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Note

Separation of 2,4-dichlorophenoxyacetic acid and its phenolic derivatives by reversed-phase high-performance liquid chromatography

ARBOR D. DRINKWINE*, DOUGLAS W. BRISTOL** and JAMES R. FLEEKER***

Residue Research Laboratory, Biochemistry Department, North Dakota State University, Fargo, N.D. 58105 (U.S.A.)

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The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) appears to be inactivated at least partially in all plants by ring hydroxylation to form phenolic acids. The amounts and types of hydroxylated products vary among plant species[1–3]. This laboratory has undertaken a study of comparing the hydroxylation of 2,4-D in differentiated plant tissue to that in callus tissue, with the eventual goal of determining whether callus tissue serves as a useful and valid model system for the study of pesticide metabolism[1].

Several methods have been used to quantitate hydroxylated 2,4-D compounds, but they are either tedious or offer inadequate resolution, or both[1–4]. High-performance liquid chromatography (HPLC) offers the potential of overcoming the difficulties encountered by these methods, since other investigators have successfully used HPLC to separate phenolic acids on reversed-phase columns[5–7]. A related study is the separation of 2,4-D and similar acids from halogenated phenols by reversed-phase chromatography[8]. Other laboratories have reported the use of HPLC in separating 2,4-D from amino acid derivatives by the ion-pair technique[9], and from structurally similar herbicides by adsorption methods[10,11]. We report here the use of a reversed-phase column in separating a mixture of 2,4-D and seven isomeric, ring-hydroxylated derivatives. In contrast to previous methods[1–4], the procedure can be carried out rapidly, requires no derivatization and the compounds are completely resolved.

EXPERIMENTAL

The compounds 2,3-dichloro-4-hydroxyphenoxyacetic acid (4-OH-2,3-D) and 2,5-dichloro-4-hydroxyphenoxyacetic acid (4-OH-2,5-D) were prepared by the method of Fleeker and Steen[3]. The procedures of Faulkner and Woodcock[12] were employed to prepare 2,4-dichloro-5-hydroxyphenoxyacetic acid (5-OH-2,4-D). The methods of Brown and McCall[13] were used to prepare 2-chloro-4-hydroxyphenoxyacetic acid

* Present address: Midwest Research Institute, Chemical Sciences Division, 425 Volker Boulevard, Kansas City, Mo. 64110, U.S.A.

** Present address: Food and Drug Administration HFF-147, 200 C Street, S.W., Washington. D.C. 20204, U.S.A.

*** To whom correspondence should be addressed.

(4-OH-2CPA), 2-hydroxy-4-chlorophenoxyacetic acid (2-OH-4CPA) and 2,4-dichloro-6-hydroxyphenoxyacetic acid (6-OH-2,4-D). A combined two-step diazotization and hydrolysis of ethyl 3-amino-2,4-dichlorophenoxyacetate, prepared by catalytic hydrogenation of the corresponding nitro compound[12], gave 2,4-dichloro-3-hydroxyphenoxyacetic acid (3-HO-2,4-D). The 3-nitro derivative was prepared by substitution of ethyl bromoacetate by 2,4-dichloro-3-nitrophenol[14] in hexamethylphosphoramide after the procedure of Shaw and Kunerth[15]. The identities of the seven ring-hydroxylated derivatives of 2,4-D and their synthetic precursors were confirmed by m.p. and examination of their spectroscopic properties[16].

Methanol and acetonitrile were distilled-in-glass solvents purchased from Burdick and Jackson Labs., (Muskegon, Mich., U.S.A.). A Waters HPLC system (Waters Assoc., Milford, Mass., U.S.A.) was employed. It consisted of dual Model 6000A pumps, a Model 660 solvent programmer, a Model 440 detector set at a fixed wavelength of 280 nm, and a Model U6K injector. Two μBondapak C_{18} columns (30 $\times$ 0.4 cm I.D.) were purchased; column A in 1976 and column B in 1978.

The synthesis of [^{14}C]4-OH-2,5-D was carried out in the same manner as the nonradioactive compound. Ethyl 2-bromo[1-^{14}C]acetate and [^{14}C]2,4-D were purchased from New England Nuclear (Boston, Mass., U.S.A.). Fractions were collected manually from the HPLC system directly into scintillation-counting vials. Scintillation solvent was added to the vials and the vials assayed for ^{14}C in a Nuclear-Chicago counter, Model 6847.

RESULTS AND DISCUSSION

Mixtures of 0.01 *M* aqueous sodium phosphate buffer, pH 7.0, with methanol or acetonitrile were used initially in attempting to separate 2,4-D and the seven derivatives. A mobile phase consisting of methanol–aqueous buffer (40:60) gave the best results, although it was inadequate with respect to the separation desired. The capacity factor[17], k', for each of the eight solutes was determined as a function of pH (Fig. 1). As expected on the basis of the pK values for the acidic solutes, the respective k' values increased as the pH (and ionization) decreased[6]. The shapes of the individual curves are readily interpreted. The sharp increase in k' in the pH range of 2 to 4 for solutes 1 through 8 corresponds to protonation of the carboxylic acid group of the phenoxyacetic acid compounds. The lack of a well defined inflection point in the curve of solute 8 [6-HO-2,4-D] may be a result of the strong intramolecular hydrogen bonding associated with this compound. It is known to form a δ-lactone readily[13] and its infrared, nuclear magnetic resonance, and mass spectra are clearly indicative of intramolecular hydrogen bonding[16]. The decrease in the k' values for solutes 1 and 5 in the pH range of 6 to 7 can be attributed to ionization of their phenolic hydroxyl groups. The hydroxyl groups of these solutes are *meta* to the alkyl ether group making their pK_a values lower than the *para* or *ortho* isomers[18,19]. Thus, it can be seen that the shapes of the k' *vs.* pH curves for a series of closely related ionogenic solutes are indicative of isomeric substitution and might prove useful for qualitative identification.

The best isocratic separation for the eight solutes was achieved at pH 2.0 using an acetonitrile–water (50:50) buffer as the mobile phase. However, the relatively long retention times encountered for solutes 7 and 8 even under these conditions ($k' = 9.18$ and 12.0, respectively), and the best resolution (R_s) of the least resolved pair (solutes 3

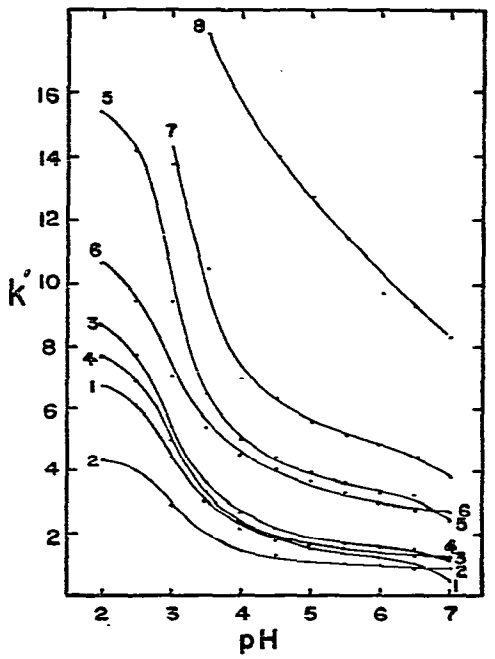

Fig. 1. Effect of mobile phase pH on capacity factor, k'. Column: μBondapak C_{18} (column A); mobile phase: methanol–0.01 *M* phosphate buffer (40:60); flow-rate: 3.0 ml/min; temperature: ambient: Solutes: 1 = 3-OH-2,4-D; 2 = 4-OH-2-CPA; 3 = 4-OH-2,3-D; 4 = 4-OH-2,5-D; 5 = 5-OH-2,4-D; 6 = 2-OH-4-CPA; 7 = 2,4-D; 8 = 6-OH-2,4-D.

and 4) of only 0.6, represented an unsatisfactory solution of the general elution problem for this mixture of isomers.

Use of a linear gradient from 100% aqueous buffer, pH 7.0 to 100% methanol in 30 min gave the desired separation (Fig. 2). The R_s value of the two closest peaks (solutes 5 and 6 under these conditions) was 1.5. Some column variation was observed using gradient elution; column A was purchased early in 1976 and column B purchased in 1978. Column A (Fig. 2), gave the best separation of the mixture at pH 7.0, while column B performed best at pH 6.5 (Fig. 3). A commercial filtration apparatus used for preparing high quality water became contaminated with microorganisms during the period when the data for Fig. 3 were gathered. The background difference in Figs. 2 and 3 is indicative of the impurities in the aqueous portion of the solvents used for elution in Fig. 3. The chromatograms shown in Fig. 2 were obtained using pure standard compounds and under these conditions the detection limit of 2,4-D was 20–25 ng. When samples of pure compounds were diluted with [^{14}C]2,4-D, the recovery of the labeled compound by collection of the HPLC eluate was greater than 95%.

This separation method is being used to separate 2,4-D and its ring hydroxylated derivatives present in extracts of plants fed [^{14}C]2,4-D. Quantitation is carried out by measuring the radioactivity under each peak. The sample extracts are supplemented before injection with the nonradioactive standard compounds. This avoids difficulties in collection and subsequent quantitation by liquid scintillation counting because of masking of the hydroxylated metabolites due to UV absorbing substances

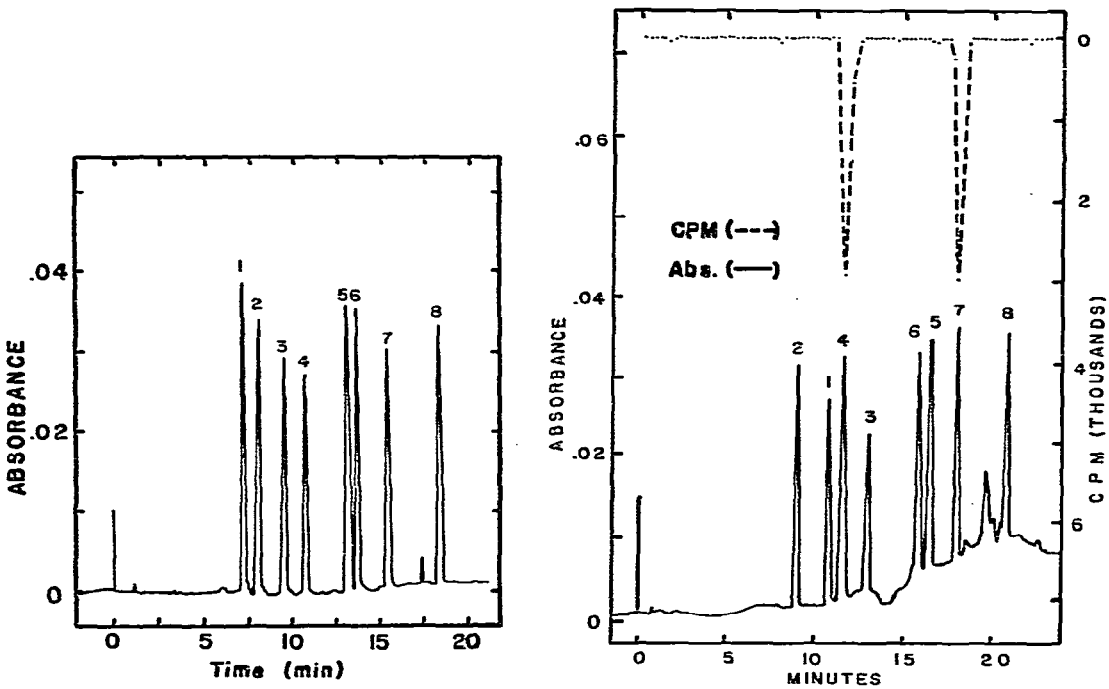

Fig. 2. Optimal separation of the hydroxylated compounds and 2,4-D. Column: μBondapak C_{18} (column A); eluent: 100% 0.01 *M* NaH_2PO_4–Na_2HPO_4 buffer pH 7.0 to 100% methanol in a 30-min linear gradient; flow-rate: 2.0 ml/min; temperature, ambient. Sample size: 50 μl of a 0.05 m*M* mixture of solutes numbered as in Fig. 1.

Fig. 3. Quantitation of [^{14}C] 4-OH-2,5-D and [^{14}C] 2,4-D by sample collection. Column: μBondapak C_{18} (column B); eluent: 100% 0.01 *M* NaH_2PO_4–Na_2HPO_4 buffer pH 6.5 to 100% methanol in a 30-min linear gradient; flow-rate: 2.0 ml/min; temperature: ambient. Sample size: 70 μl (50 μl of a 0.05 m*M* mixture of nonradioactive solutes, 10 μl of 0.2 mg/ml [^{14}C] 4-OH-2,5-D and 10 μl of 0.01 mg/ml [^{14}C] 2,4-D). Solutes are numbered as in Fig. 1.

in the sample matrix. Fig. 3 shows a chromatogram with [^{14}C]2,4-D and [^{14}C]4-OH-2,5-D added to the non-radioactive standards. The method has also been useful in detecting impurities in commercial preparations of ^{14}C-labeled 2,4-D.

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