting. Serum was isolated by centrifugation, purified by 80% saturated ammonium sulfate precipitation, lyophilized and then stored at −20 °C.

Immunoassay optimization

Various dilutions of obtained antibody were titrated against varying amounts of the coating antigen to measure the reactivity of antibody and select the appropriate concentrations of coating antigen and antibody for indirect competitive assays (checkerboard assay). The assay procedure was as follows: 96 wells polystyrene microtiter plates were coated with 100 µL/well hapten-BSA (25–1.56 µg mL^{-1}) and incubated overnight at 4 °C. The plates were washed four times with PBST, blocked with 5% skim milk (300 µL/well) and incubated at RT for 2 h. After another washing step, 100 µL/well of antibody previously diluted with 50 mM TBS, pH 9.0 (1:500–1:32000) was added. After incubation at RT for 2 h, the plates were washed and 100 µL/well of a diluted (1:3000) goat antirabbit IgG-horseradish peroxidase was added. The plates were incubated at RT for 2 h, and after another washing step, 100 µL/well of TMB substrate solution was added and incubated at RT. The reaction was stopped after 10 min by adding 50 µL/well of 2 M H_2SO_4 and absorbance was read at 450 nm using a microtiter plate reader (STAT FAX -2100). Optimal antigen concentrations and antibody dilutions which produce absorbance around 0.7–1.0 unit were chosen.