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Note

Preparative purification of alkyl methylphosphonic acid *p*-nitrophenyl esters on Sephadex LH-20

A. OSA, H. ARUKAEVU and A. AAVIKSAAR

Institute of Cybernetics of the Academy of Sciences of the Estonian S.S.R., Biochemistry Department, P.O. Box 670, Tallinn-26 (U.S.S.R.)

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Sephadex LH-20 gel chromatography in ethanol has been used for the fractionation of alkyl methylphosphonic acid *p*-nitrophenyl ester synthesis reaction mixtures in order to obtain pure esters.

Organophosphorus esters are irreversible competitive inhibitors of serine esterases and have been extensively used in the investigation of the topography of the active sites of these enzymes[1–3]. It has been shown that the preparations of the compounds may contain synthesis by-products that greatly exceed the inhibiting effectiveness of the main component and in some instances the underestimation of active by-product content had led to incorrect interpretation of experimental data[4–11]. Therefore, in enzyme studies special attention must be paid to the purification of these compounds.

It has been shown that vacuum distillation is not an effective technique for the purification of organophosphorus inhibitors as the distillation product usually contains destruction products[4,6,10,12].

Liquid chromatography on silica gel, developed in this laboratory for the fractionation of organophosphorus thioester synthesis mixtures[13], cannot be used in the purification of alkyl methylphosphonic acid *p*-nitrophenyl esters as the significant chemical activity of the silica gel surface causes the destruction of the *p*-nitrophenyl ester bond.

Gel chromatography of organophosphorus esters on Sephadex LH-20 has been used in the separation of pesticides from plant material[14,15].

EXPERIMENTAL

Materials

Absolute methanol, ethanol and diethyl ether were purified by conventional methods[16].

Alkyl *p*-nitrophenyl methylphosphonates, $(C_nH_{2n+1}O)(CH_3)P(O)OC_6H_4NO_2$-*p* with $n = 2$–8, were obtained by the reaction of freshly distilled alkyl methylphosphonyl chloride with sodium *p*-nitrophenolate in diethyl ether at 3–7° [17]. The sodium chloride precipitate was filtered off and the solvent was evaporated from the reaction mixture under vacuum at a temperature below 30°. The compounds with $n = 6$–8

have not been described earlier. The structures of all of the synthesized phosphonates were identified by ^{13}C nuclear magnetic resonance spectrometry carried out on a Bruker WH-90 spectrometer (we are indebted to Dr. T. Pehk for the spectra).

Column preparation

Dry Sephadex LH-20 (Pharmacia, Uppsala, Sweden) of particle size 25–100 μm was suspended overnight in absolute ethanol, then a 70% suspension of the gel in ethanol was prepared and packed into a vertical glass column with a diameter of 2.3 cm. The total packed bed volume (v_t) was 260 ml. Samples were applied by layering about 2 ml of synthesis mixture on the top of the column. Fractionations were carried out at room temperature (*ca.* 20°) at an ethanol flow-rate of 0.5 ml/min, and the effluent was collected in 7.5-ml fractions. After each chromatography, the column was eluted with $2v_t$ ml of absolute methanol in order to wash out traces of *p*-nitrophenol.

Examination of column effluent

Chromatograms were obtained by weighing the residue of the aliquot from each fraction after evaporation of the solvent. The contents of *p*-nitrophenol and alkyl *p*-nitrophenyl methylphosphonate in the effluent were detected spectrophotometrically at 400 nm in 0.2 *M* phosphate buffer solution at pH 7.4. With phosphonate, the *p*-nitrophenyl ester bond was hydrolyzed previously at pH 12.

RESULTS

On the chromatograms obtained from reaction mixtures on Sephadex LH-20 columns equilibrated with absolute ethanol, four peaks were obtained for all members of the series (n = 2–8). Components A and B in Fig. 1 are butyl *p*-nitrophenyl methyl-

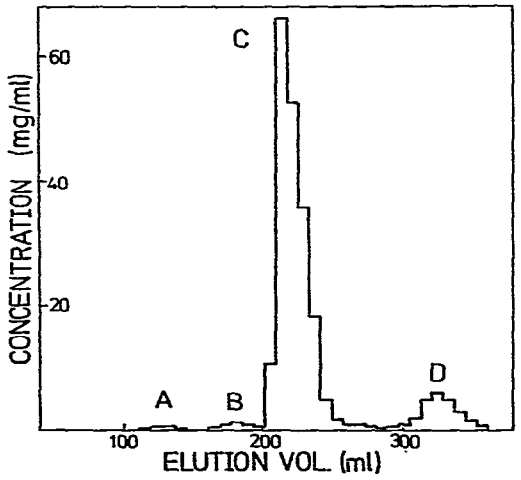

Fig. 1. Fractionation of butyl *p*-nitrophenyl methylphosphonate synthesis reaction mixture on a Sephadex LH-20 column (62.7 × 2.3 cm I.D.) equilibrated with ethanol. Sample volume, 2 ml. A and B, reaction by-products (not identified); C, the phosphonate; D, *p*-nitrophenol.

TABLE I

PURIFICATION OF ALKYL METHYLPHOSPHONIC ACID *p*-NITROPHENYL ESTERS, (n-$C_nH_{2n+1}O$) $(CH_3)P(O)OC_6H_4NO_2$-*p*, ON SEPHADEX LH-20

n	*Mol. wt.*	v_e *(ml)*	n_D^{20}	
			Exptl.	*Lit.*[18]
2	245	225.4	1.5290	1.5281
3	259	224.6	1.5240	1.5238
4	273	221.8	1.5190	1.5189
5	287	215.9	1.5145	1.5162
6	301	201.5	1.5100	—
7	315	199.0	1.5065	—
8	329	196.6	1.5023	—

phosphonate synthesis by-products, which were not identified, component C is the phosphonate and D is *p*-nitrophenol.

On going from one member of the series to another, the elution volumes (v_e) of A and B changed in such a manner that the resolution of by-products from alkyl *p*-nitrophenyl methylphosphonates did not change in the series. The elution volume of *p*-nitrophenol was sufficiently high to give a fairly good resolution of the compound from all phosphonates. The elution volumes of the alkyl *p*-nitrophenyl methylphosphonates and their refractive indices are given in Table I.

Alkaline hydrolysis of the compounds gave *p*-nitrophenol in stoichiometric amounts. In the purified products no *p*-nitrophenol was detectable spectrophotometrically.

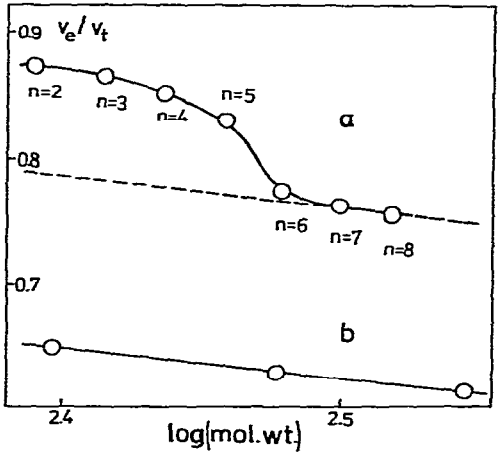

Fig. 2. Correlation between relative elution volume and molecular weight for (a) O-*n*-alkyl *p*-nitrophenyl methylphosphonates, (n-$C_nH_{2n+1}O$) $(CH_3)P(O)OC_6H_4NO_2$-*p*, and (b) polyethylene glycols[19] on Sephadex LH-20 in ethanol.

DISCUSSION

It is evident that chromatography on Sephadex LH-20 in ethanol is an effective method for the isolation of alkyl methylphosphonic acid *p*-nitrophenyl esters from their synthesis reaction mixtures.

The dependence of v_e/v_t on log (mol. wt.) in Fig. 2a shows that, in addition to the molecular sieving effect that causes the elution of the phosphonates in order of decreasing molecular weight, a significant part of retention is caused by adsorption (Fig. 2b shows the relationship between v_e/v_t and log (mol. wt.) for polyethylene glycols as reference compounds[19]).

At $n = 6$–8, the plot of v_e/v_t *versus* log (mol. wt.) for the phosphonates is a straight line, as for polyethylene glycols. The constant difference between v_e/v_t for the two types of compounds might be explained by a π-electron interaction of *p*-nitrophenyl group with the gel[20] or with a polar interaction as suggested by Determann and Lampert[21]. From the "breaking point" at $n = 6$, the plot of v_e/v_t *versus* log (mol. wt.) is non-linear with a rather sharp dependence of retention volume on the length of the alkoxyl radical in the phosphonate. We cannot give an adequate explanation of this effect, but it is worth mentioning that this specific adsorption effect significantly improves the resolution of the compounds on Sephadex LH-20 in ethanol.

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