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Development of Antibodies for the Detection of N-Acetyl-glufosinate§

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Glufosinate is a widely used herbicide, which is difficult to detect by conventional analytical techniques. For many other herbicides, suitable antibodies have been raised for immunoassay development. Unfortunately, glufosinate is a very small molecule and difficult to immunize with. Thus, a derivatization-assisted immunoassay (DAIA) using the target analyte N-acetyl-glufosinate (NAG) was constructed. The activated hapten was synthesized by a new approach, using a homobifunctional cross-linker suberic acid bis(N-hydroxysuccinimide ester). The preparation of a suitable conjugate, the immunization, and the characterization of polyclonal antibodies are shown. The determination of the conjugation density (hapten density) of the immunogens was performed by four different methods (high-performance liquid chromatography with a refractive index detector, total reflection X-ray fluorescence, inductively coupled plasma mass spectrometry, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), which gave similar results. The limit of detection was 17 μg/L NAG in water for the direct competitive enzyme immunoassay. NAG is also a main metabolite of glufosinate in resistant transgenic plants. The antibodies might be useful for the selective detection of NAG in the presence of the parent compound glufosinate (cross-reactivity 0.13%) and other metabolites.

KEYWORDS: Glufosinate; phosphinothricin; conjugate; immunization; ELISA; derivatization-assisted immunoassay; disuccinimidyl suberate; herbicide; hapten; glyphosate; amino acid

INTRODUCTION

Glufosinate (also known as PPT) is widely used in the form of the ammonium salt as a broad spectrum nonselective herbicide (*2*). The L-enantiomer is a natural amino acid (L-2-amino-4-(hydroxymethylphosphinyl)butanoic acid) containing a phosphorus−carbon bond, which is extremely rare in nature. Glufosinate is part of the tripeptide phosphinothricyl−alanyl−alanine (bialaphos), which was independently discovered by a Japanese (*3*) and a German (*4*) group in 1971 in the soil bacteria *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes*, respectively. The peptide showed some antibiotic properties but never reached medical application. The herbicidal activity of glufosinate was discovered by researchers from Hoechst AG in 1977. The racemic glufosinate ammonium salt (trade names: Basta, Buster, Challenge, Harvest, Dash, Finale, Liberty, Ignite, and Rely) was synthetically produced in amounts of 2500 tons/year in 1995 (*5*). For the year 2000, sales of 113 million euros have been reported for Basta (*6*, *7*).

A related structure features another herbicide, which is known as glyphosate (N-(phosphonomethyl)glycine). Different salts of glyphosate were patented by Monsanto (trade name: Roundup) and ICI, now Syngenta (trade name: Touchdown). Glyphosate is the world's best-selling herbicide (*8*), with estimated amounts of 200 000 tons/year in 2002 (*9*−*11*) and 74 045 tons or 1490 million U.S. dollars in 1997 (*12*). Although the chemical structures of glufosinate and glyphosate show some similarity, they exhibit different modes of action. Glyphosate belongs to the 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase inhibitors, whereas glufosinate inhibits mainly the enzyme glutamine synthetase. Only L-glufosinate shows herbicidal activity; the D-enantiomer is completely inactive. Recently, the crystal structure of a glufosinate/glutamine synthetase complex was published (*13*). Because glufosinate and glyphosate are nonselective contact herbicides, their use was limited to only a few areas of application. However, glufosinate (and glyphosate) tolerant plants have been designed by genetic engineering leading to a new approach of herbicide application. Glufosinate resistance genes (*pat* and *bar*) were obtained from bialaphos-synthesizing *Streptomyces* strains, which need these genes for self-protection. The *pat* and *bar* genes have been transferred to crops, such as corn, soybean, oilseed rape, and cotton. The transgenic plants produce a new enzyme, phosphinothricin-N-acetyltransferase (PAT), which inactivates glufosinate in the cell by acetylation. Other plants (for instance, weeds) are killed by

§ Dedicated to the late Professor Ernst Bayer (*1*).

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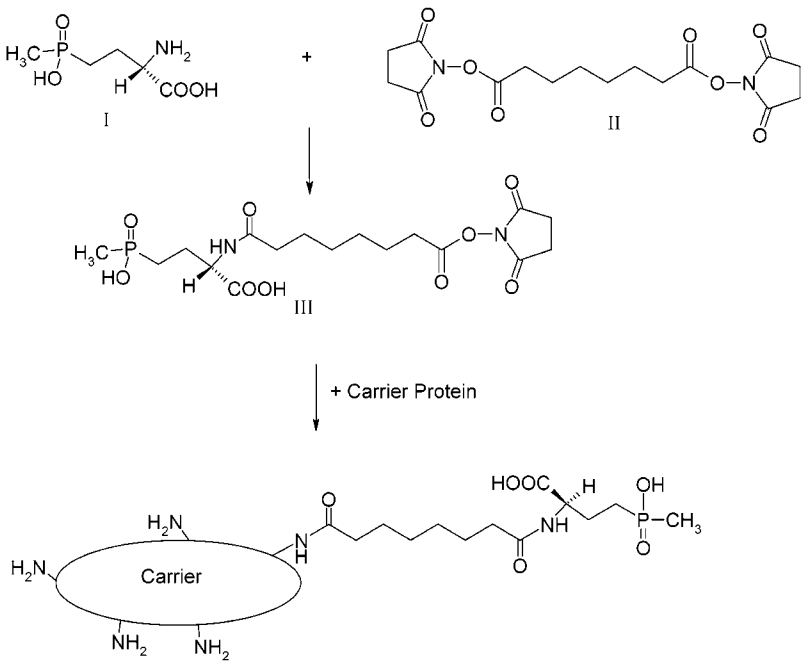

Figure 1. Synthetic scheme for immunogen preparation. The reaction of L-glufosinate (I) with the bifunctional compound (II) leads to a mixture of products from which compound (III) is isolated by chromatography (**Figure 2**). Conjugation of III with the carrier protein leads to a pure conjugate, since the only byproduct NHS can be easily separated from the conjugate by gel chromatography or washing of insoluble conjugates.

glufosinate, since they do not possess such a protecting enzyme. Plants with the respective genes show NAG as the predominant metabolite, in contrast to sensitive crop plants, which exhibit MPP and MPA as metabolites (*14*−*16*).

Glufosinate ammonium (salt) is extremely water soluble (∼1370 g/L), whereas the solubility in organic solvents is very low. In addition, glufosinate forms strong complexes with multivalent metal cations (*17*). Glufosinate is a small molecule of a molecular mass of only 181 g/mol. Similar to other amino acids, the volatility is very low and the melting point is given as 241−242 °C for the racemate (*18*). The dissociation constants have been determined by ^{1}H NMR titration (*19*). Surprisingly, the acidity of the carboxylic group and the phosphinic group (**Figure 1**, compound I) is essentially the same; the pk_a is about 2.2 for both.

The analysis of glufosinate can be accomplished by different means; however, all of them are laborious and complicated. Because glufosinate exhibits extreme water solubility, enrichment on RP material or with liquid−liquid extraction fails in most cases. Furthermore, there is no significant retention on usual RP columns in HPLC. In addition, glufosinate does not possess chromophores useful for UV detection. Therefore, nearly all chromatographic methods described in the literature are based on precolumn derivatization. Even enantioselective variants have been described (*20*). The derivatization with *p*-nitrobenzoyl chloride (*21*) or FMOC chloride was reported (*22*). A commercial system based on the postcolumn derivatization with OPA and *N*,*N*-dimethyl-2-mercaptoethylamine became available (*23*). In this case, an ion exchange column is used for separation. It is also possible to detect glufosinate with gas chromatographic techniques (*24*). The most popular method uses acetic acid and trimethylorthoacetate as a derivatization reagent (*24*, *25*). Mass spectrometric detection (MS/MS) seems to improve the performance of this approach (*25*). Furthermore, a procedure based on micellar electrokinetic chromatography and laser-induced fluorescence detection was proposed (*26*). In addition, anion exchange chromatography was coupled to pulsed amperometric detection (*27*) to avoid any derivatization. Recently, a review was published, comparing several chromatographic techniques for the analysis of glufosinate (*28*).

It would be desirable to have a fast and simple analytical method for glufosinate, since the increasing cultivation of genetically modified plants leads to an increased use of the corresponding herbicides. Immunoassays are well-suited for the screening of water, soil, and biological material. Furthermore, they are very cost effective and rapid. Many applications of immunoassays do not need any or very little sample preparation. Immunoassays for glufosinate have not been described, in contrast to immunoassays for glyphosate (*29*−*31*). The low molecular mass and the weak immunogenicity might be reasons for this situation. For most haptens, a chemical derivatization is needed to make them suitable for the conjugation to a carrier protein. This conjugation is crucial to elicit an immune reaction. Substances of low molecular mass do not show any direct immunogenicity if injected in an animal. The method and the site of conjugation are critical for the success of an immunization. The main rule of conjugation is to minimize the changes of the properties to the target molecule. Otherwise, one would obtain antibodies against the derivative only. In the case of glufosinate, this rule can hardly be met, because the molecule is so small. Therefore, we decided to target a derivatized analyte, acetyl glufosinate (*32*). Recently, a similar approach was published for glyphosate (*29*, *31*), which was termed linker-assisted enzyme immunoassay by one group (*29*). We see this as a special case of the more general approach of a DAIA, since not only the masking of the conjugation site may be beneficial but also the increased molecule size or altered molecular properties may improve the binding of the antibody to its corresponding analyte by extension of their contact area. This effect is not limited to the linker-connected site but can be applied to all other parts of the molecule, which are accessible to a chemical derivatization reaction. This approach has been known for quite a long time, for example (*33*−*35*), but has achieved only limited application, yet. For the determination of polychlorinated biphenyls, a DAIA was shown, using a catalytic dechlorination to obtain a single product (biphenyl),

which was detected by immunoassay (*36*). In other immunoassays (e.g., for indole-3-acetic acid) the methylation by diazomethane of the analyte was used to improve selectivity and sensitivity of the analytical procedure (*37*, *38*). In this paper, the generation of antibodies to NAG and their characterization are described. The conjugation via acylation of the amino group of glufosinate with suberic acid bis(NHS ester) leads to a strong preference of the antibodies for N-acyl derivatives of glufosinate in relation to glufosinate. Hence, NAG can be measured directly with the immunoassay described in this paper. If glufosinate instead of NAG should be measured, a preceding acylation step would be necessary (DAIA). This derivatization, which would be part of some analytical procedures, will not be dealt with in this paper. In brief, acylation might be performed by several kinds of acylating reagents, such as acetic acid anhydride, acetic acid NHS ester, or by enzymatic means, such as the PAT/AcetylCoA system (*32*).

MATERIALS AND METHODS

Chemicals and Immunoreagents. Most chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany) in the highest purity available. N-Acetyl-DL-glufosinate disodium salt, DL-glufosinate, L-glufosinate ammonium salt, 3-(hydroxymethylphosphinyl)propanoic acid, and 2-(hydroxymethylphosphinyl)acetic acid were supplied by Bayer CropScience (Frankfurt, Germany). DL-2-Amino-4-phosphonobutanoic acid was obtained from Tocris (Bristol, U.K.), and bialaphos was obtained from ifb Halle GmbH (Halle, Germany). Keyhole limpet hemocyanine (KLH) was purchased from Biosyn (Fellbach, Germany), horse anti-mouse IgG (H + L) peroxidase-labeled was from Vector (Burlingame, CA), and horseradish peroxidase (EIA grade) was obtained from Roche Diagnostics (Mannheim, Germany).

Synthesis of Hapten Carrier Conjugates (Immunogens and Coating Antigens). *Synthesis of Glufosinate-C8 NHS Ester.* Four grams of L-glufosinate ammonium salt was dissolved in 4.5 mL of water. A 0.8 g amount of NaOH was added, and the solution was dried with a rotary evaporator at 50 °C. Reconstitution in water and drying was repeated three times. The removal of ammonia can be checked with a wet pH paper over the aqueous solution. A 200 mg amount of boric acid was added and brought to pH 9 by HCl. The solution was diluted to 50 mL by water, which leads to a 0.4 M solution of L-glufosinate (L-phosphinothricin, L-PPT) in borate buffer. A 200 mg (0.54 mmol) amount of suberic acid bis(NHS ester) was dissolved in a mixture of 2 mL of dry dimethyl sulfoxide and 1 mL of dry dioxane. Then, 200 μL of the borate-buffered glufosinate solution (0.08 mmol) was added at room temperature. After 3 h of stirring, 13 mL of water was added. The colorless precipitate was removed by filtration. The product was isolated by anion exchange chromatography with a flow rate of 0.8 mL/min (isocratic) on a Spherisorb S5 SAX column (Waters, 4.6 mm × 250 mm) with refractive index detection. Two milliliters of the product solution was injected per run. The eluent consisted of a methanolic phosphate buffer pH 3.0 (6.8 g of KH_2PO_4, 850 mL of water, 100 mL of methanol, adjusted with H_3PO_4 (85.5%) to pH 3.0, made up to 1000 mL). The product was the largest peak at about 13 min. To minimize hydrolysis, the product fraction was collected in a flask cooled with liquid nitrogen and freeze-dried subsequently. A colorless mixture of glufosinate-C8 NHS ester and phosphate salts was obtained.

KLH Conjugates. Twenty milligrams of KLH was dissolved in 4 mL of borate buffer (pH 9.3). Eight milliliters (= four batches, see above) of glufosinate-C8 NHS ester fractions was lyophilized, and the residue was added to the KLH solution. The mixture was adjusted to pH 8.5 by sodium hydroxide solution. The solution was stirred overnight at room temperature. Precipitates were removed by centrifugation. The supernatant was concentrated by evaporation under a gentle nitrogen stream to about 2 mL. This solution was applied to a short gel chromatographic column (PD-10, Pharmacia) and fractionated into a MTP. Small aliquots of the wells were tested for protein with a commercial BCA protein test (Sigma). This procedure lead to a conjugation density of 88%, assuming an amount of 182 nmol lysine per mg KLH (Biosyn). In a second batch, a 10 times lower ratio of hapten:protein was used, which leads to a conjugation density of 3%. In this case, 10 mg of KLH was dissolved in 2 mL of borate buffer (pH 8.5). A 0.4 mL amount of lyophilized glufosinate-C8 NHS ester was added. No adjustment of pH was necessary. The further steps were performed as described for the high density conjugate.

HRP Conjugate, Enzyme Tracer. A 2.5 mg amount of horseradish peroxidase (EIA grade) was dissolved in 3 mL of borate buffer (pH 8.5). Six milliliters of lyophilized glufosinate-C8 NHS ester solution (= three batches, see above) was added and stirred for 24 h at room temperature. The product was purified on a gel chromatographic column (PD-10, Pharmacia) with phosphate buffer as eluent. Fractions with high absorbance at 405 nm were pooled and stored at 4 °C.

Synthesis of N-Hexanoyl-DL-glufosinate. A 10.5 g amount of DL-glufosinate (70%) was dissolved in 45 mL of 2 M NaOH and 20 mL of dioxane. The mixture was cooled to 5 °C. Small portions of capronic acid anhydride (20.5 mL in total) were added, while adjusting the pH to >9 by additional NaOH. The solution was warmed to room temperature and stirred overnight. The volume of the mixture was reduced at 45 °C under vacuum until a clear solution was formed, which was acidified with HCl and four times extracted with diethyl ether (50 mL) at pH 6, 4, 2.5, and 0. At pH 0, three layers were formed. The oily phase in the middle was separated and dried at 45 °C in vacuo. A 1.7 g (15%) amount of a glassy, colorless substance was obtained. The structure was confirmed by ^{1}H NMR (D_2O, normalized to 4.66 ppm, HDO): 0.74 (t, 3 H), 1.17 (m, 4 H), 1.41 (d, 3 H), 1.48 (m, 2 H), 1.68−2.1 (m, 4 H), 2.18 (t, 2 H), 4.32 (dd, 1 H).

Determination of Conjugation Density. Four techniques were tested for their suitability to determine the conjugation density of hapten−protein conjugates. Because the carrier proteins are essentially free of phosphorus, even elemental analysis was an option. Previous work showed that elemental analysis is a powerful technique for the characterization of bound residues (*39*).

Conjugation Density by HPLC-RI. A soluble or insoluble sample of a protein conjugate was hydrolyzed in 6 N HCl (24 h, 110 °C, vacuum). By this procedure, glufosinate was released and could be determined by HPLC (conditions see above). The sample of the acidic solution was evaporated to dryness by a nitrogen stream and reconstituted by water before injection.

Conjugation Density by TXRF. The content of phosphorus was directly proportional to the conjugation density, if the hapten contained this element. It could be verified that the desalted proteins did not contain significant amounts of phosphorus (data not shown). Soluble conjugates were analyzed directly; insoluble ones needed a digestion step (nitric acid). Vanadium was used as internal standard. Unfortunately, the response factor of phosphorus was low and the sulfur peak may overlap partially with the phosphorus signal. Therefore, the determination may be hampered in sulfur rich proteins. The limit of detection was estimated to be in the range of 1 mg/L phosphorus.

Conjugation Density by ICP-MS. The sample pretreatment was similar to TXRF; however, larger volumes were needed for ICP-MS. The limit of detection for P was in the range of 5 μg/L (calibration with phosphoric acid).

Conjugation Density by MALDI-TOF-MS. This is a well-established technique for measuring the molecular mass of a protein. The difference between the mass of the unconjugated protein and the conjugate leads directly to the mean conjugation density, considering the mass of the hapten molecules (*40*). It is typical that the different species cannot be resolved; therefore, a relatively broad peak is obtained for the conjugate. Unfortunately, the technique is limited in practice to soluble proteins of medium mass. KLH, one of the most important carrier proteins in immunological work, seems to not be suitable for MALDI-TOF analysis. However, the sensitivity of the technique is excellent; only minute amounts of the conjugate are needed.

Immunization. All immunizations were carried out at Davids Biotechnologie (Regensburg, Germany). Two New Zealand White rabbits (~2 kg, male) were immunized with 50 μg of glufosinate-C8 KLH (insoluble, high density, 88% conjugation). The first three immunizations were performed with water-in-oil emulsions (TiterMax),

once intradermally and twice subcutaneously. The following booster injections were performed with glufosinate-C8 KLH (soluble, low density, 3% conjugation) intramuscularly without adjuvant. The booster injections were done 28, 63, 91, 126, 154, and 198 days after the primary immunization. Sera were taken after the 4th and the 7th immunization, 1 week after injection. The serum after the 7th immunization was used for the immunoassays described here.

Immunoassay Procedure. A direct competitive enzyme immunoassay was used throughout (*32*, *41*). A MTP (Maxisorp, Greiner, Germany) was directly coated (200 μL per well) with antiserum diluted (1:5000) in carbonate buffer (pH 9.6) for 4 h. The coated MTP was washed thrice with washing buffer (pH 7.4, HEPES; Tween 20, 0.09%). The plate was blocked with 0.5% bovine serum albumin (BSA) over 30 min (250 μL/well). Then, the plate was washed again. Subsequently, 200 μL of (neutral) sample or standard solution was pipetted into the wells. A preincubation of 15 min was sufficient in all cases. Then, 50 μL of enzyme tracer (in HEPES buffer or diluted horse serum) was added. After 15 min of incubation, the MTP was washed and filled with chromogenic enzyme substrate (citrate buffer, pH 4.3, tetramethylbenzidine, hydrogen peroxide). The color reaction was stopped after a suitable time (about 5−30 min) with diluted (5%) sulfuric acid. The resulting absorbance should not exceed 1.5 at 450 nm. The calibration curves were fitted with a (sigmoidal) four parametric equation (*41*). The conditions used for **Figures 3** and **4** were as follows: coating: rabbit serum no. 1 (1:5000), 3 h; blocking with 0.5% BSA, 30 min; preincubation: 200 μL of standard (or solvent mixture, **Figure 4**), 15 min; tracer incubation: 50 μL of glufosinate-C8−HRP (1:10,000), 15 min; development: 5 min.

RESULTS AND DISCUSSION

Synthesis of Hapten Conjugates of High Purity. For haptens containing a free amino group, several approaches are available (*42*). The most popular might be the glutaraldehyde method (homobifunctional) or the use of heterobifunctional linkers. However, both approaches have their drawbacks. Glutaraldehyde conjugates are of unspecified structure and might even be unstable. Heterobifunctional linkers are expensive and of limited applicability, since two different chemistries have to be applied. Other homobifunctional linkers are also popular but typically lead to some byproducts. This might cause the induction of unwanted antibodies in immunization. This can be largely avoided by the isolation of the activated species, which is then reacted with the carrier protein or enzyme. A novel approach was tested (**Figure 1**), which might be generally applicable for many haptens possessing a free amino group, such as other amino acids and peptides. The activated hapten was prepared by the reaction of a limited amount of the hapten with an excess of a homobifunctional reagent (disuccinimidyl suberate, 1,8-disuccinimidyl octane diacid, suberic acid bis(NHS ester)). The monoester was isolated by HPLC (**Figure 2**). Although NHS esters are sensitive to hydrolysis, an aqueous gradient could be used for separation. However, the eluate was frozen immediately and lyophilized. This approach avoids the formation of conjugates containing the sole linker and cross-linked proteins, which would be unwanted in most cases. Traces of monoester hydrolyzed after purification would not harm, since the formed carboxylic acid is not capable of forming any conjugates. It would be finally removed together with the NHS, which is set free by hydrolysis or conjugation by the usual gel chromatographic step at the end of the procedure. Therefore, it can be expected that this approach leads to conjugates of extraordinary structural purity, which is of crucial importance for the generation of polyclonal antibodies of high selectivity.

The conjugation density of the glufosinate-C8−BSA conjugates has been analyzed by four independent methods (**Table 1**). The lowest density is obtained by ICP-MS. Liquid chromatography of the hydrolyzed conjugate with refractive index detection gives nearly the same results than MALDI-TOF-MS, which seems to be the most popular and simple method. TXRF is another variant to measure the phosphorus content of the conjugate. However, heavier elements are preferable for this technique. With all approaches, it could be confirmed that a suitable conjugation density was achieved by the described procedure.

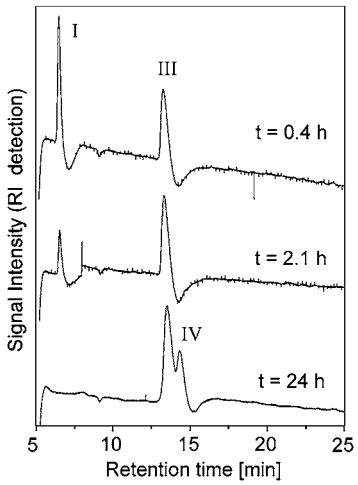

Figure 2. Chromatographic isolation of glufosinate-C8 NHS ester. I, L-glufosinate (reactant); III, glufosinate-C8 NHS ester (product); IV, glufosinate-C8 carboxylic acid (hydrolysis product of III).

Table 1. Comparison of Conjugation Density of Glufosinate-C8−BSA Conjugate by Different Methods[a]

method	analyte	conjugation density (molecules/carrier)
TXRF	P	32 ± 0.3
ICP-MS	P	21 ± 0.5
HPLC-RI	glufosinate	26 ± 1.2
MALDI-TOF-MS	conjugate	26 ± 0.5

[a] A maximum of 30−35 available lysine groups is reported in the literature (*42*).

Immunization. Two rabbits have been immunized with KLH hapten conjugates. First, a glufosinate-C8−KLH immunogen of high conjugation density (88%, 50 μg, intradermally) has been used for two immunizations. Subsequently, a conjugate of low density (3%, 50 μg, subcutaneously and intramuscularly) was used to boost the generation of high affinity antibodies. This should reinforce the mechanism of affinity maturation, which might be suppressed by the use of a high density conjugate capable of forming strong multivalent antibody complexes (bonus effect of multivalency (*43*)) even with antibodies of medium or low (monovalent) affinity. Both rabbits showed a good response to the immunogens. Because rabbit 1 showed slightly better affinities (~factor of 2), an antiserum of rabbit 1 was used throughout this study. In **Figure 3**, a calibration curve of the optimized assay is shown. The limit of detection (3s) was about 17 μg/L N-acetyl-DL-glufosinate, which is the molar equivalent to 14 μg/L DL-glufosinate or 7 μg/L L-glufosinate, if 100% enantioselectivity is assumed. In molar concentrations, the detection limit of the racemate is below 80 nmol/L and the midpoint (IC_{50}) at ~1.2 μmol/L.

In accordance with other immunoassay developments, this assay was tested for the influence of important matrix components. First of all, the pH dependence was examined. However, except for a change in signal intensity, probably caused by the instability of horseradish peroxidase at nonneutral pH, no

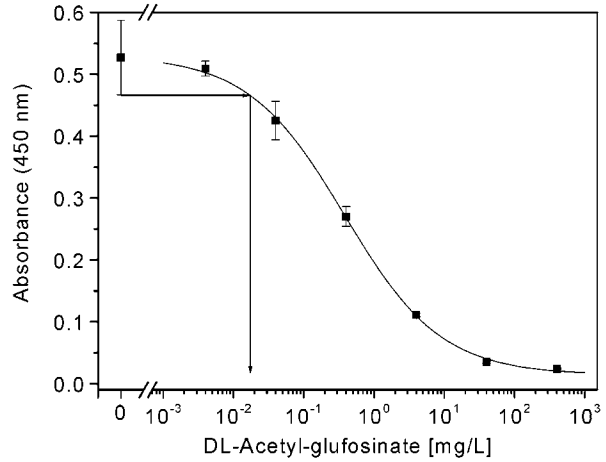

Figure 3. Optimized immunoassay for the determination of N-acetyl-DL-glufosinate. The limit of detection (3s, see arrow) was about 17 μg/L N-acetyl-DL-glufosinate. Error bars: standard deviation (except blank, 3s).

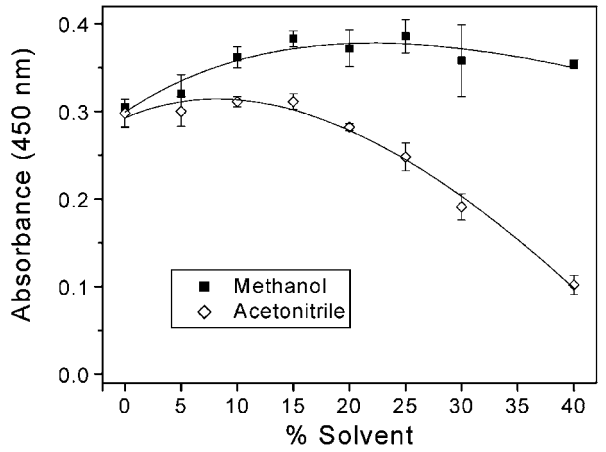

Figure 4. Stability of antibodies and tracer against organic solvents. Midpoints: 2.6 ± 0.4 (0% solvent), 2.7 ± 0.6 (7.5% methanol), 2.6 ± 0.3 (15% methanol), 2.4 ± 0.3 (7.5% acetonitrile), and 1.7 ± 0.3 mg/L (15% acetonitrile). Error bars: standard deviation.

changes in the midpoint were detected between pH 3 and pH 9 (data not shown).

A significant influence of the ionic strength on the absorbance could be observed; an optimal salt (NaCl) concentration of about 75 mM was determined. However, the effect on the antibody and on the enzyme tracer could not be separated. The IC_{50} values were constant at different salt concentrations, which is an indication that the binding site itself is not dependent on the ionic strengths but more likely the overall stability of the antibody or the enzyme label. Finally, the influence of ethylenediaminetetraacetic acid in combination with horse serum was tested to reveal any metal ion dependencies. However, no influence on IC_{50} was detected (data not shown).

Chemical derivatization reactions are often performed in organic solvents. Therefore, the influence of solvents was examined (**Figure 4**). Similar to many other immunoassays, water/methanol mixtures seem to be relatively uncritical (up to 40% methanol), in contrast to acetonitrile, which makes problems at lower percentages (>60% signal loss at 40% solvent). Some of the effects might also be due to the viscosity changes of solvent mixtures. The midpoint was not influenced by addition of up to 15% solvent (methanol or acetonitrile), which is an indication that hydrophobic interactions are of minor importance for this antibody.

Table 2. CRs of Rabbit Serum against L-Glufosinate-C8 Conjugate

compound	CR [%][a]
N-hexanoyl-DL-glufosinate	590
N-acetyl-L-glufosinate	**100**
N-acetyl-DL-glufosinate	64
N-(3-nitro-4-methoxy-benzoyl)-DL-glufosinate	51
3-(hydroxymethylphosphinyl)propanoic acid	0.29
L-glufosinate	0.13
N-acetyl-L-glutamic acid	0.12
N-acetyl-DL-glutamic acid	0.07
2-(hydroxymethylphosphinyl)acetic acid	<0.03
N-acetyl-DL-aspartic acid	<0.03
N-acetyl-DL-alanine	<0.03
N-acetyl-DL-phenylalanine	<0.03
N-acetyl-DL-phenylglycine	<0.03
N-acetyl-DL-serine	<0.03
DL-2-amino-4-phosphono-butanoic acid	<0.03
glyphosate	<0.03
bialaphos	<0.03

[a] Estimate of relative error 10–25% (based on IC_{50} values); CR calculated from molar concentrations CR [%] = 100 × $IC_{50,molar}$(standard)/$IC_{50,molar}$(compound).

CRs and Enantioselectivity. The determination of CRs is a crucial step in immunoassay development, since unfavorable CRs can prevent the useful application of an otherwise powerful assay. It has to be considered which compounds might be present in the anticipated matrices and whether a group selective ("generic") or a compound selective ("specific") assay is desired. High CRs to compounds, which are unlikely to occur in real samples (e.g., rare laboratory chemicals), do not have a negative influence on the applicability of an immunoassay and often are tested only for the examination of structure/affinity relationships or to explore the binding site of the antibody in more detail (*44*). The most popular method for the determination of cross-reactivities is the comparison of sigmoidal curve midpoints (IC_{50}) expressed in mol/L related to a reference compound. It should be noted that the selection of this reference compound is quite arbitrary and might be application dependent. In many cases, the most important analyte is chosen as the standard compound. This does not necessarily mean that this is the compound with the highest affinity. Hence, CRs of >100% may occur. It is advisable to run a calibration curve of the standard compound on each MTP used for CR determination and to calculate the CRs relative to this IC_{50}. Most antibodies are enantioselective per se, since their binding site is three-dimensional. It is quite difficult to make antibodies, which do not distinguish enantiomers, even if a racemate or a nonchiral hapten is used for immunization. The measurement of the enantioselectivity is not different to other CRs, if the pure enantiomers are available. In our case, only one enantiomer (higher CR, homologous to immunogen) and the racemate were available. The accuracy of the measurement of enantioselectivity based on this comparison is relatively poor. In the best case, only a factor of 2 can be expected, if one enantiomer shows a CR of 100% and the other one of 0%. The situation would be better if the enantiomer of low CR would be available. However, it should be noted that the CR measured is highly dependent on the enantiopurity of the compound of low CR.

In **Table 2**, CR data of a serum of rabbit no. 1 are listed. The respective structural formulas are given in **Figure 5**. Considering the chemical structure of the immunogen (**Figure 1**), the results can be interpreted quite smoothly. L-Acetyl glufosinate was chosen as the reference substance, since this is the main target substance and a homologous structure to the immunogen. The CR of the racemate is about 50% of the standard compound, which is in accordance with the assumption

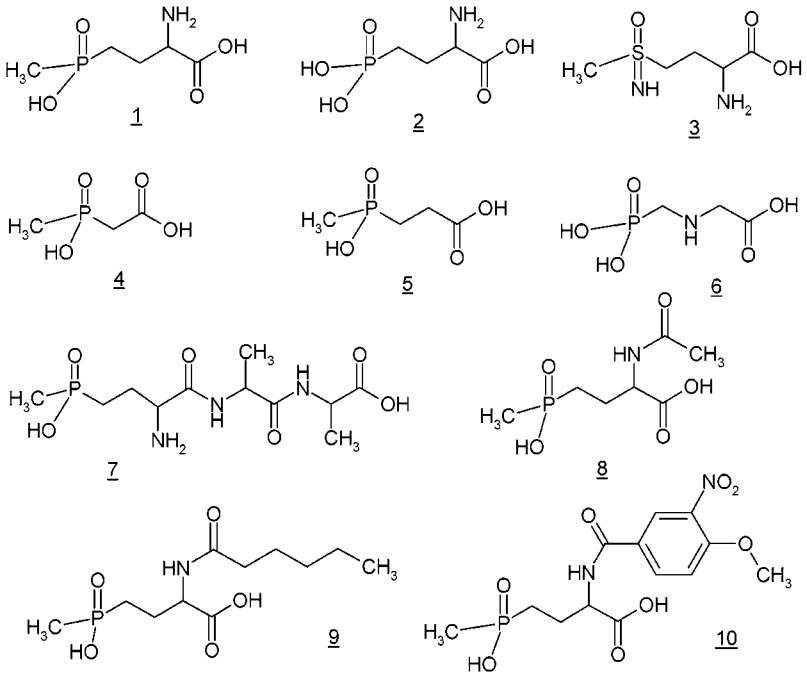

Figure 5. Structural formulas. Compound **1**, glufosinate (phosphinothricin, PPT, 2-amino-4-(hydroxymethylphosphinyl)butanoic acid); **2**, 2-amino-4-phosphonobutanoic acid (DL-AP4); **3**, L-methionine sulfoximine; **4**, 2-(hydroxymethylphosphinyl)acetic acid; **5**, 3-(hydroxymethylphosphinyl)propanoic acid; **6**, glyphosate (N-(phosphonomethyl)glycine); **7**, bialaphos (L-2-amino-4-(hydroxymethylphosphinyl)butanoyl-L-alanyl-L-alanine); **8**, N-acetyl-L-glufosinate; **9**, N-hexanoyl-DL-glufosinate; and **10**, N-(3-nitro-4-methoxy-benzoyl)-DL-glufosinate.

that the serum is enantioselective and shows only weak or no CR to D-acetyl-glufosinate. The latter substance was not available (D-glufosinate, neither) and therefore could not be tested for CR. N-Hexanoyl-DL-glufosinate is the most similar compound in relation to the immunogen. Hence, it was not a surprise that this compound exhibits the highest CR of all of compounds tested (about 600%), which corresponds to a 10 times higher affinity in relation to the acetyl compound (also racemate). More surprising is the fact that the 3-nitro-4-methoxy-benzoyl-glufosinate showed no difference in CR to the acetyl compound. There seems to be a compensation between the higher sterical demand and an affinity bonus gained at the linker site. Because glufosinate is an analogue of glutamic acid, it was also expected that acetyl-glutamic acid might be a cross-reactant. Amino acids are ubiquitous compounds in the environment. Therefore, their CRs are highly relevant for the measurement of real samples. Fortunately, the CR of acetyl-glutamic acid is very low (about 0.1% of the standard), which emphasizes the high selectivity of this serum, despite the small size of the analyte(s). The enantioselectivity between the acetyl-glutamic acids is similar to those obtained from the acetyl-glufosinates. The herbicide L-glufosinate itself also shows a very low CR of only about 0.13%, ~770 times lower than the acetylated compound. This stresses the importance of correct charges in the molecule (*44*) and the effect of the linker bonus (binding enhancement by the presence of the linker or a part thereof). The metabolite 3-(hydroxymethylphosphinyl)propanoic acid shows an even higher CR than L-glufosinate. This can be explained by the disruptive positive charge at the amino group of glufosinate, which is lacking in the case of the propanoic acid. Even the loss of one carbon atom in the chain could not compensate for this effect. However, the loss of a second carbon atom in 2-(hydroxymethylphosphinyl)acetic acid leads to a CR < 0.03%. Other tested N-acetyl amino acids, such as N-acetyl-aspartic acid, N-acetyl-alanine, N-acetyl-phenylalanine, N-acetyl-phenylglycine, and N-acetyl-serine showed no significant CR. The comparison between L-glufosinate and 2-amino-4-phosphono-butanoic acid shows that the methyl group located at the phosphorus makes a measurable contribution to the affinity of the antibodies. The modification of the carboxyl function of glufosinate in bialaphos eliminates binding completely (**Table 2**). This might be caused by steric hindrance, as it is unlikely that the binding site tolerates any larger substituents at this position opposite to the linker site. Furthermore, the amino group of bialaphos was not acetylated.

CONCLUSIONS

A highly selective serum for NAG and other N-acylated glufosinate derivatives has been generated by immunization with a KLH−L-glufosinate conjugate (glufosinate-C8−KLH). The conjugation of the amino group of glufosinate with suberic acid bis(NHS ester) leads to a strong preference for N-acyl derivatives of glufosinate in relation to glufosinate. This seems to be caused by the difference in charge and size of the analytes. N-Hexanoyl-glufosinate shows the highest CR of all of the compounds tested. This is in accordance with the concept that the compound of highest homology with the immunogen shows the highest CR. This even can be used for the identification of an unknown immunogen structure (*44*). The serum displayed enantioselectivity for N-acetyl-L-glufosinate and N-acetyl-L-glutamic acid, which should be taken into consideration for real samples, which might contain the technical racemate. Non-acylated glufosinate shows an ~770 or 4600 times lower CR in relation to N-acetyl- or N-hexanoyl-glufosinate, respectively, which can be considered as irrelevant in most cases. The developed antibodies are well-suited for the development of a DAIA for the detection of glufosinate. Assuming an essentially quantitative derivatization step, a limit of detection in the low μg/L range might be reached for glufosinate. Although this sensitivity is not satisfactory for direct drinking water analysis, immunoaffinity enrichment and the examination of food, soil,

and plant material may be suitable applications. In this regard, the extremely low CR to other amino acids is favorable to avoid unwanted signals from biological material. It is not surprising that other compounds, which are not similar enough in structure, such as glyphosate, do not show any CR. This can be expected for any other structurally nonrelated herbicide, also. However, these antibodies might be not only useful for the setup of DAIAs for glufosinate but also for the direct and selective detection of the major metabolite NAG even in the presence of the parent compound.

ABBREVIATIONS USED

CR, cross-reactivity; DAIA, derivatization-assisted immunoassay; FMOC, 9-fluorenylmethoxycarbonyl group; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC-RI, high-performance liquid chromatography with refractive index detector; ICP-MS, inductively coupled plasma mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MPA, 2-(methylphosphinyl)acetic acid; MPP, 3-(methylphosphinyl)propionic acid; NAG, N-acetyl-glufosinate; MTP, microtitration plate; NHS, N-hydroxysuccinimide; OPA, *o*-phthalaldehyde; PPT, phosphinothricine (glufosinate); RP, reversed phase; TXRF, total reflection X-ray fluorescence.

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