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Note

Separation of parathion metabolites by reversed-phase ion-pair high-performance liquid chromatography

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Basic to the proper assessment of potential health hazards of organophosphorus pesticides is the knowledge of their fate in biological systems and in the environment. Because studies on the disposition and metabolism of these compounds depend on the characterization of a number of degradation products, efficient chromatographic procedures are essential. All types of chromatography have been utilized, including gas–liquid chromatography[1–3], thin-layer[4,5] and column chromatography[6,7] and more recently high-performance liquid chromatography (HPLC)[8–10].

During our study of the hepatic disposition of the organophosphate, parathion, it became necessary to quantitate a large number of samples containing radiolabeled phosphorus acid metabolites. However, available analytical procedures suffer from lengthy elution times, the use of corrosive and dangerous chemicals, or low accuracy in quantitation and sensitivity. We therefore investigated several HPLC procedures including ion-exchange and normal- and reversed-phase chromatography. This paper summarizes performances of these systems and describes a reversed-phase ion-pair chromatographic procedure for the rapid isocratic separation and quantitation of the strongly acidic, anionic metabolites of parathion.

EXPERIMENTAL

Chemicals

Phenyl[1-^{14}C]paraoxon (O,O-diethyl O-4-nitrophenyl phosphate) was synthesized by coupling sodium 4-nitro[1-^{14}C]phenate (22.5 mCi/mmole, 99% pure, Amersham/Searl, Arlington Heights, IL, U.S.A.) with diethyl phosphoryl chloride (supplied by American Cyanamid, Princeton, NJ, U.S.A.)[11]. Ethyl[1-^{3}H]parathion (1430 mCi/mmole, 98% pure, Amersham) was oxidized to ethyl[1-^{3}H]paraoxon with bromine water[11]. Both [^{3}H]parathion and [^{3}H]paraoxon were purified by HPLC (LiChrosorb Si-60, 10 μm, MC/B Manufacturing Chemists, Gibbstown, NJ, U.S.A.) with hexane–isopropanol (70:30) as the eluent. Ethyl[1-^{3}H]diethyl phosphorothioic acid ([^{3}H]DEPTA) and ethyl[1-^{3}H]diethyl phosphoric acid ([^{3}H]DEPA) were obtained by hydrolyzing [^{3}H]parathion and [^{3}H]paraoxon, respectively[11]. Labeled paraoxon was enzymatically deethylated to monoethyl paraoxon ([^{14}C]MEP or [^{3}H]MEP) by incubating with rat liver microsomes and $2 \cdot 10^{-3}$ M NADPH in the presence of 1 ·

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10^{-3} *M* EDTA to suppress enzymatic hydrolysis of paraoxon. The labeled chemicals were stored at -20°C in acetone ([^{14}C]paraoxon), toluene ([^{3}H]parathion and [^{3}H]paraoxon) or 0.1 *M* ammonia (acidic derivatives). Other chemicals used include 4-nitro-[1-^{14}C]phenol (22.5 mCi/mmole, 99% pure, Amersham), tetrabutylammonium phosphate (TBAP, Eastman Kodak, Rochester, NY, U.S.A.), acetonitrile (CH_3CN, Nanograde, Mallinckrodt, St. Louis, MO, U.S.A.) and [2-^{14}C]uracil (50 mCi/mmole, 98% pure, Sigma, St. Louis, MO, U.S.A.). All chemicals were reagent grade and all solvents were spectrophotometric grade.

Apparatus and procedures

The chromatographic system was a Hewlett-Packard 1084B dual solvent-pump liquid chromatograph equipped with a keyboard terminal, variable volume injector, autosampler, fixed-wavelength UV (254 nm) detector and automatic fraction collector. Radiolabeled chemicals were detected by using a Berthold LB 503 radioactivity monitor, equipped with a 130-μl glass-scintillator cell (manufacturer's listed counting efficiency: 4% for ^{3}H and 50% for ^{14}C). A 90-cm piece of stainless-steel tubing connecting the detector and the column had a total volume of 0.57 ml. Columns of Type 316 stainless-steel (15 × 0.46 cm I.D.) were either purchased prepacked or home-packed by using a column slurry packer (Jones Chromatography, Columbus, OH, U.S.A.). The columns used in ion-exchange chromatography were a strong anion exchanger (Partisil-10 SAX, 10 μm, Whatman, Clifton, NJ, U.S.A.) and Ion Analyzer (model 269-001, Wescan Instrument, Santa Clara, CA, U.S.A.). Also tested were a cyanopropyl bonded silica (RSil CN, 10 μm, Alltech, Deerfield, IL, U.S.A.) and a silica column (LiChrosorb Si-60, 10 μm), and, for ion-pair chromatography, a reversed-phase column (RSil C_{18}, HL, 10 μm, Alltech). Packing conditions were: slurry medium, toluene–carbon tetrachloride (1:1, v/v); slurry concentration, 16.5% (w/v); packing pressure, 41 MPa (6000 psi). To retard dissolution of the stationary phase, a pre-column (15 × 0.46 cm, I.D., pre-column gel, 37–53 μm, Whatman) was used with the strong anion-exchange and silica columns. For ion-pair chromatography, a guard column (5 × 0.46 cm I.D.) packed with the same C_{18} material was used.

Aqueous solutions used for the mobile phase were filtered through a 0.22-μm membrane filter (Millipore, Bedford, MA, U.S.A.). Columns were maintained at 40°C during the runs while flow-rates between 0.5 and 1.5 ml/min were tested. Radiolabeled DEPTA, DEPA and MEP, representing the three major phosphorus acid metabolites of parathion, were tested in all the systems examined, both individually and as a composite sample. In some cases, 4-nitro-[1-^{14}C]phenol (4-NP) and paraoxon were also tested. ^{14}C- and ^{3}H-labeled compounds were used interchangeably when both were available. The injection volume was typically 10 μl, but up to 200 μl was occasionally used. The amount of sample per injection ranged from $4 \cdot 10^{-12}$ to $8 \cdot 10^{-11}$ mole for ^{14}C-chemicals and $6 \cdot 10^{-11}$ to $5 \cdot 10^{-10}$ mole for ^{3}H-samples. All calculations related to radiochromatograms were made with the corrected retention time obtained by subtracting from the observed retention time a transit time (0.57 min for the flow-rate of 1 ml/min) attrbituable to the 90-cm tubing between the column outlet and the radioactivity detector.

RESULTS AND DISCUSSION

Because the phosphorus acid metabolites of parathion are strongly acidic anions of pK < 2 (ref. 12), an obvious choice for their separation was ion-exchange chromatography with a strong anion-exchange resin[7,10]. Therefore, initially a Partisil-10 SAX column was tested with 0.01 to 0.25 M acetate, benzoate or cacodylate buffers as the eluent at pH 4.8 to 6.5 using both isocratic and gradient elutions. These buffers of monobasic anions have the optimal pH range for the stability of both samples and the chromatographic system and provided good elution strength whereas the excessive elution strength of di- and trivalent anions was unsuitable for the monobasic metabolites. In all instances, regardless of the flow-rate, ionic strength of the mobile phase or buffers tested, separation of the peak was unsatisfactory. In addition, peak symmetry was generally poor and the clustered elution of metabolites occurred only after a long lead time. Further testing with an analogous column, a Wescan ion analyzer, eluted with 0.005 M phthalate, produced similarly poor results. A silica column and a cyanosilica column were also tested with various solvent mixtures without success.

As another method to exploit the physicochemical differences between the strongly acidic metabolites, ion-pair chromatography was then investigated using a reversed-phase C_{18} silica column and acetonitrile containing TBAP to provide the counter-ion. Following a preliminary test, 0.005 M TBAP was selected for its consistently excellent effect. To provide a constant concentration 0.005 M TBAP was added to both solvent reservoirs, one containing 95% acetonitrile and the other distilled water, which were used to produce the desired solvent strengths. DEPA, DEPTA and MEP were all eluted within 0.5 min of each other at about 3 min in the absence of TBAP in 35% acetonitrile in water at a flow-rate of 1.0 ml/min. The unretained solute, [2-^{14}C]uracil, used to determine the void volume, was eluted at 2.00 min at 1.0-ml/min flow. However, with TBAP clear separation of the three metabolites was obtained with 30 to 40% acetonitrile in water as the eluent at a flow-rate of 1.0 ml/min. Other acetonitrile concentrations and flow-rates produced suboptimal results. The relationship between the solvent strength (% acetonitrile) and the retention (capacity factor k') of the metabolites with the 1.0 ml/min flow-rate is shown in Fig. 1.

The eluent containing 30 to 40% acetonitrile also allowed simultaneous analyses of two other major metabolites of parathion, *i.e.*, 4-NP and paraoxon, although the latter eluted too slowly for these conditions to be practical for its separation (40 min at 35% acetonitrile). A typical radiochromatogram for DEPA, DEPTA, 4-NP and MEP is shown in Fig. 2. Salient features of the optimal system employing 30% acetonitrile are summarized in Table I. The retention time (t_R) is corrected for the time attributable to the tubing between the column and radioactivity detector. k' was calculated using the corrected retention time. Resolution (R_s) and separation factor (α) refer to the actual separation and relative position, respectively, for the pairs: DEPTA–DEPA, 4-NP–DEPTA and MEP–4-NP. Symmetry is expressed as the ratio of the distance from the peak midpoint to the front and back of the peak at 10% peak height. Distortion of peaks due to the large detector cell volume and a somewhat long time constant (30 sec) is evident in the constantly broad and slightly tailing peaks, giving the impression that the theoretical plate number is very low especially

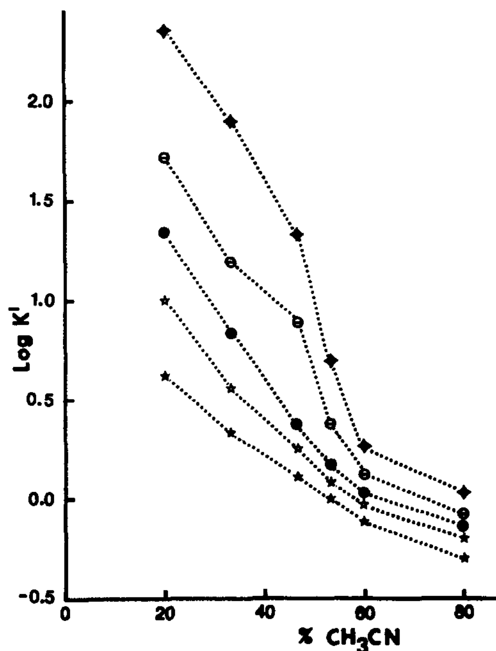

Fig. 1. Effect of solvent strength on the retention of parathion metabolites. See Table I for HPLC conditions. Compounds: ★, DEPA; ☆, DEPTA; ●, 4-NP; ⊖, MEP; ◆, paraoxon.

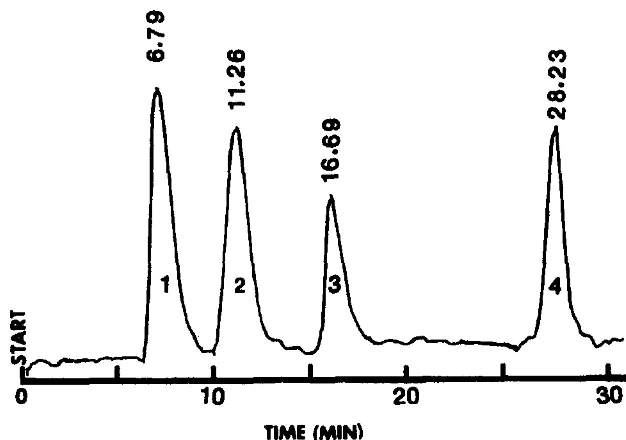

Fig. 2. A radiochromatogram showing the typical separation of the anionic parathion metabolites. See Table I for HPLC conditions. Compounds: 1 = DEPA; 2 = DEPTA; 3 = 4-NP; 4 = MEP. The retention time indicated above each peak includes the time attributable to the tubing between the column and the radioactivity detector.

TABLE I

RETENTION TIME, CAPACITY FACTOR, RESOLUTION AND SYMMETRY FOR LABELED PARATHION METABOLITES

R_s and α refer to the compound in question and the one preceding. Column (15 × 0.46 cm I.D.) and guard column (5 × 0.46 cm I.D.) were both packed with RSil C_{18}, 10 μm. Mobile phase: acetonitrile–water (30:70) containing 5 m*M* TBAP. Flow-rate: 1 ml/min. Temperature: 40°C.

	Metabolite	t_R	*k'*	R_s	*Symmetry*	α
1	[^{3}H]DEPA	6.22	3.35	–	1.33	–
2	[^{3}H]DEPTA	10.69	6.48	1.64	1.14	1.93
3	[^{14}C]4-NP	16.12	10.28	2.13	1.63	1.58
4	[^{3}H]MEP	27.66	18.34	4.91	1.11	1.79

for earlier peaks. However, a separate experiment in which the elution of MEP with 35% acetonitrile–0.005 *M* TBAP was monitored with the 254-nm UV detector (cell volume: 16 μl) gave a peak width at half height of 0.47 min and a corrected retention time of 13.24 min which translated to a theoretical plate number of about 29,150/m, essentially matching the value furnished by the manufacturer (29,500/m). It is likely that all the metabolites would separate in the system with similarly sharp elution, and that the poor appearance of the radiochromatogram is a detector artifact. These chromatographic features are far superior to those observed with ion-exchange chromatography and allow for a greater degree of flexibility in meeting requirements of actual analyses. Despite the limitation of the detector, therefore, the system provides a reproducible and rapid analysis (within 30 min) of the anionic parathion metabolites. The metabolites were stable either in a sodium cacodylate buffer (pH 6.2) or in 35% acetonitrile in water at room temperature (about 25°C) up to 72 h. No degradation of these metabolites occurred on the column since 92 to 103% was recovered when each metabolite ranging from $4 \cdot 10^{-12}$ to $5 \cdot 10^{-10}$ mole was injected. The injection volume could be varied from 10 to 200 μl without adverse effects.

This procedure has been applied to the analysis of parathion metabolism in the rat *in vivo*, the liver perfused *in situ*, in isolated hepatocytes, and in cell-free systems. While clean samples could be prepared by extraction of the metabolites with diethyl ether, direct analyses provided excellent speed and quantitation. For example, urine collected after 4 h from rats injected intraperitoneally (i.p.) with either 0.8 or 2.0 mg ethyl-[1-^{3}H]parathion/kg of body weight, was analyzed for radiolabeled DEPA, DEPTA, and MEP. The relative standard deviation of triplicate experiments was less than 8% with all the radioactivity accounted for as either DEPA (15–20%), DEPTA (40–60%), MEP (4.8–6.2%), or unknown (18–32%). The unknowns could be secondary metabolites or conjugated compounds. Thus, all the major metabolites of parathion were analyzed within 30 min. Even though the rat urine contains a considerable amount of extraneous waste materials, repeated direct analyses of up to 40 unextracted samples yielded essentially identical data. Occasional changes of the guard column should further prolong the column life, allowing simple and accurate analyses of a large number of samples. Since many organophosphorus insecticides yield closely related metabolites, this ion-pair chromatographic procedure should be useful for a wide variety of these pesticides with little modification.

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