A Radioimmunoassay for Benomyl and Methyl 2-Benzimidazolecarbamate on Food Crops

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A radioimmunoassay was developed which determines methyl 2-benzimidazolecarbamate (MBC) in evaporated ethyl acetate extracts of food crops without prior cleanup. Benomyl was converted quantitatively by the extraction procedure to MBC. Average recoveries from eight commodities fortified with benomyl exceeded 90% and correlated well (r = 0.99) with those determined by cation-exchange liquid chromatography.

Several methods are available for the determination of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] residues and include fluorometry (Pease and Holt, 1971; Aharonson and Ben-Aziz, 1973), cation-exchange liquid chromatography (Kirkland et al., 1973), reverse-phase liquid chromatography (Farrow et al., 1977), and gas–liquid chromatogaphy after acetylation (Pyysalo, 1977), pentafluorobenzylation (Tjan and Jansen, 1979), or trifluoroacetylation (Rouchard and Decallonne, 1974). While these methods are sensitive and specific, they are time consuming. Immunochemical methods have been developed for pesticides such as dieldrin and aldrin (Langone and Van Vunakis, 1975), S-bioallethrin (Wing and Hammock, 1979), warfarin (Cook et al., 1979), paraquat (Levitt, 1979; Fatori and Hunter, 1980), and the benomyl metabolite 2-aminobenzimidazole (Lukens et al., 1977), but few have been applied to the determination of residues on crops in which they may occur. Since radioimmunoassay (RIA) appeared to offer adequate sensitivity, often exhibited a high degree of specificity, and could be used to analyze relatively large numbers of samples simultaneously, the technique was applied to the determination of benomyl residues on food crops.

EXPERIMENTAL SECTION

Materials. 2-Aminobenzimidazole (97%), benzimidazole (98%), 2-benzimidazolylurea (95%), *N*-methylurea (97%), urea (98%), *N,N'*-dimethylurea (98%), succinic anhydride (99%), succinamide, and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (98%) were purchased from Aldrich Chemical Co., Inc. Milwaukee, WI. Human albumin, 4 × crystallized, was supplied by ICN Pharmaceuticals, Inc., Cleveland, OH. Ethyl carbamate, mp 48–50 °C, was puchased from Brickman and Co., Montreal, Quebec. Thiabendazole was obtained from Merck Sharp + Dohme Canada Ltd., Montreal, Quebec, while benomyl and methyl 2-benzimidazolecarbamate (MBC) analytical standards were obtained from Du Pont of Canada Ltd., Montreal, Quebec. Bovine γ-globulin, Cohn fraction II, and dextran, average M_r 79400, were supplied by Sigma Chemical Co., St. Louis, MO. Freund's complete adjuvant was purchased from Difco Laboratories, Detroit Mi, and charcoal, Darco grade G-60, was from Atlas Chemical Industries Inc., Wilmington, DE. [^{14}C]Thiourea (54.3 mCi/mmol) and Aquasol scintillation fluid were obtained from New England Nuclear, Boston, MA.

Instruments. High-pressure cation-exchange liquid chromatography was carried out on a 2.1 mm × 100 cm Zipax SCX column (Du Pont Instruments, Wilmington, DE) maintained at 50 °C. Solvent (0.1 M tetramethylammonium acetate, pH 4.1) was delivered to the column at 1.1 mL/min by an Altex Model 110 pump. A Waters Model 440 detector containing a 280-nm filter was used to monitor column effluent. Centrifugation was carried out on an International Model CS centrifuge maintained at 4 °C. ^{14}C was counted on a Beckman LS-230 scintillation counter using 10-min counts. Mass spectra were determined on a Varian MAT 311A double-focusing spectrometer.

Buffers. Phosphate-buffered saline (PBS) contained 20 mmol of NaH_2PO_4, 140 mmol of NaCl, and 3 mmol of NaN_3 per L of distilled water. The pH was adjusted to 7.15 with NaOH before making to volume. Antiserum diluent contained 0.1% bovine γ-globulin in PBS. Charcoal suspension contained 1 mg/mL charcoal and 0.1 mg/mL dextran in antiserum diluent. Tetramethylammonium acetate buffer was prepared by adjusting 0.1 mol of acetic acid to pH 4.1 with 20% tetramethylammonium hydroxide in methanol and diluting to 1 L with water.

Syntheses. *2-Succinamidobenzimidazole.* A solution of 2-aminobenzimidazole (13.3 g, 0.1 mol) and succinic anhydride (10.0 g, 0.1 mol) in acetonitrile (400 mL) was stirred at ~40 °C for 3 h. The heavy precipitate was filtered on a medium porosity Buchner filter and washed thoroughly with water. The solid was then suspended in boiling methanol (500 mL) and filtered. After being washed with a further portion of boiling methanol (500 mL), the solid was air-dried. The yield was 13.9 g (59.7%), mp >300 °C. The mass spectrum showed a molecular ion at m/e 233.

Immunogen. Immunogen consisted of 2-succinamidobenzimidazole coupled to human albumin. 2-Succinamidobenzimidazole (23 mg) was dissolved in dilute NaOH (1.0 mL), pH 10–11, and added to a stirred solution of human albumin (70 mg) in water (1.0 mL). 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (57 mg) in water (0.5 mL) was added and the pH adjusted to 7 with dilute HCl. Stirring was continued for 20 h at room temperature. The solution was then dialyzed at 4 °C against several changes of 0.05 M $NaHCO_3$, followed by several changes of distilled water. The immunogen was freeze-dried and stored at −20 °C. From the absorption at 285 nm in 0.14 N NaCl, it was calculated that the albumin contained 3.7 residues/molecule of BSA.

Methyl [2-^{14}C]Benzimidazolecarbamate. The procedure was modified from that described by Gardiner et al. (1974). [^{14}C]Thiourea (3 mCi, 4.2 mg) was dissolved in methanol (0.5 mL) containing thiourea (11 mg) and transferred to a tarred 12 × 75 mm tube. After removal of the solvent on a rotary evaporator, water (50 μL) and dimethyl sulfate

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(20 μL) were added. The contents of the tube were mixed thouroughly on a vortex mixer and the bottom 1 cm of the tube was placed in a 90 °C water bath for 1 h. After the reaction solution was cooled, methyl chloroformate (30 μL) was added and the tube contents were mixed on a vortex mixer. Ten percent NaOH (250 μL) was added rapidly and, after mixing, was followed by glacial acetic acid (25 μL). *o*-Phenylenediamine (22 mg) was dissolved in the reaction solution, and the tube was heated slowly to 60 °C and then more rapidly to 85–90 °C and maintained at the latter temperature for 30 min. After the mixture was cooled on ice, crystallization occurred. The product was washed 3 times with cold water (1 mL) by suspension of the crystals, centifugation, and aspiration of the supernatant. The procedure was repeated 3 times with cold acetone, and the tube and contents were dried in an evacuated desiccator. The yield was 15.9 mg (41%) of material with a chemical purity as determined by high-pressure liquid chromatography of 102%. The specific activity was determined to be 15.3 mCi/mmol.

Antisera. Each of six New Zealand rabbits was injected subcutaneously at multiple sites with 0.5 mL of an emulsion consisting of immunogen (7 mg) dissolved in 0.14 N NaCl (1.1 mL) and mixed with Freund's complete adjuvant (2.4 mL). Booster injections were given 2 weeks later and at monthly intervals thereafter. Blood was collected from the marginal ear vein 5 weeks after the initial injections and serum prepared. Before immunization, binding of the radioligand equalled that of nonspecific binding by the assay components. Determination of the titer at weekly intervals showed that useful antisera were produced as early as 8 weeks after the initial immunization. Antiserum was stored frozen (−20 °C) in aliquots (200 μL) until immediately before use. When treated in this manner, binding activity remained constant for at least 6 months.

Sample Preparation and Extraction. Samples were cut into small pieces and homogenized in a Waring blender. Subsamples (2.0 g, 5.0 g of cucumber or melon) were mixed at high speed in a Sorvall Omni Mixer with ethyl acetate (40 mL) and saturated aqueous sodium carbonate (2 mL). The mixture was transferred to a 125-mL boiling flask with ethyl acetate (10 mL) and a reflux condenser fitted. The mixture was boiled for 15 min, cooled, and filtered through Whatman No. 1 paper on a Buchner funnel by using gentle vacuum. The filtrate was transferred to a 50-mL volumetric flask and made to volume with ethyl acetate. For radioimmunoassay, duplicate aliquots (100 μL) were placed in 12 × 75 mm tubes and the solvent was removed in an evacuated desiccator.

Radioimmunoassay. The dried sample extract was taken up to PBS (200 μL). Standards consisting of 0, 5, 12.5, 25, 50, and 160 ng/mL MBC in PBS (200 μL) were added to another series of 12 × 75 mm tubes. Antisera diluted 1:20 in PBS (200 μL) was added to samples and standards, and after they were mixed, the tubes were refrigerated at 4 °C for 45 min. [^{14}C]MBC in PBS (100 μL, 3100 cpm) was added, and after a further 15 min at 4 °C, cold (4 °C) charcoal suspension (1.0 mL) added. After the samples were mixed in a vortex mixer and allowed to stand for 10 min at 4 °C, the tubes were centrifuged at 1200*g* for 10 min. The supernatant was decanted into scintillation vials containing Aquasol (10 mL), and the vials were counted in a scintillation counter. A standard curve of cpm of bound ligand plotted against ng of MBC/tube was prepared, and the samples were quantitated by comparison of the cpm obtained with those of the curve.

Table I. Cross-Reactivity of Various Compounds with Antiserum to 2-Succinamidobenzimidazole

compd	ng required for 20% inhibition of binding of [^{14}C]MBC[a]
MBC	2
2-benzimidazolylurea	3
2-aminobenzimidazole	26
benzimidazole	>100
thiabendazole	>100
succinamide	>100
ethyl carbamate	>100
urea	>100
N-methylurea	>100
N,N'-dimethylurea	>100

[a] Those values designated as >100 produced no inhibition at the 100-ng level.

Determination of MBC by Cation-Exchange Liquid Chromatography (LC). The cleanup procedure involving acid–base partitioning was modified from that described by Pease and Holt (1971). The liquid chromatographic step is similar to that of Kirkland (1973) except that the column temperature was reduced to 50 °C and a 0.1 N tetramethylammonium acetate buffer, pH 4.1, at a constant flow rate of 1.1 mL/min was used.

The ethyl acetate sample extract was transferred to a 125-mL separatory funnel and extracted with 0.1 N HCl (2 × 25 mL). The aqueous phase was combined and extracted with ethyl acetate (25 mL). The ethyl acetate wash was discarded and the aqueous phase made alkaline by the addition of 10 N NaOH (2 mL). MBC was extracted into ethyl acetate (2 × 25 mL) and an aliquot (25 mL) of the combined extracts transferred to a 50-mL round-bottomed flask containing 1 N acetic acid (1 mL). The ethyl acetate was removed by evaporation on a rotary evaporator with a 30 °C water bath. After transferring the aqueous residue to a 10-mL volumetric and making the volume with 0.1 N HCl, we subjected an aliquot (100 μL) to analysis by high-pressure cation-exchange liquid chromatography. Samples were quantited by comparison of the peak height to that of a 0.1 μg/mL standard of MBC.

RESULTS AND DISCUSSION

Benomyl degrades to MBC in aqueous (Clemons and Sisler, 1969) and organic (Chiba and Doornbas, 1974) solvents and in plants (Peterson and Edgington, 1970). With the present analytical scheme, the amount of MBC found is a measure of the total MBC and benomyl in the original sample, since benomyl is converted quantitatively to MBC by heating the ethyl acetate extract. Similarly, thiophanate methyl is partially converted to MBC in plants and aqueous solution (Selling et al., 1970; Soeda et al., 1972). However, a yield of only 35–40% MBC was obtained by heating ethyl acetate extracts containing thiophanate methyl, so the MBC found does not represent the total thiophanate methyl present.

Coextractives do not affect the binding of the radioligand to the antiserum, so the assay was performed without cleanup of the initial extract. With apple or tomato, it was found that a 5-fold increase in sample concentration could be used without detrimental effect. The dilution of antiserum used (1:20) permitted a 38% binding of the radioligand in the absence of inhibitor. Nonspecific binding was 2%. A typical RIA standard curve is shown in Figure 1. Samples containing 10 ppm or more of MBC must be diluted by at least 50% for accurate determination.

The specificity of the antiserum is indicated by the data in Table I. 2-Benzimidazolylurea and, to a lesser extent, 2-aminobenzimidazole cross-react with MBC. Since the

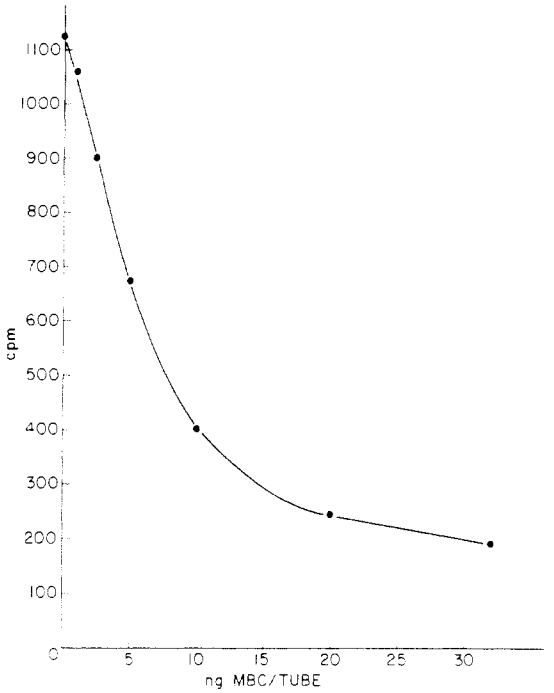

Figure 1. Standard curve for the radioimmunoassay of MBC. Cpm are corrected for nonspecific binding and are the means of duplicate determinations.

Table II. Recovery[a] of MBC from Several Commodities Fortified with Benomyl by Radioimmunoassay (RIA) and by Liquid Chromatography (LC)

benomyl added, ppm	recovery, % RIA	recovery, % LC	benomyl added, ppm	recovery, % RIA	recovery, % LC
	Orange			Grape	
1.0	100	79	0.5	101	91
5.0	101	85	2.5	95	88
10	95	88	5.0	99	88
	Lemon			Apple	
1.0	117	91	0.5	109	91
5.0	102	86	2.5	86	87
10	94	87	5.0	90	89
	Grapefruit			Tomato	
1.0	88	88	0.25	69	69
5.0	94	83	1.25	92	93
10	102	82	2.50	93	92
	Peach			Cucumber	
1.0	69	69	0.05	101	82
5.0	92	93	0.25	91	83
10	93	92	0.50	88	82

[a] Values are the means of duplicate determinations.

former has not been identified as a metabolite of benomyl or MBC, and since 2-aminobenzimidazole is a very minor (less than 2% of the soluble constituents) metabolite (Baude et al., 1973; Siegel and Zabbia, 1972), neither compound would be expected to interfere with residue determinations. Other ureas, benzimidazole or thiabendazole, do not react significantly.

Using both RIA and LC, recovery studies were carried out on several commodities fortified with benomyl at the maximum residue limit established in Canada and half and one-tenth of the maximum residue limit. As shown by the data in Table II, good recoveries (mean > 90%) were obtained with either method. The amount of MBC found by RIA also correlated well ($r = 0.99$) with that found by LC. Sample blanks did not contain material which interfered with either method at the sample concentrations used. Although the cleanup procedure for LC was abbreviated from that originally described (Pease and Holt, 1971), this method is better suited for confirmation purposes than routine screening, since RIA is much simpler and capable of processing samples approximately 5 times faster than LC.

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