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THE SEPARATION OF THE COLOURED DERIVATIVES OF SOME ORGANIC COMPOUNDS USING LIQUID CHROMATOGRAPHY IN SMALL-BORE COLUMNS PACKED WITH ION-EXCHANGE RESINS

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SUMMARY

A possibility of applying liquid chromatography to the separation of coloured homologous N,N-dimethyl-*p*-aminobenzeneazobenzoyl esters and amides has been studied using apparatus consisting of a pulse-free plunger feeding pump, a narrow bore column, a spectrophotometer with a flow-through measuring cell of our own design, and a recorder. These compounds can be sorbed on sulphonated styrene-divinylbenzene cation-exchange resins with a low degree of cross-linking and separated by elution with hydrochloric acid solutions in aqueous–organic solvents. The influence of the eluent composition, the molecular size of the solute and functional groups upon chromatographic behaviour have been studied. Good resolution can also be achieved by adsorption chromatography on silica columns.

INTRODUCTION

Liquid column chromatography has become an invaluable tool in biochemical research, namely in the analysis of amino acids, proteins, enzymes, hormones, nucleosides, sugars and carboxylic acids. Gel chromatography, closely related to the above method, is now the most convenient technique for the determination of the molecular weight distribution of synthetic and natural polymers. Recently a few fundamental papers have appeared dealing with increasing the speed and efficiency of liquid chromatography[1–8], which may stimulate its application to the separation of other classes of compounds. We have tried to combine the outstanding properties of N,N-dimethyl-*p*-aminobenzeneazobenzoyl derivatives with the advantages of liquid chromatography.

N,N-dimethyl-*p*-aminobenzeneazobenzoyl esters and amides have proved their outstanding qualities for the separation and identification of aliphatic alcohols, glycols and amines by means of paper and thin-layer chromatography[9–12]. N,N-dimethyl-*p*-aminobenzeneazobenzoyl chloride, which is used for their preparation, is very stable and highly reactive. The preparation is very simple and rapid. Coloured derivatives of phenols, mercaptans and possibly other compounds may also be prepared. Detection of these derivatives is possible without any addition of a colour-developing agent, and has a high sensitivity.

Liquid chromatography of N,N-dimethyl-*p*-aminobenzeneazobenzoyl derivatives would have some special advantages: a wide range of organic compounds can react with this single reagent forming coloured products differing in polarities (basicities), which makes it possible, in principle, for both ion-exchange and adsorption chromatographic separations to be carried out in conjunction with continuous spectrophotometric effluent monitoring which would yield a record of the elution pattern. In addition to its better accuracy in comparison with paper and thin-layer chromatography, this method is easy to automate. An apparatus for the column liquid chromatography of microgram quantities of these coloured compounds was designed using available laboratory instruments, in order to investigate the liquid chromatography of these derivatives.

MATERIALS AND METHODS

Liquid chromatography system

Straight glass columns of narrow bore (0.8–3 mm in diameter, 10–100 cm long, jacketed) are used. Their lower end is fixed into a polyethylene tube reduced to capillary size (0.5 mm I.D.) connecting the column to a flow-through detector measur-

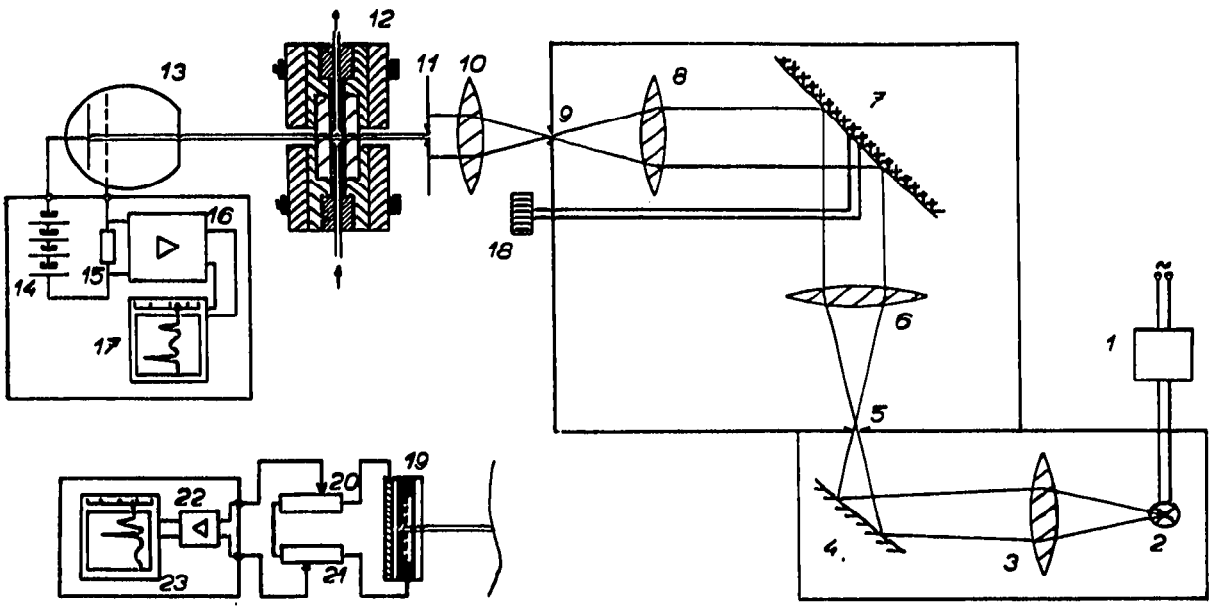

Fig. 1. Diagram of the photometric detector. A spectrophotometer Spekol (Zeiss, Jena) serves as a monochromatic light source. Light from a lamp (2) with a stabilised direct current power supply (1) is focused by the optical system (3), (4) on the monochromator entrance slit (5). A narrow monochromatic beam of light is selected by a slit (9) from spectrum reflected by a dispersion grating (7) and enters an adaptor of our own design screwed on to the Spekol housing. A lens (10) and a screen (11) of the objective fixed in the adaptor cap direct the light beam into the centre of a flow-through cell (12) which is fixed on the adaptor cover and can be removed with it from the duralumin adaptor cap. The light emerging from the cell falls on a photoelectric element connected to the adaptor cap. A gas filled phototube (13) can be used in conjunction with a compensation recorder G1B1 (Zeiss, Jena) (17) having its own phototube potential source (14) and amplifier (16). A selenium cell (19) can also be used, its potential being recorded (23) after sensitivity reduction by resistors. (20), (21) and amplification (22) by an EZ 3 or EZ 4 recorder (Laboratory Instruments, Prague). The wavelength required can be adjusted by a micrometric screw (18) controlling the dispersion grating position.

ing cell. The eluent is delivered at a constant, reproducible flow rate by a pulse-free pump (Linear proportioner, ZSNP, Žiar n. Hronom) with a constant speed motor driving a plunger by means of an adjustable gear system. The plunger pushes the eluting liquid out from a precision graduated glass syringe (10 or 20 ml capacity) through a polyethylene capillary (1 mm I.D.) connected to the upper end of the column.

A photometric detector to give continuous effluent stream monitoring was designed. A single-beam spectrophotometer Spekol (Zeiss, Jena) is used to provide a monochromatic light source. A narrow monochromatic beam of light selected by a slit from the spectrum reflected by a dispersion grating is focussed into the centre of a flow-through cell of our own design, placed in an adaptor screwed on to the Spekol housing. The light emerging from the cell falls on the photoelectric element. A gas filled phototube can be used in conjunction with a compensation recorder G1 B1 (Zeiss, Jena) having its own phototube potential source and amplifier. A selenium cell potential recording by an EZ 3 or EZ 4 recorder (Laboratory Instruments, Prague) has also given satisfactory results.

The flow-through measuring microcell is made out of a small teflon cylinder which has been bored through the centre. The channel forming the optical path is sealed at both ends perpendicularly to the optical path by glass windows (2 mm thick). Liquid enters the channel from a teflon capillary (0.5 mm I.D.), at the bottom end and flows through the channel, leaving it at the other end to flow out through another teflon capillary. The cell is placed between two metal sheets and screwed tight by means of four bolts. Plastic gaskets bored in the centre are inserted between the teflon cylinder and the glass windows making the cell leak tight. Two bored rubber pieces inserted between the metal sheets and the windows protect the windows from cracking.

The cell is resistant to corrosive agents (only teflon and glass come in contact with liquid) and easy to take apart and clean.

The cell has been designed in two sizes for measuring at different sensitivities — one 20 μl volume (with feeding capillary), 1.5 mm optical path length, 2 mm I.D., the other 35 μl volume, 10 mm path length and 1 mm I.D.

The base-line shift during an 8 h run did not exceed 1.5 %, the recorder scale corresponding to the full absorbance value range. A tenfold absorbance scale expansion is possible (in the range from 450 to 550 nm).

The linear relationship between the peak areas evaluated as peak widths at their half peak heights multiplied by the corresponding absorbance values and the amount of coloured compound was determined experimentally for both inert and retarded components (Ponceau 6R and *n*-amyl ester of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid) on a Dowex 50W-X2 column. The random error of peak area measurement was about 3–5 % rel. An increase in the eluent flow rate causes a slight increase in the peak areas, whereas the response (peak height) falls off exponentially.

Separations by liquid chromatography

A strongly acidic sulphonated styrene-divinylbenzene cation-exchange resin, Dowex 50W-X2 (H^+ form) was used for the separation of the coloured derivatives. Their protonised forms are distributed between the external solution (eluent) and the solution in the resin particles in accordance with the basicities of the non-ionic forms.

The equilibrium depends on the H^+ ion activity in the external solution and in the resin particles.

Because of the negligible solubility of these compounds in aqueous–acid solutions, it is necessary to employ mixed aqueous-organic media. The amount of the organic solvent present obviously affects the distribution equilibrium by its solubility and solvation effects, and also by the dielectric constant effect.

Cation-exchange resin with a low degree of cross linking (X2) was used in the separation in order to improve the accessibility of the ion-exchange phase to the rather large molecules of the derivatives and to accelerate the diffusion rate in the resin.

The quantitative sorption of coloured esters and amides on Dowex 50W-X2 (H^+ form), 200–400 mesh, from mixed aqueous–organic solutions (80% ethanol; 80% methanol) has been established. Sorbed compounds can be eluted by aqueous–ethanolic or aqueous–methanolic solutions of hydrochloric acid. The effect of the eluent composition on the chromatographic behaviour of some homologous esters and amides has been studied, and the volume distribution coefficients D_v have been determined by the dynamic method[13]. Both homologous esters and amides are eluted in order of increasing basicities; *i.e.*, in order of decreasing molecular weights. Amides with a higher basicity have a higher distribution coefficient than esters, whose basicity is lower. Secondary amides are sorbed more strongly than the less basic primary ones, their elution curves showing greater broadening than those of the esters.

The contribution of the CH_2-group to the logarithm of the distribution coefficient has proved to be about the same for the homologous esters of aliphatic alcohols and primary aliphatic amides. It increases to some extent with decreasing hydrocarbon chain length. Secondary amides have shown greater changes corresponding to the same molecular weight contribution. D_v values of iso-derivatives are slightly lower compared with the normal ones. Multiple bond contribution to D_v values does not seem to be significant (Table I).

The hydrochloric acid concentration in the eluent influences the equilibrium

TABLE I

VOLUME DISTRIBUTION COEFFICIENTS D_v OF SOME DERIVATIVES OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOIC ACID ON CATION EXCHANGER DOWEX 50W-X2 IN 0.925 *M* HYDROCHLORIC ACID SOLUTION IN 80.5% ETHANOL

D_v has been defined as a ratio of the amount of compound in a unit volume of the ion-exchanger phase to the same volume of external solution.

Derivative	D_v	*Derivative*	D_v
Methyl ester	6.3	Methyl amide	8.6
Ethyl ester	5.2	Ethyl amide	7.4
n-Propyl ester	4.5	*n*-Propyl amide	6.5
n-Butyl ester	3.9	*n*-Butyl amide	5.6
n-Amyl ester	3.5	*n*-Hexyl amide	4.7
n-Hexyl ester	3.0	Allyl amide	6.4
n-Octyl ester	2.3	Dimethyl amide	9.9
n-Nonyl ester	2.1	Diethyl amide	6.6
n-Decyl ester	1.9	Di-(*n*-propyl) amide	4.7
Isopropyl ester	4.3	Di-(*n*-butyl) amide	3.5
Isobutyl ester	3.6		

between the protonised and non-ionic form of these compounds. A higher concentration decreases the volume distribution coefficients of the compounds under study and the corresponding retention volumes (and also their differences). This effect becomes more significant at lower hydrochloric acid concentrations (Fig. 2).

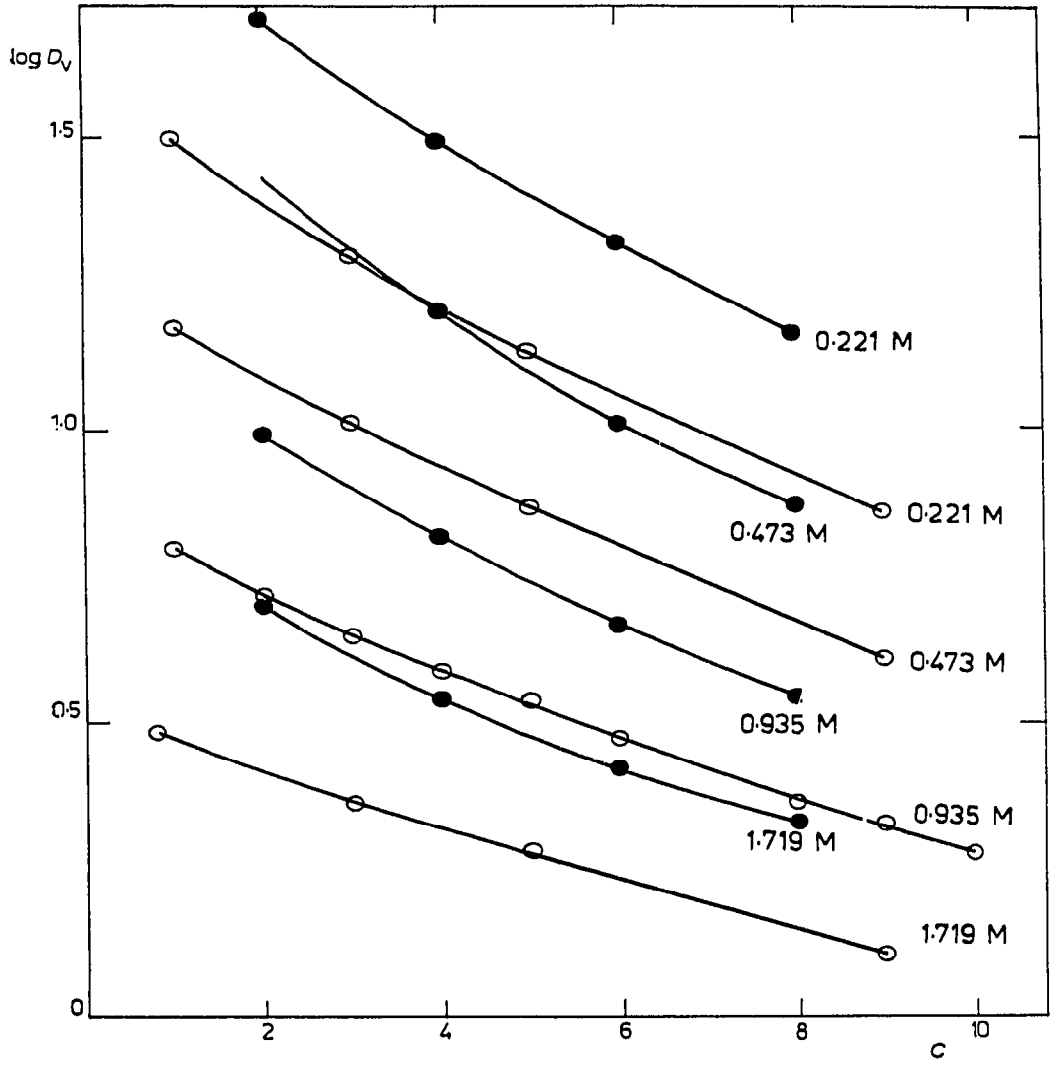

Fig. 2. The effect of the hydrochloric acid concentration in the eluent. Volume distribution coefficients D_v for esters and amides are plotted *versus* the number C of carbon atoms in a primary aliphatic alcohol or secondary amine molecule. Ethanol concentration in eluent, 80.5 % by weight. ○, Esters of homologous primary aliphatic *n*-alcohols. ●, Amides of homologous secondary aliphatic *n*-amines.

Ethanol or methanol concentration has a similar influence (Fig. 3). In solutions with an alcohol concentration $\leqslant 50$ % these derivatives are sorbed too strongly by the ion-exchanger, with only small differences in D_v values. A higher concentration enhances solubility in the external solution, especially that of the higher homologous esters and amides. The distribution coefficients decrease and their differences increase. The external alcoholic solutions have an optimum composition at about 80–90 wt. %. The differences in the distribution coefficients of homologous derivatives reach a

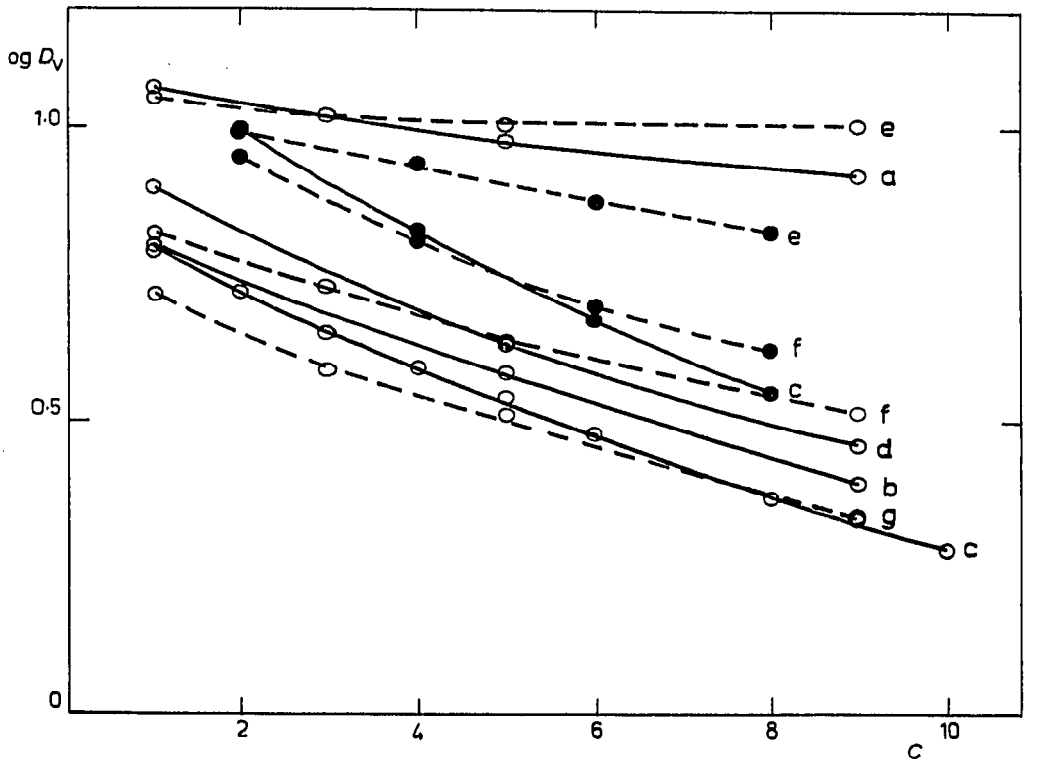

Fig. 3. The effect of the alcohol concentration in eluent. Solid lines — ethanol; broken lines — methanol. Hydrochloric acid concentration in eluent — 0.935 *M*. Other symbols have the same meaning as in Fig. 2. Ethanol concentration: (a) 47.0 % by weight; (b) 63.1 %; (c) 80.5 %; (d 91.0 %; methanol concentration: (e) 58.5 % by weight; (f) 74.9 %; (g) 88.0 %.

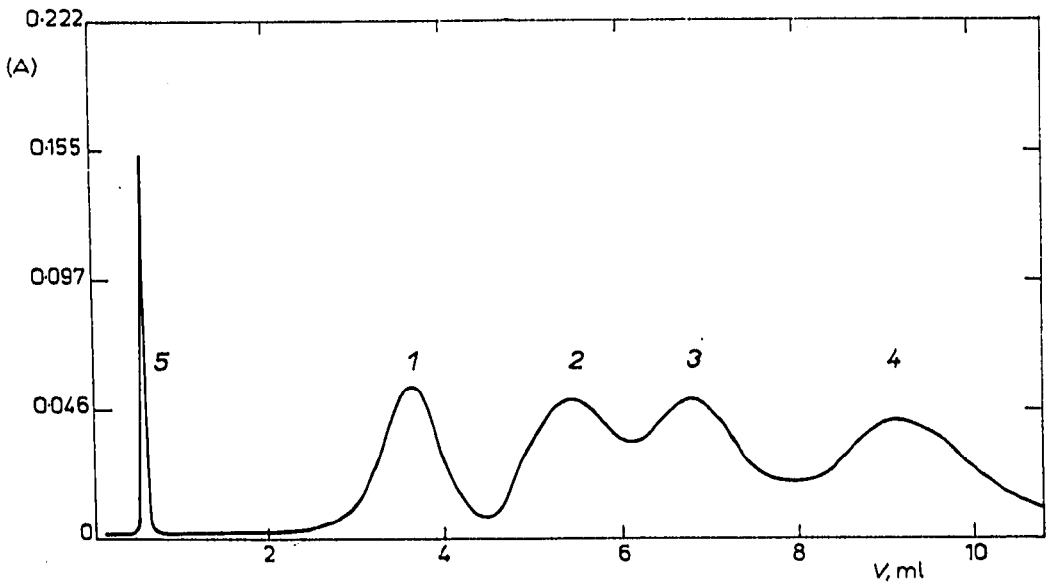

Fig. 4. Chromatographic separation of some primary aliphatic esters of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid. (A)absorbance. Column 240 × 2.7 mm, Dowex 50W-X2 (200-400 mesh) H^+ form. Sample volume 20 μl. Eluent — 0.925 *M* HCl in 80.5 % ethanol. Flow rate 0.016 ml/min, 10 mm optical path length cell, λ = 510 nm. (1) 0.5 μg of *n*-nonyl ester; (2) 0.5 μg of *n*-amyl ester; (3) 0.5 μg of *n*-propyl ester; (4) 0.5 μg of methyl ester; (5) inert compound (Ponceau 6R).

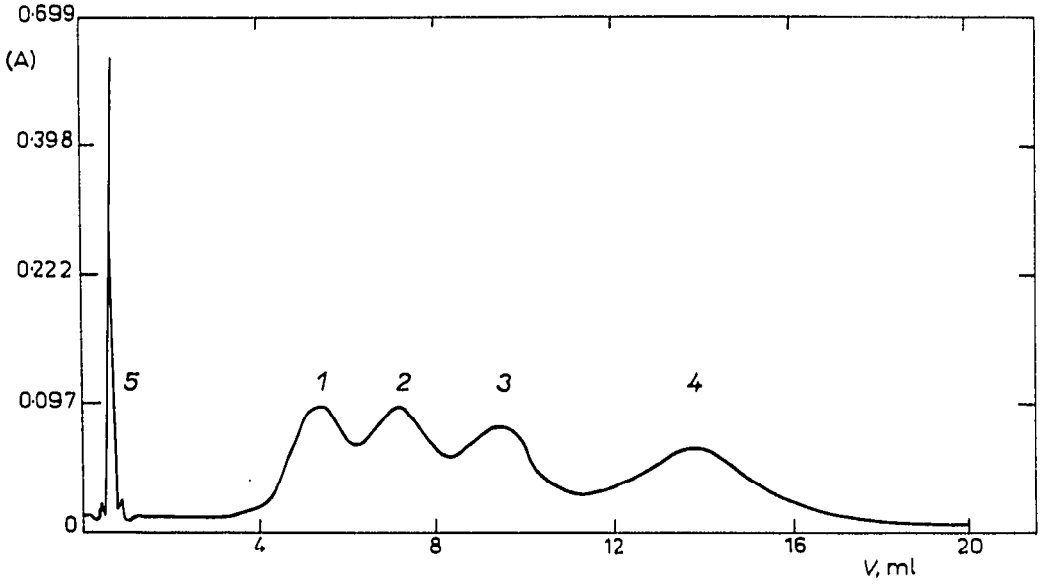

Fig. 5. Chromatographic separation of some secondary aliphatic amides of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid. (A)absorbance. Flow rate 0.032 ml/min; sample volume = 60 μl, other conditions as in ester separation .(1) 1.5 μg of di-(*n*-butyl) amide; (2) 1.5 μg of di-(*n*-propyl) amide; (3) 1.5 μg of diethyl amide; (4) 1.5 μg of dimethyl amide; (5) inert compound (Ponceau 6R).

maximum in these solutions whereas the D_v values are minimal; solvation and dielectric properties controlled by the alcohol ratio in both phases are obviously most advantageous for chromatographic separation.

In these experiments the hydrochloric acid concentration was kept constant at 0.925 *M*.

An example of the separation of esters of lower aliphatic alcohols on a Dowex 50W-X2 column by elution with aqueous–ethanolic hydrochloric acid solution is shown in Fig. 4; Fig. 5 illustrates the chromatographic separation of some lower secondary amides under similar conditions.

Further improvement of separation efficiency and time can be expected from elution with a hydrochloric acid gradient, which is the subject of our investigations now.

Good resolution of coloured derivatives was also achieved by adsorption chromatography on Silica CH (5 – 40 μm) columns. Some homologous amides and esters can be resolved by elution with cyclohexane–ethyl acetate mixtures in the order of increasing polarities.

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