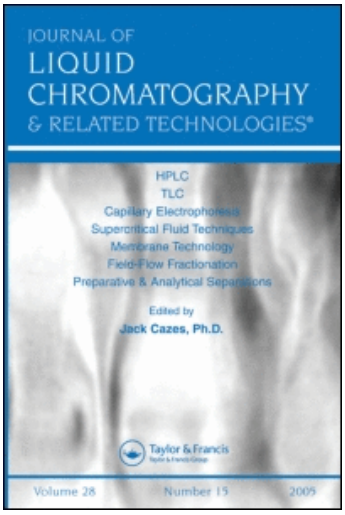

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Fractionation of [^{14}C]Metolachlor Metabolites by Centrifugal Partition Chromatography

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FRACTIONATION OF [^{14}C]METOLACHLOR METABOLITES BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

Metolachlor (2-chloro-N-(2'-ethyl-6'-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide) is one of the most widely used and important herbicides throughout the world. In order to understand the biodegradation pathways for this compound it is essential to develop large scale separation and purification procedures for its metabolites. However, analyzing crude plant extracts for these compounds poses several problems. In particular, the complex matrix and low levels of many metabolites requires a preparative separation and detection procedure that is both sensitive and robust. The use of [^{14}C] labeled compounds and scintillation counting satisfies the sensitivity requirement but creates an additional problem when using preparative LC. Irreversible adsorption to the stationary phase frequently occurs when analyzing "real-world" biological samples or extracts thereof. This is particularly undesirable if the matrix contains radioactive components. This problem can be avoided by using a technique with a liquid stationary phase such as centrifugal partition chromatography (CPC). CPC is used for the preparative fractionation of [^{14}C]metolachlor metabolites contained in crude corn plant extract. Also, a rapid analytical HPLC method is developed for the separation of standard non-radiolabeled metolachlor metabolites on a dimethylphenyl-derivatized β-cyclodextrin stationary phase.

INTRODUCTION

Metolachlor (2-chloro-N-(2'-ethyl-6'-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide) is one of the α-chloroacetamide herbicides that is used extensively in the cultivation of corn and soybeans as a pre-emergence control for most annual grasses and many broadleaf weeds. It is one of the most widely employed herbicides and frequently it is applied in combination with atrazine (1). Residues of metolachlor and its metabolites may occur in crops as a result of labeled uses and in environmental water as a result of run-off, spills or improper application(s). The most common methods used to measure metolachlor residues in water are gas chromatography and immunoassays (2-4). The detection of metolachlor in aqueous media has been reported in the range of 0.1 to 0.9 ppb by enzyme-linked immunosorbent assay (ELISA) (4).

Metabolism studies of specific α-chloroacetamide herbicides have been reported previously (5-8). An investigation of the metabolic fate of [^{14}C]propachlor (2-chloro-N-isopropylacetanilide) in the leaves of corn, sorghum, sugarcane, and barley was conducted by thin-layer chromatography (TLC). Evidence was found that one of the pathways for metabolism of α-chloroacetamide herbicides in higher plants involved glutathione conjugation (5). Recently, the initial metabolism of [^{14}C]acetochlor (2-chloro-N-(ethoxymethyl)-N-(2'-ethyl-6'-methylphenyl)acetamide) in corn and soybean seedlings was studied by high-performance liquid chromatography (HPLC) combined with structural assignments by fast atom bombardment mass spectrometry (FAB-MS) (6). At least eight

extractable products from the metabolism of metolachlor by the soil fungus *Chaetomium globosum* were resolved by gas-liquid chromatographic analysis (GLC) (7). Several key metolachlor metabolites in corn have been identified and a possible metabolic pathway has been proposed (8,9). Two-dimensional TLC and ionic exchange chromatography (IEC) have been used to fractionate and characterize metolachlor metabolites contained in crude extracts from corn (10).

All of the aforementioned studies made effective use of a variety of analytical methodologies. However, as is often the case when working with complex extracts of biological materials; larger, pure or partially pure sample fractions are often needed for more extensive multiple analyses. Preparative HPLC is often useful in this respect but irreversible retention is often a problem with biological samples. Complete sample recovery is often desirable, particularly when working with radioactively labeled compounds. Incomplete recovery in HPLC converts costly stationary phase into radioactive solid waste.

Centrifugal partition chromatography (CPC), a form of countercurrent chromatography (CCC), has been shown to be particularly useful for preparative separations (11-13). For example, it was determined that approximately 880 mg of D,L-tryptophan methyl ester can be separated into enantiomerically pure fractions with one injection (13). We have employed CPC to fractionate petroleum catalytic cracker feeds and asphalts in amounts between 1 and 10 grams per injection (14). Since a liquid rather than a solid stationary phase is used, strongly retained solutes can be completely recovered by flushing the system. The ability

to use dual-mode operation (*i.e.*, both normal-phase and reversed-phase separations in a single run) makes CPC well suited for the fractionation of samples that contain compounds with a wide range of polarities. In addition, separation efficiencies improve with increased flow rate (15). This trend is opposite to that observed for other chromatographic techniques. To our knowledge, there have been no reports on the use of CPC for the separation of radioactive pesticide metabolites.

In this work we evaluate the utility of CPC in the fractionation and characterization of [^{14}C]metolachlor metabolites contained in crude extract of mature corn plants. Both UV detection and liquid scintillation counting detection were used. Also, we demonstrate a rapid analytical HPLC method for the separation and characterization of standard non-radiolabeled metolachlor metabolites on a dimethylphenyl-derivatized β-cyclodextrin stationary phase.

EXPERIMENTAL

Materials

The following materials were obtained from CIBA-GEIGY Corporation (Greensboro, NC): ground mature corn plants (control material), standards of non-radiolabeled metolachlor metabolites (C-25702, CGA-37735, CGA-50720, CGA-13656, CGA-37913, CGA-40919, CGA-41507, CGA-46127, CGA-40172, CGA-51202, CGA-46576, CGA-110186, and CGA-118243), and [^{14}C]radioactive crude corn plant extract. The structures of all metabolites are given in Figure 1. A

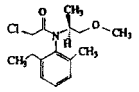
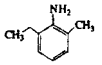
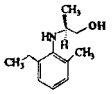
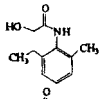
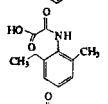
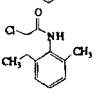
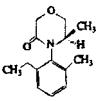
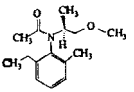
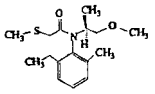
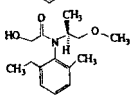
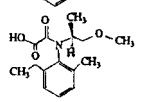
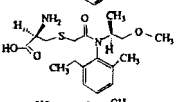
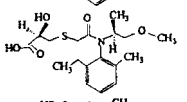
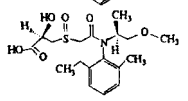
Structure	Abbrev
	Metolachlor
	C-25702
	CGA-37913
	CGA-37735
	CGA-50720
	CGA-13656
	CGA-40919
	CGA-41507
	CGA-46127
	CGA-40172
	CGA-51202
	CGA-46576
	CGA-110186
	CGA-118243

Figure 1. Structure of Metolachlor and its metabolites

biodegradable liquid scintillation cocktail, Ecolume, was purchased from ICN Biomedicals Inc. (Irvine, CA). HPLC grade 1-butanol, methanol, hexane, chloroform, triethylamine, trifluoroacetic acid, acetic acid and 7 ml scintillation vials were purchased from Fisher (Fairlawn, NJ). HPLC grade dichloroacetic acid was purchased from Aldrich (Milwaukee, WI). All water used was distilled and passed through a Barnstead D8922 cartridge to trap organics and filtered through a 0.45 μm Alltech nylon 66 membrane to remove particles.

Centrifugal Partition Chromatography

The CPC apparatus, Model CPC-NMF, equipped with 6 partition cartridges Type 250W (resulting in a total volume of 120 ml) from Sanki Laboratories Inc. (Mount Laurel, NJ). was used. Up to 12 cartridges (with 240 ml total volume) can be used in this instrument as has been described previously (11). A Rheodyne Model 7010 valve with 1000 μl sample loop was used to introduce the sample and a Rheodyne Model 7000 valve was used for mode selection when changing between descending and ascending modes. A Shimadzu LC-6A pump and a Shimadzu SPD-6A spectrophotometric UV detector were used with the CPC. The Recorder Company Series 4500 strip chart recorder, was used to record the CPC chromatogram. After elution, samples were collected with an Isco Cygnet fraction collector (Lincoln, NE).

Corn plants grown in a greenhouse were treated with [^{14}C]doped metolachlor herbicide. The corn was harvested when mature. The mature corn stalks were

mixed with methanol/water (8:2) on a shaker for approximately 30 minutes. The filtrate from three extractions were combined and concentrated using a rotary evaporator. The CPC experiments were performed using a two-phase ternary solvent system: 1-butanol/acetic acid/water. This solvent system was made by mixing 1860ml 1-butanol, 280ml acetic acid, and 1860ml water by volume. This forms a two phase mixture, each saturated with the other. The two phases (one butanol rich and one water rich) can then be separated from one another and used in the CPC. Fractionation was optimized on a nonradioactive corn extract control using UV (254 nm) detection. A 500 μl aliquot the [^{14}C]radioactive crude extract was injected directly into the CPC in the descending mode (*i.e.*, predominantly aqueous mobile phase, switching to the ascending mode (*i.e.*, predominantly butanol mobile phase) after two hours. Fractions were collected and 5 ml of scintillation cocktail was added to a 1 ml portion of each fraction which had been pipetted into a scintillation vial. A Beckman LS 7500 system, Beckman Instruments, Inc., Fullerton, CA was used to record the β-decay events. Blank solutions consisted of 5 ml scintillator cocktail and 1 ml water saturated with 1-butanol. All fractions and blanks were counted for 5 minutes.

High Performance Liquid Chromatography

An HPLC method was developed for the separation of metolachlor metabolite standards (C-25702, CGA-37735, CGA-50720, CGA-13656, CGA-37913, CGA-40919, CGA-41507, CGA-46127, CGA-40172, CGA-51202, CGA-

46576, CGA-110186, and CGA-118243). A system controller, Shimadzu SCL-6B, two Shimadzu LC-6B pumps, and a Shimadzu SPD-2AM detector were used in the gradient mode. The metabolite standards were separated using a CYCLOBOND I-DMP column obtained from Advanced Separation Technologies (Whippany, NJ). The elution program contained three steps. First, a mobile phase of 30:70, methanol:buffer (by volume) was employed for 10 minutes. This was followed by a linear gradient from 30:70 to 50:50 methanol:buffer in 10 minutes. An isocratic elution with 50:50, methanol:buffer was continued for 20 minutes. The buffer consisted of 0.1% triethylammonium acetate (pH=7.1), the flow rate was 1 ml/min and UV detection was at 254 nm.

Results and Discussion

Correct choice of the solvent system plays a significant role in the optimization of CPC separations (16). In this study, three solvent systems were evaluated: hexane/water, chloroform/water, and 1-butanol/water systems. Although a third component, methanol, was added to the hexane/water and chloroform/water systems to adjust the partitioning of the sample between the stationary phase and the mobile phase, no desirable fractionations were obtained with these solvents. The best results were obtained with the 1-butanol/water solvent system. This was optimized by adding various ternary components such as: 2-propanol, triethylamine, dichloroacetic acid, trifluoroacetic acid, and acetic acid. The best CPC fractionation of the corn plant sample was

obtained with a 1-butanol/acetic acid/water (1860ml/280ml/1860ml) solvent system (see Experimental).

As can be seen in Figure 1, two of the metolachlor metabolites are weak bases, *i.e.*, a primary amine (C-25702) and an amino alcohol (CGA-37913). All the rest of the compounds are amides. However, three of the amide compounds also contain carboxylic acid functional groups (*i.e.*, CGA-118243, CGA-110186, and CGA-51202) while one compound contains an amino acid moiety (CGA-46576). Using UV detection (Figure 2A, top) the CPC fractionation of the crude corn plant extract was optimized so that the total peak area in the descending mode was roughly equivalent to that in the ascending mode. It appears that there are at least four distinct peaks in the descending mode (at 15, 19, 30 and 45 minutes). The first peak at 15 min also represents the dead time (t_o) for this separation. It is highly likely that each peak is composed of several closely or co-eluting compounds. This is particularly apparent in the ascending mode (Figure 2A) where many of the peaks are so close together (after the initial one at 117 min) that they are often difficult to distinguish one from another.

Using exactly the same chromatographic conditions that were used to generate Figure 2A, crude corn plant extract previously treated with [^{14}C]metolachlor was fractionated (see Experimental). The resulting radiochromatogram shown in Figure 2B. It is apparent from this figure that over two thirds of the radioactive compounds are eluted in the ascending mode. When the radiochromatogram is directly compared to the above UV chromatogram, it is

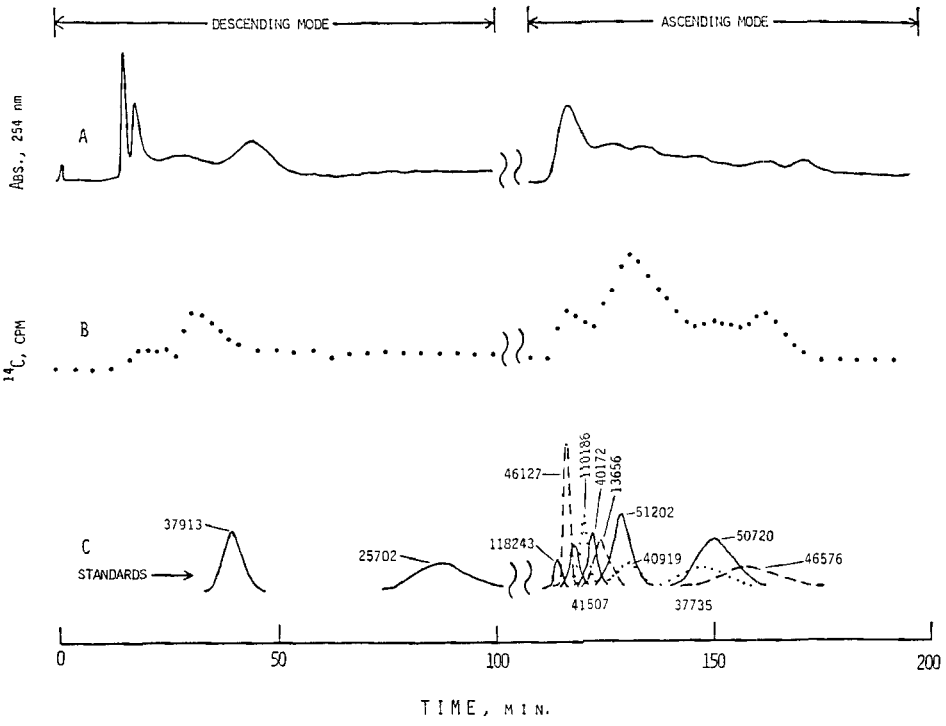


Figure 2. CPC fractionations of [^{14}C]metolachlor metabolites contained in crude corn plant extract using (A) UV detection and (B) liquid scintillation counting detection. Chromatogram (C) is a composite showing the retention of 13 metolachlor metabolite standards (structures given in Fig. 1). Identical conditions were used for all these CPC separations (see Experimental for details).

apparent the relative magnitudes of similarly retained peaks are very different. Also peaks sometimes appear in one chromatogram but not the other. Even though essentially the same material was fractionated in both cases, this is not an unexpected result given the very different selectivities of the detection methods. Clearly there are a number of UV absorbing pigments and compounds that elute in the descending mode (*i.e.*, which has a mobile phase predominantly of water

plus acetic acid) that are completely unassociated with [^{14}C]metolachlor or its degradation products. It seems that there may be a higher correlation between the UV peaks and those of the radiochromatogram in the ascending mode (Figures 2A and 2B). However, it appears that the radiochromatogram, like the UV chromatogram is made up of a large number of overlapping peaks.

Figure 2C shows the elution order of 13 known metolachlor metabolites when separated by CPC using conditions identical to those employed for the corn plant extract. Only the two amine compounds are eluted in the descending mode. Compound 25702 has no corresponding peak in either the UV chromatogram or the radiochromatogram. Interestingly, a major portion of both the UV absorbance and radioactivity in the descending mode chromatogram does not correspond to any of the tested metolachlor degradation products.

Eleven of the thirteen metolachlor metabolite standards elute in the ascending mode. The retention times of the standards correspond to many of the peaks seen in the radiochromatogram and UV chromatogram. The largest peak in the radiochromatogram has a maximum at about 130 minutes. It is broad, and has shoulders indicating that it includes several compounds. Two metolachlor metabolites (CGA-51202 and CGA-40919) elute at about this time. Both compounds result from the dehalogenation of metolachlor (Figure 1), which seems to be an important early step in its biodegradation. Clearly in the ascending mode there are similarities between the retention of the metabolite standards (Figure 2C) and the various maxima in the radiochromatogram (Figure 2B). This was not the case in the descending mode.

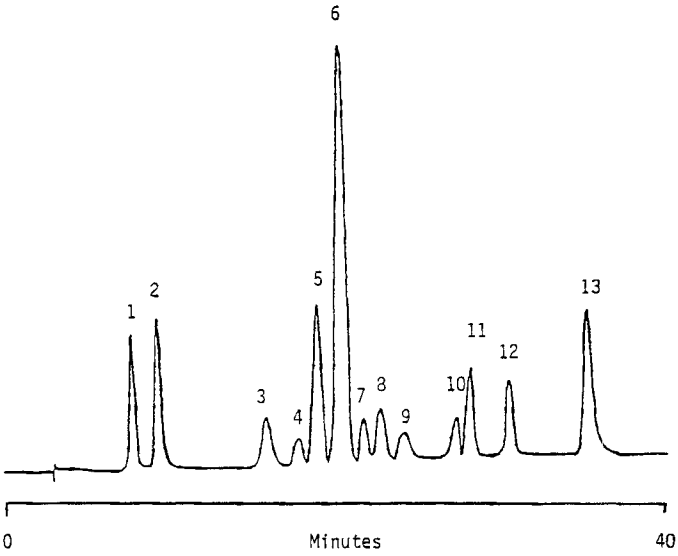


Figure 3. HPLC chromatogram of non-radiolabeled metolachlor metabolite standards on the dimethylphenyl-β-cyclodextrin stationary phase. A background subtraction approach was used along with gradient elution (see Experimental for details). The peaks are as follows: 1. CGA-37735, 2. CGA-50720, 3. CGA-13656, 4. CGA-51202, 5. C-25702, 6. CGA-37913, 7. CGA-118243, 8. CGA-40919, 9. CGA-46576, 10.CGA-40172, 11. CGA-110186, 12. CGA-41507, and 13. CGA-46127.

Gradient HPLC Separation of Metabolite Standards

Currently there are few effective analytical methods for chromatographic separation of the 13 metolachlor metabolites shown in Figure 1. It was found that these metabolite standards could be separated by HPLC on a dimethylphenyl-β-cyclodextrin (DMP-β-CD) stationary phase in the reversed phase mode using gradient elution. The results are shown in Figure 3. Interestingly, the elution order was found to be different than reported on a conventional C_{18} stationary

phase (*i.e.* CGA-50720 < CGA-37735 < CGA-37913 < CGA-51202 < CGA-118243 < CGA-46576 < C-25702 < CGA-13656 < CGA-40919 < CGA-40172 < CGA-110186 < CGA-41507 < CGA-46127) (10). This selectivity difference occurs because the DMP-β-CD stationary phase interacts with solutes via a combination of hydrophobic, hydrogen-bonding and π-π interactions. In the reversed phase mode, the hydrophobic character of DMP-β-CD stationary phase is known to be the result of the dimethylphenyl substituents as well as the interior of the cyclodextrin cavity. pH plays an important role in controlling both the retention and selectivity of the reversed phase separation on the DMP-β-CD stationary phase, just it does with a C_{18} column. It should be noted that the separation of metabolite standards on a DMP-β-CD phase is complete in 40 min. This is approximately twice as fast as the analogous separation on a C_{18} column (10). When running a gradient a broad "solvent-hump" appears over which the peaks are superimposed. This is eliminated with a baseline subtraction's method that is available with most HPLC instruments.

ACKNOWLEDGEMENT

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