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Chapter 9

Development of Immunoassays for Thiocarbamate Herbicides

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Immunoassays for the thiocarbamate herbicides, molinate, thiobencarb and EPTC (Eptam) are described. Using hapten synthesis strategies similar to those reported earlier for molinate, several haptens were synthesized for EPTC and thiobencarb. Rabbits were immunized with conjugates of two haptens for each target compound. Lower titer antibodies were produced against EPTC haptens, resulting in less sensitive assays (I_{50} for EPTC = 35 uM; 6.6 ppm). Cross reactivity with related thiocarbamates was 9-50%. The antibodies raised against thiobencarb haptens were of higher titer and of similar sensitivity (I_{50} for thiobencarb = 0.3 uM; 158 ppb) to the molinate assay. With thiobencarb, three assays were characterized using different combinations of antibodies and antigens. Antibodies against an azophenyl hapten of thiobencarb used in a homologous assay showed very high specificity for thiobencarb (cross reactivity by other thiocarbamates was below 0.1%). In the other two assays related thiocarbamates cross reacted less than 2%. This assay has been applied to the analysis of split samples from a field study to evaluate assay performance and to compare to gas chromatographic analysis.

The thiocarbamate herbicides, molinate and thiobencarb (Figure 1), are used as pre-emergence herbicides in rice culture in California. Between early May and the end of June each year, about 900,000 kg of these materials are applied in California alone (1). Molinate has been implicated in fish kills in drainage canals (2) and thiobencarb imparts an off taste to the drinking water that can be tasted by some people at very low concentrations. Because of these problems, the California Department of Food and Agriculture (CDFA), in conjunction with the California Department of Fish and Game, the local Water Quality Control board and manufacturers of these thiocarbamates, has a program to monitor drainage canals and river water for these two compounds. In collaboration with CDFA, our laboratory was asked to develop immunoassays for molinate and

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thiobencarb. CDFA's primary goal was to be able to analyze the samples from this monitoring program by a quicker more cost effective method than the currently used gas liquid chromatography (GC) method. A second, longer term goal of CDFA was to develop an understanding of immunoassays and determine the potential contribution of this analytical method to the Environmental Monitoring and Pest Management Branch's analytical program (Stoddard, ACS Symposium Series, in press).

The history, principles and justification for development and use of immunoassays are discussed elsewhere in this volume (Vanderlaan et al.) and in reviews from this laboratory (3-8, Hammock et al. ACS Symposium Series, in press) and others (9-10). The former also include examples and references to many of the assays which we have developed. The thiocarbamates, in particular, were of interest to us because they present a challenge for assay development. For example, molinate is a low molecular weight, relatively volatile, and somewhat hydrolytically unstable compound. Thus the very properties that make molinate easy to analyze by GC make it a difficult candidate for immunoassay. Some of the information presented has been published, but is condensed here to serve as background to explain the unified development strategy for the thiocarbamate class. It also provides a basis for comparison of previously obtained data to those newly reported here.

Molinate Hapten Synthesis, Conjugate Preparation and Antibody Screening

Details on strategies for thiocarbamate hapten design can be found in Gee et al. (11) and Harrison et al. (this volume). Haptens were synthesized by a thio replacement reaction in which the parent compound was oxidized to the sulfone using 3-chloroperoxybenzoic acid. The sulfone was then displaced with the appropriate thiol to yield either a carboxylic acid hapten or a nitrophenyl hapten with varying aliphatic chain spacers (Figure 2). The nitrophenyl haptens were reduced to aminophenyl haptens using dodecacarbonyltriiron and then coupled to carrier proteins by diazotization. Carboxylic acid haptens were coupled to carrier proteins by the mixed anhydride method using isobutylchloroformate and tributylamine (11).

Rabbits were immunized with conjugates of the carboxylic acid (Ia) and aminophenyl (Ic) haptens. The resulting polyclonal antibodies were screened for spacer recognition and target specificity. Antibodies raised against hapten Ia bound strongly to the coating antigen having a homologous hapten. This binding could not be inhibited by the target analyte, molinate. However this binding could be inhibited by molinate when the coating antigen contained a heterologous hapten, i.e. antigens containing haptens Ib, Ic or Id. Antibodies raised against hapten Ic also bound strongly to the homologous coating antigen and could not be competed off by molinate, presumably due to strong linker recognition (Harrison et al., this volume). In addition, molinate only slightly inhibited the binding of this antibody to the heterologous haptens. Thus, the most useful antibodies were raised against hapten Ia. These antibodies were used in an indirect competition enzyme linked immunosorbent assay (ELISA) (11-13). Details of the synthesis (11) and assay development (13) have been reported previously.

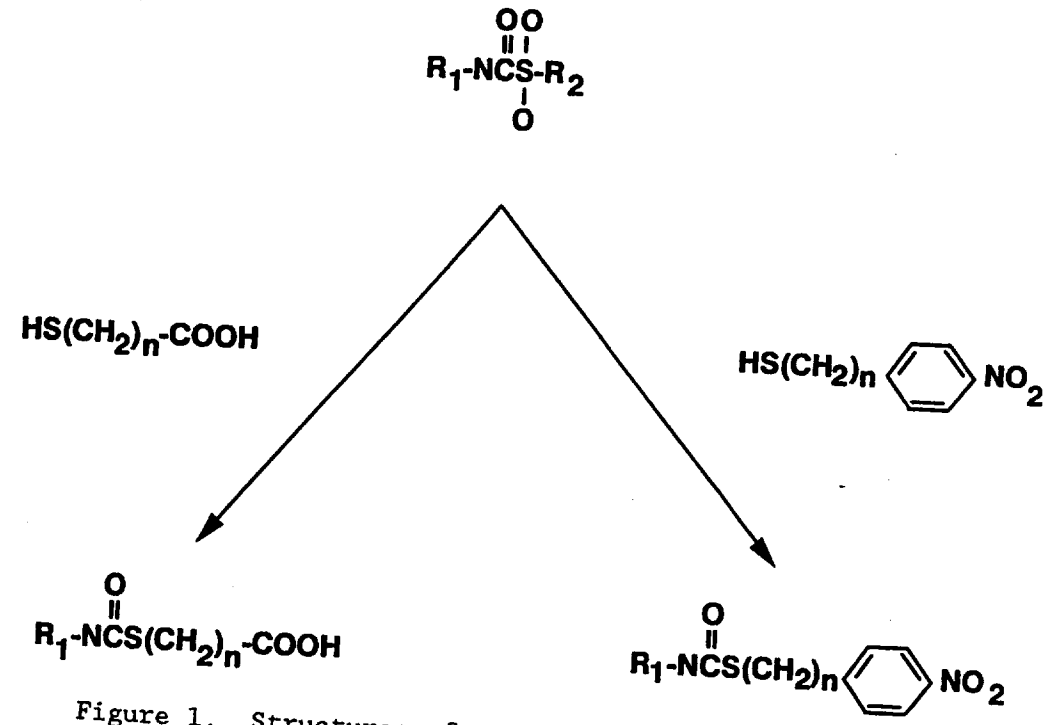
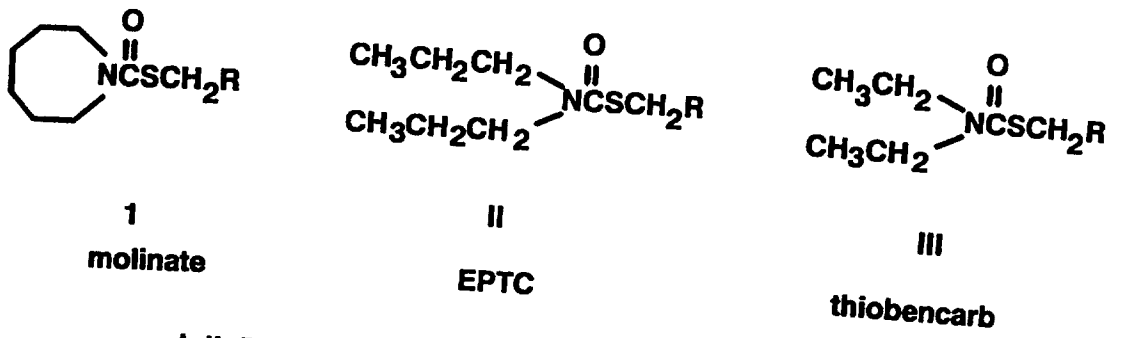


Figure 1. Structures of molinate, thiobencarb, EPTC and haptens.

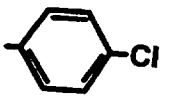


1
molinate

II
EPTC

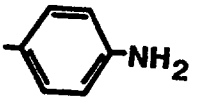
III
thiobencarb

I, II: R = $-CH_3$

III: R = 

Ia, IIa, IIIa: R = $-CH_2CH_2COOH$

Ib, IIb, IIIb: R = $-(CH_2)_5COOH$

Ic, IIc, IIIc: R = 

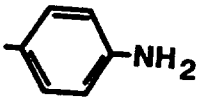
Id, IIId: R = 

Figure 2. Synthetic routes for the thiocarbamate haptens.

Molinate Field Study Validation

This assay was validated in a field study which examined assay performance and compared data obtained on the same samples by GC analysis. These data have been published (12), and to our knowledge, is the first study of its kind which addresses procedural error and data handling for real world samples in immunoassay for pesticides. Thus, it seems appropriate to reiterate some of the salient points here.

Samples obtained from a treated rice field were analyzed by both GC and ELISA. Samples for GC analysis were extracted with toluene and the extracts analyzed. Samples for ELISA analysis were buffered, diluted and analyzed without further workup.

The limit of reliable measurement of the ELISA was 21 ppb. The limit of reliable measurement is a calculated concentration that is approximately the mean plus two standard deviations, multiplied by two, of a negative control sample (12,14). This measurement is probably more meaningful than a conventional limit of detection because it provides a conservative and statistically well supported estimate of the operating characteristics of the assay, obtained under realistic conditions. The I_{50}, which is the amount of molinate needed to inhibit the assay by 50%, was 106 ± 32 ppb. The working range was approximately 35 to 500 ppb.

We used this study to assess the importance of a number of potential sources of error in the ELISA procedure. The variability of the baseline ELISA signal (interwell coefficient of variation at the absorbance for the zero dose control) was 4%, of which 0.3% was reader error (instrument imprecision plus inaccuracy; 15). The between well variability is akin to the variability in baseline signal in GC analysis. Pipetting error was measured gravimetrically to be less than 1%. Two procedural variables were tested for their effect on reproducibility. Shaking the plate before reading decreased the average coefficient of variation for quadruplicate wells by almost two fold. Similar improvements in readings were obtained by Kemp et al. (16). Reading in dual wavelength mode (405-650 nm) accounted for a small but reproducible decrease in the coefficient of variation.

A nested analysis of variance (ANOVA) was conducted for two control samples analyzed in 37 assays. Variability among replicate wells (within plates) accounted for more than 90% of the total variability for both controls. Among day and among plate (within day) variability constituted 5-10% and less than 1% of the total, respectively. This interwell variability is a characteristic of microplate ELISAs which is not widely documented, but it is known to those who work in this field. This variability is due to several compounded factors, including intrinsic variability in the binding characteristics of the plates, pipetting error, thermal variations across the plate and interwell variability of washing. The relative contributions of these factors have not been studied adequately.

The ELISA data obtained for field samples and spiked samples compared favorably to data obtained by GC. The correlation coefficient (r) exceeded 0.90 in each of two separate comparisons. Other issues addressed in this study were development of quality assurance criteria, analysis of four parameter standard curves and interpretation of resulting data, pipetting techniques, and study

design. Complete details are given in the report by Harrison et al. (12).

Assay Development for EPTC and Thiobencarb

The thiocarbamates, thiobencarb and EPTC, were also targets for immunoassay development. Using a synthetic strategy similar to that used for molinate, several haptens were synthesized (Figure 1) and the resulting antibodies were evaluated. Rabbits were immunized with mercaptopropanoic acid haptens IIa, IIIa, coupled to keyhole limpet hemocyanin (KLH) via mixed anhydrides, and p-aminophenyl haptens IIc and IIId, coupled to KLH by diazotization. The resulting antibodies were tested in both homologous and heterologous assay systems. In general, antibody titers in homologous assays were similar for each antigen. Combinations of coating antigens and antibodies resulting in titers of less than 1000 from a checkerboard titration were not screened further. The coating antigen and antibody combinations that passed this first screen were then tested for the ability of the target analyte to inhibit binding.

Antibodies directed against the EPTC hapten IIa could only be inhibited by EPTC when used in a heterologous system. The amount of EPTC needed to inhibit one of these indirect ELISAs by 50% (I_{50}) was 35 uM (6.6 ppm) under optimized conditions. Other structurally related thiocarbamates such as vernolate, pebulate, butylate and cycloate cross reacted from 9-50% (Table 1) relative to EPTC. There was high cross reactivity with EPTC haptens IIa and IIc, as expected (I_{50}s were 0.4 and 0.5 uM, respectively). Antibodies against hapten IIc were not inhibited by levels of EPTC up to 2 ppm, regardless of the coating antigen used. Apparently binding of antibodies to the linker moiety of EPTC conjugate IIc-BSA was stronger than to the N,N-dipropyl substituent of the thiocarbamate in homologous assays. Similar results were obtained with molinate (11). In heterologous assays, the antibody recognition of the N,N-dipropyl substituent of the thiocarbamate was so poor as to make the assay unusable. Further discussion of this and related problems in hapten design can be found in Harrison et al. (this volume).

Antibodies made against thiobencarb hapten IIIa could not be competed off in a homologous assay, probably due to poor recognition of the N,N-diethyl substituent on the thiocarbamate. Thiobencarb effectively inhibited the binding of this antibody to the heterologous antigen. After optimization, these assays had good sensitivity for thiobencarb (I_{50} = 0.7 uM) and cross reactivity with structurally related compounds of less than 2% (Table 2) relative to thiobencarb. The maximum absorbance of this assay was very small, but could be improved using signal amplifying systems. Antibodies against thiobencarb hapten IIId gave the best assays with both homologous and heterologous coating antigens due to the close structural similarity of immunizing hapten, coating hapten and target compound. This result implies that the aromatic ring is important to antibody binding and is certainly important to antibody specificity. The I_{50}s with this antibody in heterologous and homologous assays were 0.6 and 0.3 uM, respectively (Table 2). Cross reactivities of other thiocarbamates in the heterologous assay were less than 2% and strikingly, in the homologous assay was less than 0.07%, denoting a particularly specific antibody.

Table 1. Relative Cross Reactivity of Some Thiocarbamates in the EPTC Assay

Inhibitor	I_{50} (uM) Assay I[a]	Assay II[b]
II (EPTC)	35 (6.6 ppm)	67 (12.6 ppm)
IIa	0.5	0.9
IIb	-	-
IIc	0.4	0.6
pebulate	400	800
cycloate	200	400
butylate	c	c
vernam	75	130
thiobencarb (III)	c	c
molinate (I)	c	c

Mean I_{50} values for 2 experiments; for each experiment standard curves for each inhibitor were prepared using quadruplicate wells at each of 10 concentrations. Dashes indicate compound was not tested.
[a]Assay I: Coating antigen: IIc-BSA; Immunizing antigen: IIa-KLH
[b]Assay II: Coating antigen: IIc-CONA; Immunizing antigen: IIa-KLH
[c]The I_{50} is greater than 5 X 10^{-4}M, the highest concentration tested.
Abbreviations: BSA = bovine serum albumin; KLH = keyhole limpet hemocyanin; CONA = conalbumin

Table 2. Relative Cross Reactivity of Some Thiocarbamates in Three Thiobencarb Assays

Inhibitor	I_{50} (uM) Assay I[a] (heterologous)	Assay II[b] (heterologous)	Assay III[c] (homologous)
III (thiobencarb)	0.60 ± 0.03 (0.3 ppm)	0.70 ± 0.21 (0.4 ppm)	0.3 (0.2 ppm)
IIIa	421 ± 162	1.91 ± 0.14	350
IIIb	13.2 ± 3.9	1.28 ± 0.34	9
IIIc	0.90 ± 0.33	0.50 ± 0.15	1.1
IIId	0.22 ± 0.07	0.42 ± 0.22	0.1
EPTC (II)	25 ± 4.3 (4.7 ppm)	54 ± 24.5 (10.2 ppm)	d
vernolate	54 ± 1	62 ± 35.2	500
pebulate	97 ± 0.5	198 ± 74	2500
cycloate	361 ± 0.1	198[e]	d
molinate	462 ± 71 (87 ppm)	d	500
butylate	d	d	d

Mean I_{50} values for 3 experiments ± SD unless otherwise indicated; for each experiment, standard curves for each inhibitor were prepared using quadruplicate wells at each of 10 concentrations.

[a]Assay I: Coating antigen: IIIb-OA; Immunizing antigen: IIId-KLH.
[b]Assay II: Coating antigen: IIIc-THY; Immunizing antigen: IIIa-KLH.
[c]Assay III: Coating antigen: IIId-OA; Immunizing antigen: IIId-KLH, n = 2
[d]The I_{50} is greater than 5 X 10^{-4}M, the highest concentration tested.
[e] N = 1

Conclusions

We have shown that the thiol replacement reaction chemistry for the thiocarbamates can be applied to other members of that class for development of antigens and successful antibody production. The use of this reaction to synthesize haptens with different spacers and coupling chemistries was crucial to the production of useful assays for molinate and EPTC because of strong spacer recognition. Assays for the three compounds had different sensitivities. The EPTC assay was 100 fold less sensitive compared to the molinate assay, likely due to the lack of the rigid structure found in molinate. The EPTC assay also lacked sensitivity compared to the thiobencarb assay since EPTC lacks structures capable of pi stacking or dipole-dipole interactions (Harrison et al., this volume). The assays described here demonstrate that antibodies can be made against haptens that are relatively hydrolytically unstable. The molinate assay has clearly been demonstrated to be useful for quantitative analysis of environmental samples. The data analyzed therein provides guidelines for the optimization, validation, quality control and assurance of other assays.

Acknowledgments

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