Journal of Chromatography, 218 (1981) 209–216
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,178

HIGH-RESOLUTION SEPARATIONS BASED ON ELECTROPHORESIS AND ELECTROOSMOSIS

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SUMMARY

The related electrokinetic effects of electrophoresis and electroosmosis may be used to achieve high resolution separations. An "instrumental" version of zone electrophoresis carried out in 75 μm I.D. glass capillaries is described. This technique demonstrates excellent resolution of charged substances in a short time. Using virtually the same apparatus, reversed-phase chromatography is performed with electroosmotic pumping of an acetonitrile mobile phase. This technique also produces separations with excellent efficiency. Very low values of reduced plate height are found, which suggests that column packing irregularities are less important when electroosmotic flow is used instead of flow generated by pressure.

INTRODUCTION

Electrophoresis and electroosmosis are related electrokinetic effects. Electrophoresis is the familiar phenomenon of migration of charged particles in solution in an electric field gradient. The velocity of migration, v_{ep}, is given by:

$$v_{ep} = \mu_{ep} E \tag{1}$$

where μ_{ep} is the electrophoretic mobility and E the electric field gradient. The related phenomenon of electroosmosis is perhaps a little less familiar. If charged particles are held stationary (as, for instance, silica particles packed tightly in a tube), then in the presence of an applied electric field, instead of the particles migrating through the liquid, the liquid migrates or flows through the bed of particles. The velocity of this electroosmotic flow, v_{eo}, is given by:

$$v_{eo} = \mu_{eo} E \tag{2}$$

where μ_{eo} is the coefficient of electroosmotic flow. In 1952 Mould and Synge[1,2] first demonstrated the use of electroosmotic flow in a thin-layer chromatographic system for the separation of polysaccharides in collodion membrane strips. This method of generating flow was applied to both thin-layer and column chromatographic systems in 1974 by Pretorius *et al.*[3]. In their column work, these authors reported only on

their results with unretained solutes. Their initial data on column efficiency were promising, and electroosmotic flow appeared to offer some interesting possibilities from a chromatographic point of view.

The purpose of this paper is to describe two forms of high-efficiency separations based on these two electrokinetic effects. First we will consider zone electrophoresis in open tubes, and second, chromatography in packed columns with flow generated by electroosmosis. The equipment used in the two separation systems is essentially the same. However, for the sake of clarity, the topics will be treated in two separate sections.

ZONE ELECTROPHORESIS IN OPEN TUBES

Theory

Zone electrophoresis is fundamentally a simple process of migration of zones of charged substances in an electric field. Certainly molecular diffusion contributes to some of the broadening of an initially narrow zone. However, it is by no means the only cause, and in most cases it is actually a fairly insignificant contributor to zone broadening. The real situation is generally more complex. Heat is generated uniformly throughout the separation medium by the passage of electric current, but is only removed at the edges of the medium. The inevitable result is a temperature gradient within the medium, and associated with this, a density gradient[4]. Some means must be used to suppress convective flows associated with density gradients or else zone broadening will result. The most common approach is to perform zone electrophoresis in a gel. Although gels solve the problem of convection, they introduce new zone-broadening phenomena such as eddy migration and adsorptive interactions between solutes and gel[4]. A more sophisticated solution to the problem of convection which circumvents the difficulties associated with gels is the free zone electrophoresis technique developed by Hjertén[5]. In this method, stabilization is achieved by the continuous rotation of the electrophoresis tube about its longitudinal axis. Another approach is the group of methods known as continuous-flow deviation electrophoresis. This ingenious group of techniques uses flow in "serpentine" or "helical" paths to combat convection, and has been reviewed by Kolin[6]. A still simpler approach involves the use of capillary tubes as the separation chamber, as described by Mikkers *et al.*[7]. The walls of a tube act with the liquid's viscosity to counteract flow. As the tube diameter is decreased this stabilizing "wall effect" is enhanced.

Although these various approaches are effective ways to minimize convection they do not eliminate the temperature gradient. Since electrophoretic mobility increases at an approximate rate of 2% per °C (ref. 4), solute molecules in the hotter interior of the medium will migrate faster than solute at the cooler edges, resulting in significant spreading of the zones. According to Wieme[4], when electrophoresis is performed in tubes of circular cross-section the magnitude of the temperature difference from the center to the wall of the tube is proportional to the square of the tube diameter. Thus a reduction in tube diameter will result in a significant decrease in temperature differences across the tube. Even with tubes of very small diameter some temperature gradient will persist, and thus some zone broadening should result owing to the temperature dependence of migration velocity. However, if the tube diameter is

small enough, the solute molecules will be able to diffuse back and forth across the tube's diameter many times during their migration down the length of the tube. In this way they randomize their occupancy of the tube's cross-section, which tends to average-out any radially dependent velocity differences. This will tend to minimize the importance of any remaining temperature gradient.

If zone electrophoresis were carried out in an open tube of small diameter, a situation may develop where zone broadening occurs predominantly by molecular diffusion. If electrophoresis is carried out in tubes, substances could be introduced at one end and would migrate under the influence of the electric field to the far end. At the far end they could be detected as they migrated past a detection device. This would yield a plot of solute concentration as a function of time, which is called an electropherogram. The migration velocity, v_{ep}, was given by eqn. 1. This equation may be rewritten as:

$$v_{ep} = \mu_{ep} E = \mu_{ep} V/L \tag{3}$$

where V is the voltage applied across the length, L, of the tube. The time, t, that a solute takes to migrate the entire length of the tube is given by:

$$t = \frac{L}{v_{ep}} = \frac{L^2}{\mu_{ep} V} \tag{4}$$

Diffusion will be occurring during this time. If an initially infinitely thin zone is allowed to diffuse for a time, t, the spatial variance, σ_L^2, of the zone will be:

$$\sigma_L^2 = 2Dt = \frac{2DL^2}{\mu_{ep} V} \tag{5}$$

where D is the diffusion coefficient of the solute. We can express the separation efficiency of an electrophoretic system in terms of the number of theoretical plates, as suggested by Giddings[8]. The number of theoretical plates, N, is defined as:

$$N = \frac{L^2}{\sigma_L^2} \tag{6}$$

and by substituting eqn. 5 into eqn. 6,

$$N = \frac{\mu_{ep} V}{2D} \tag{7}$$

This simple result suggests that the most direct approach to high separation efficiencies in zone electrophoresis is the use of very high voltages. Interestingly, as long as heat dissipation is adequate, tube length plays no direct role in separation efficiency. If very high voltages are applied to narrow-bore capillary tubes, high separation efficiencies should be obtained.

Experimental

Glass capillary tubes were drawn from Pyrex borosilicate glass to an I.D. of 75 μm, an O.D. of 550 μm, and a length of 1 m, using a Shimadzu (Kyoto, Japan) GDM-1B glass-drawing machine. These tubes were filled with a $5 \cdot 10^{-2}$ *M* phosphate buffer (pH 7) as the electrophoresis medium. A Megavolt (Hackensack, NJ, U.S.A.) Model RDC-30-10 high-voltage d.c. power supply, delivering from 0 to +30 kV, provided the electric field for electrophoresis. Accidental contact with the high-voltage end of the system was prevented with an interlock mechanism. The high-voltage end was encased in a plexiglass box, and opening this box automatically shut off the high voltage. Graphite electrodes were used to connect the power supply to the buffer reservoirs located at each end of the capillary. Samples were introduced at the positive (high-voltage) end and the detector was located at the negative (ground) end of the system. Samples were introduced into the capillary by replacing the buffer reservoir with a sample container. A brief application of high voltage caused the migration of a small amount of sample into the tube. The buffer reservoir was then replaced, voltage applied, and electrophoresis proceeded. The detector used was a home-made on-column fluorescence detector. This device permitted the sensitive detection of solutes while still in the capillary and thus avoided extracolumn zone broadening. However, only fluorescent solutes were detectable. Details of the design of this detector will be published. In all instances, 30 kV potential was applied to the system, and the current measured under these circumstances was $1 \cdot 10^{-4}$ A.

Results and discussion

Fig. 1 is an electropherogram of a series of *n*-alkylamines labeled with fluorescamine (note the truncated time axis). Each amine derivative differs from its neighbor by only one methylene unit and yet all are well resolved. Each addition of a methylene

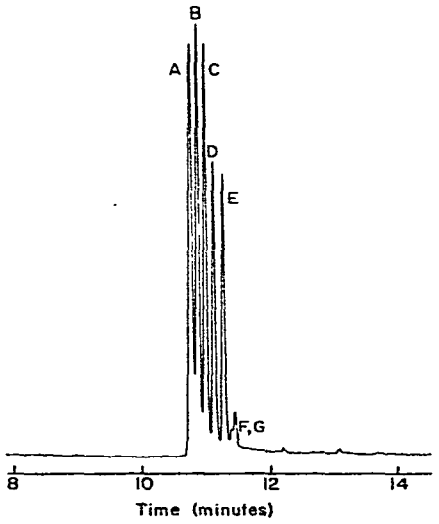

Fig. 1. Electropherogram of *n*-alkylamines as fluorescamine derivatives. Peaks: A = octyl; B = heptyl; C = hexyl; D = pentyl; E = butyl; F = unknown impurity; G = propyl. Approximately 7 pmoles of each derivative, except for propylamine.

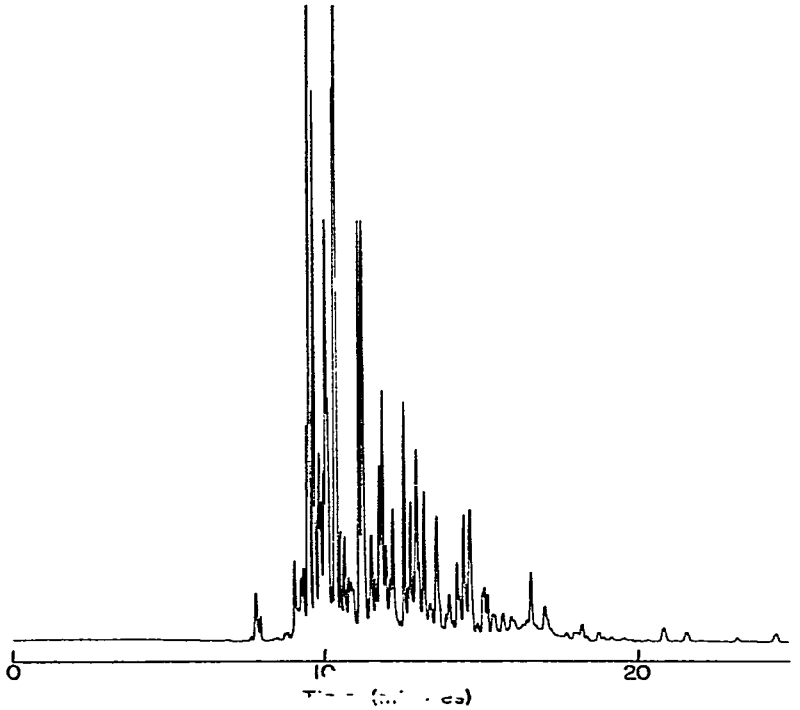

Fig. 2. Electropherogram of fluorescamine labeled peptides obtained from a tryptic digest of chicken ovalbumin.

unit represents roughly a 4% increase in molecular mass or a 1% increase in molecular diameter of the derivative. This is an indication of the very high efficiency of this electrophoresis system. An interesting aspect of this separation is that all these derivatives are negative ions and yet migrate toward the negative electrode. This apparent paradox is explained by the fact that the buffer is undergoing a very strong electroosmotic flow, and is carrying the negative ions with it toward the negative electrode. The solute ions are migrating against this flow, but are eventually carried by it to the detector. This also explains why the larger amine derivatives appear at the detector first. The larger derivatives have lower mobilities, and thus are less effective in migrating against the electroosmotic flow. Electroosmotic flow modifies the equations describing the separation process somewhat. However, owing to the flat flow profile of electroosmotic flow[3,9], the basic linear dependence of separation efficiency on applied voltage remains correct[10]. Electroosmotic flow is an advantage in this system in that it permits analysis of both positive and negative ions in a single run. Fig. 2 is an electropherogram of fluorescamine labeled peptides. The peptides were obtained from a tryptic digest of heat-denatured chicken ovalbumin[11]. Both very high resolution and speed of analysis are evident in this separation of a complex mixture.

The potential of this technique for yielding rapid and efficient separations has been clearly demonstrated. However, fluorescence detection is rather limiting, and a wider range of detection devices will be desirable. The possibilities for protein analysis

are particularly promising, and continuing development in this direction will be pursued.

ELECTROOSMOTIC FLOW IN CHROMATOGRAPHY

Theory

As previously mentioned, electroosmosis is the flow of liquid through a porous medium under the influence of an applied electric field. This phenomenon was well reviewed by Pretorius *et al.*[3], as well as in standard texts[12]. The flow velocity was given in a simple form by eqn. 2. An extended form gives the flow velocity as:

$$v_{eo} = \left[\frac{\varepsilon\zeta}{4\pi\eta}\right] E \tag{8}$$

where ε is the dielectric constant and η the viscosity of the solvent, and ζ is the zeta potential of the liquid–solid interface[3]. One interesting aspect of electroosmotic flow is that unlike other forms of flow it generates a flat flow profile[3,9]. In a chromatographic system this should result in a lower resistance to mass transfer in the mobile phase. Perhaps a more important facet of electroosmosis is that, according to eqn. 8, the velocity of flow is independent of the geometry and size of the channels in the packing. This means that, in a packed column, flow should be uniform across the column regardless of packing irregularities. Lower values of the height equivalent of a theoretical plate (HETP) should thus be obtained with electroosmotic flow than with other forms of flow. Indeed this was found to be the case for unretained solutes by Pretorius *et al.*[3]. The purpose of this investigation was to study the performance of electroosmotic flow in realistic chromatographic systems.

Experimental

A straight length of Pyrex glass tube was drawn to an I.D. of 170 μm, an O.D. of 650 μm, and a length of 68 cm on a glass-drawing machine (Shimadzu GDM-1B). This column was to be packed with a 10 μm packing, and thus the column end had to be "plugged" with a porous plug. To accomplish this, *ca.* 5 mm of one end of the tube was filled with Permaphase ODS pellicular packing (DuPont, Wilmington, DE, U.S.A.) of *ca.* 30-μm diameter. This end was then heated in a flame, sintering the pellicular packing into the end of the tube and forming a porous plug. Partisil-10 ODS-2 (Whatman, Clifton, NJ, U.S.A.), a 10-μm reversed-phase packing, was then pumped at 1000 p.s.i.g. as an acetonitrile slurry into the column in a "down-flow" manner. This was continued until the column appeared fully packed. The acetonitrile was then forced from the column with gas pressure, and the remaining end "sealed" by sintering another 5-mm plug of pellicular packing in this end. Once sealed, the column was again filled with acetonitrile and was ready for use. This column was operated in essentially the same manner as described for the electrophoresis. Samples were introduced to the column by replacing a solvent reservoir with sample and applying voltage briefly. Flow of solvent was in the direction of the negative electrode. The on-column fluorescence detector was again used to detect solutes. The distance from the column inlet to the detector was 58 cm, which represents the portion of the column available for separation prior to detection.

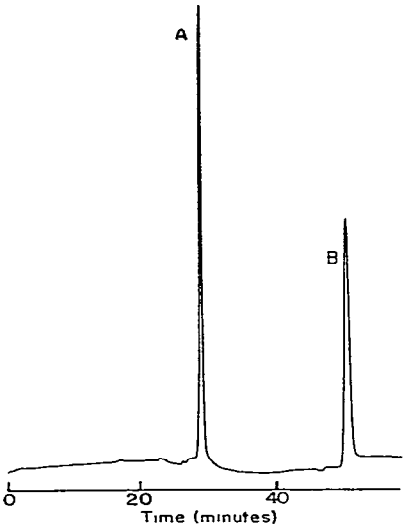

Fig. 3. Chromatogram obtained with electroosmotic flow. Peaks: A = 9-methylanthracene; B = perylene.

Results and discussion

With acetonitrile as the mobile phase, and with 30 kV applied across the column, a current of *ca.* 50 nA was observed. Fig. 3 shows a chromatogram of 9-methylanthracene and perylene obtained in this system. The efficiency for 9-methylanthracene is 31,000 theoretical plates while for perylene it is 23,000 theoretical plates. These efficiences are relatively good although retention times are long. The values of the HETP for the two peaks are 19 μm and 25 μm, respectively. This leads to values for the reduced plate heights of 1.9 and 2.5, respectively. In view of the crude methods and low pressure used in packing this column, these plate heights are quite good[13]. Current theories of column packing would predict that this column is poorly packed, and should give mediocre performance. However, if the flow is uniform and independent of packing irregularities, as predicted by the theory of electroosmotic flow, then these good results are to be expected.

Our experience in using electroosmotic flow in a chromatographic system has been that the method is a bit difficult and inconvenient to work with. Certainly part of this is due to the novelty of this approach to chromatography. However, electroosmosis lacks the direct and simple control over mobile phase flow that we are accustomed to with conventional pumping systems. The performance of these columns appears to offer a modest improvement over conventional flow, but may not justify the increased difficulty in working with electroosmotic flow.

The possibility that packing irregularities cause little peak broadening is interesting. One might infer from this that a narrow particle size distribution is also not essential to good performance when electroosmotic flow is used. An ideal application of electroosmotic flow may be in large diameter preparative-scale columns. The low power dissipation of 1.5 mW for our column indicates that columns of several centimeters inside diameter could be operated without overheating problems. The great expense of high-performance small-particle packing virtually precludes their use in

columns of large volume. Also, application of the necessary high pressure to large diameter tubes is potentially dangerous, although not an insurmountable problem. Furthermore, uniform packing of a large diameter column may prove difficult. If small-particle packings with a broader size distribution were used, the expense of the packing would drop dramatically, and if packing irregularities are less significant, column packing would be less of a problem. It appears that preparative-scale separations may be the area of most potential impact for electroosmotic flow in chromatography.

ACKNOWLEDGEMENT

Support for this work was provided by the Petroleum Research Fund of the American Chemical Society and the University Research Council of the University of North Carolina.

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