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CAPILLARY HYDRODYNAMIC CHROMATOGRAPHY

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SUMMARY

Liquid chromatographic separation in capillary tubing of particles ranging from 0.5 to 30 μm is reported. Elution volumes are inversely related to particle diameter for materials of diverse composition such as latex, pollen, bacterial spores, silica, and whole cells. Relative elution times of particles are dependent on column diameter and both the velocity and viscosity of the mobile phase. Viscosity appears to affect the relative retention of small particles more than particles over 1 μm in diameter. Addition of ethylene glycol to aqueous mobile phases diminishes peak trailing with latex particles. The practical limit on column length is approximately 300 ft.

INTRODUCTION

Rapid determination of particle size is often difficult in the case of organic polymers. Intuitively one would speculate that some type of sieving or permeation chromatographic system should be capable of resolving particles by size. The concept of differential permeation of solutes into porous supports is well understood and extensively used in the resolution of molecules with molecular weights below several million daltons[1]. Introduction of controlled porosity macroporous glass[2,3] and silica supports in the past decade have indeed resulted in the rapid size separation of viruses and latex particles. Fractionation of particles up to 500 Å in diameter requires large pore diameter supports and therefore rather large support particles (37–128 μm). Although macroparticulate supports are necessary, they have very poor mass transfer characteristics in the separation of particles. The combination of deep pools of stagnant mobile phase in the pores of the support and the very small diffusion coefficients of particles requires low mobile phase velocities to approach solute equilibrium between mobile and stationary phases.

A different type of particle resolution became possible when Small[4] discovered hydrodynamic chromatography. He reported that the rate of colloid transport in an aqueous suspension through a bed of impermeable spherical particles was a function of the size of colloidal particles, support particle size, and ionic strength of the mobile phase. Particles eluted by size with large particles eluting first. Resolution of particles from hundreds to thousands of angstroms in size was achieved. Small concluded that the forces causing the separation were derived exclusively from factors operating in the interstitial volume of the packed column. Resolution was attributed to the partitioning of particles between the mobile phase and a stagnant layer of liquid at the surface of packing materials. The depth of particle penetration into this thin layer of stagnant liquid would depend on the size of the particle. Large particles would spend less time in this stationary phase than small particles and would consequently elute from columns first.

This paper examines a third type of chromatographic separation of particles in which resolution is achieved in capillary columns. Forces necessary for resolution in capillary columns are also generated entirely by the movement of mobile phase through the interstitial volume of the column; however, the separation mechanism appears to be more complex than that reported by Small[4]. Operation of capillary hydrodynamic systems and factors that control resolution are described below.

MATERIALS

Colloids

All polystyrene (latex) particles and pollens were obtained from Polyscience (Warrington, Pa., U.S.A.). LiChrospher was purchased from EM Labs. (Elmsford, N.Y., U.S.A.). Organic reagents were supplied by Aldrich (Milwaukee, Wisc., U.S.A.).

Equipment

Chromatographic apparatus consisted of a Model 314 syringe pump fitted with a UA-5 absorbance monitor from Instrument Specialties Company (Lincoln, Nebr., U.S.A.) and a Micromeritics Model 7000 liquid chromatograph. Capillary tubing was obtained from Handy and Harmon Tube Company (Norristown, Pa., U.S.A.). The Isco liquid chromatograph was fitted with a Model 706 injection valve from Disc Instruments (Monrovia, Calif., U.S.A.).

RESULTS

When a mixture of 1- and 10-μm latex particles was chromatographed in a 200 ft. $\times$ 0.015 in. I.D. capillary column, the chromatogram shown in Fig. 1 was obtained. Samples collected at points 1, 2, and 3 along the chromatogram were subjected to microscopic examination and yielded the micrographs in Figs. 2–4, respectively. It will be seen that samples 2 and 3 were essentially homogeneous preparations with regard to size while the sample collected in the valley between the two peaks was a mixture. It may be concluded from these data that resolution of microparticulate material is possible in a capillary liquid chromatography system.

Resolution of several different-sized particles and a dipeptide, glycyl-L-tyrosine, is seen in Fig. 5. The first material to elute from the column was 25.7-μm latex

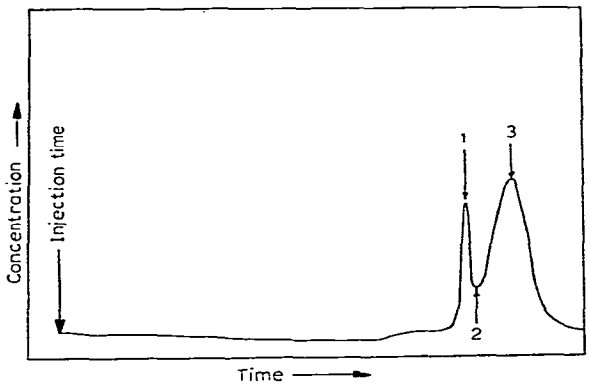
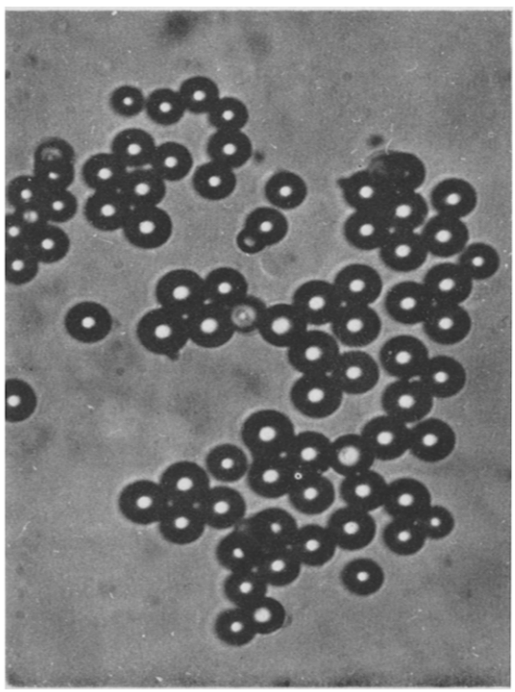


Fig. 1. Separation of latex particles with fractions collected at the indicated times. The peak at 1 is composed of 10-μm particles and that at 3 of 1-μm particles. Mobile phase, methanol; column, 200 ft. × 0.015 in. I.D.



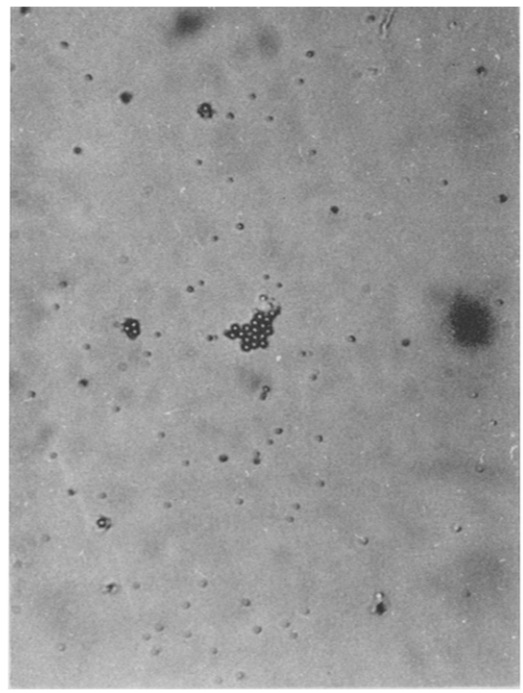
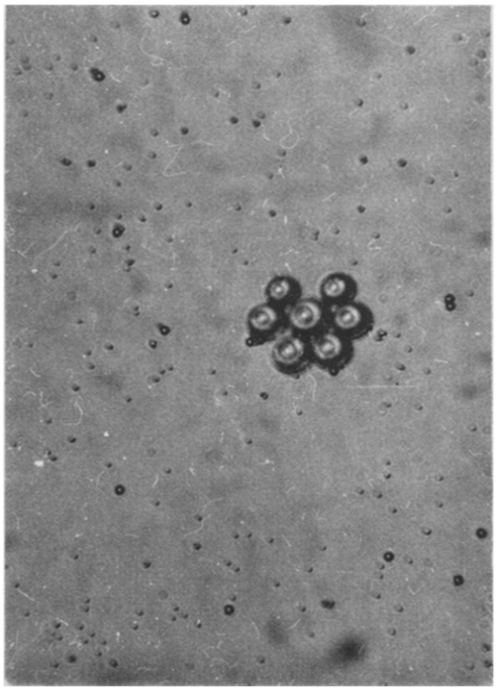


Fig. 2. Electron micrograph of fraction collected at 1 in Fig. 1.

Fig. 3. Electron micrograph of fraction collected at 3 in Fig. 1.

particles (A) followed by 10-μm silica (B) and glycyl-L-tyrosine (C) in declining order of size. After examining many different types of polymeric material it was found that all species under 10^4 daltons co-eluted at an elution volume designated as V_m. In no case was a material found that had an elution volume greater than V_m. The relative elution position of a substance was described as V_e/V_m, where V_e is the elution volume



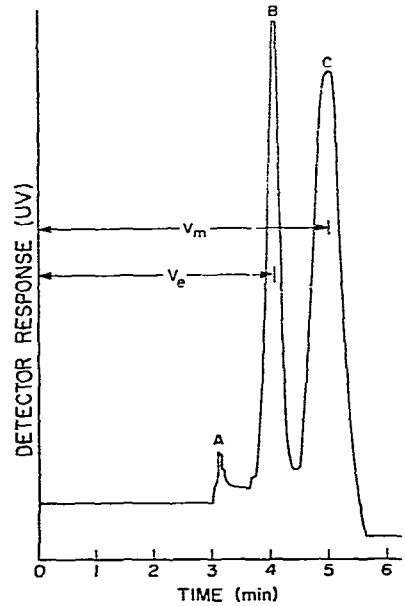


Fig. 4. Electron micrograph of fraction collected at 2 in Fig. 1.

Fig. 5. Separation of 25.7-μm and 10-μm latex particles from glycyl-L-tyrosine. Column, 300 ft. × 0.015 in. I.D.; mobile phase, 1% ethylene glycol in water; flow-rate, 125 ml/h. A = 25.7-μm latex particles; B = 10-μm silica particles; C = glycyl-L-tyrosine.

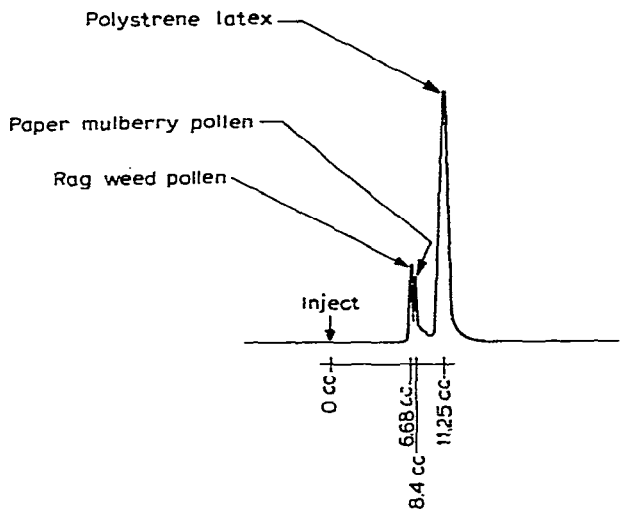


Fig. 6. Separation of ragweed pollen (19–20 μm), paper mulberry pollen (11–12 μm) and polystyrene latex (0.866 μm). Column, 100 m × 0.381 mm I.D.; mobile phase, methanol; flow-rate, 1 ml/min; pressure, 230 p.s.i.

of the material and V_m is the elution volume of a small marker as described above. Resolution of several pollen specimens and polystyrene particles is shown in Fig. 6.

Several problems were encountered in the initial separation of latex particles. When water was used as the mobile phase, particle aggregation and chromatographic peak trailing was observed. Addition of polyethylene glycol to the mobile phase decreased trailing but caused a shift in the V_e/V_m ratio of some particles. Both 0.1% albumin and 1% ethylene glycol were superior to polyethylene glycol in controlling aggregation and trailing without changing the V_e/V_m ratio. Owing to the ready availability of ethylene glycol, it was used in all further experiments for the control of surface adsorption.

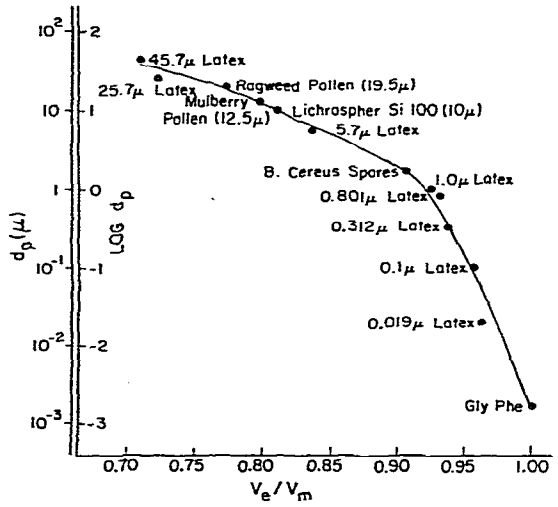
It is implied in Fig. 5 that there is a relationship between the elution volume of a particle and its size. A plot of log d_p *versus* V_e/V_m is shown in Fig. 7, where d_p is the diameter of a particle and V_e/V_m is as described above. From this calibration curve it is seen that there is a definite relationship between the hydrodynamic volume of a particle and its elution volume. Maximum resolution on a 0.02 in. I.D. capillary is in the range of 1–25 μm particles. Columns ranging in diameter down to 0.010 in. I.D. extended the range to below 1 μm in size. Although the resolution of particles less than 0.5 μm in diameter is very poor, their elution volume is still indicative of their size. The effect of column diameter on V_e/V_m ratio is seen in Fig. 8. The major difference is seen in the separation of latex particles less than 1 μm in diameter.

The effect of flow-rate (F_r) on V_e/V_m for a 10-μm silica particle is shown in Fig. 9. The variation in V_e/V_m with F_r makes it apparent that when one is determining the size of an unknown particle, the particle diameter calibration curve used to determine particle size must be made at the same velocity. It should also be noted in Fig. 9 that there is a minimum flow-rate below which particles will no longer focus. Apparently the generation of separation forces sufficient to resolve particles does not occur until mobile phase velocities of approximately 0.33 ft./sec are used.

Plate heights (H) are quite large for capillary hydrodynamic chromatography compared to those obtained in high-performance liquid chromatography (HPLC). When water is used as the mobile phase, the plate height of capillary columns was approximately 100 mm compared to 0.02 mm in HPLC columns. Fig. 10 shows the relationship between H and F_r for 10-μm silica particles and a dissolved solute. It will be noted that an increase in mobile phase velocity decreased band spreading of 10-μm particles but increased H for low-molecular-weight species. The opposite effects of velocity on band spreading with large and small particles suggest that they are being separated by different mechanisms. Fig. 10 again indicates that there is a minimum velocity below which the resolution of particles decays rapidly. Presumably this is because low mobile phase velocities do not generate forces sufficient to separate particles.

It was observed that approximately three times more theoretical plates were generated when methanol was substituted for water as the mobile phase. A 650 ft. × 0.01 in. I.D. capillary produced 5921 plates for 10-μm latex particles with a mobile phase velocity of 2.29 ml/min.

Since pressure drops in the open tubular columns used were less than 4 p.s.i./ft., it was reasoned that large numbers of theoretical plates could be generated by using 1000-ft. columns. Using a 1% aqueous ethylene glycol mobile phase and latex particles, a coupled 500 ft. × 0.020 in. I.D. and 500 ft. × 0.015 in. I.D. column



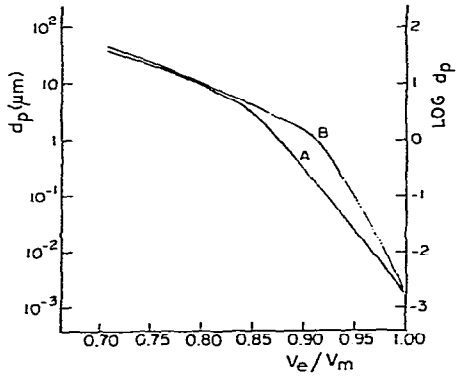
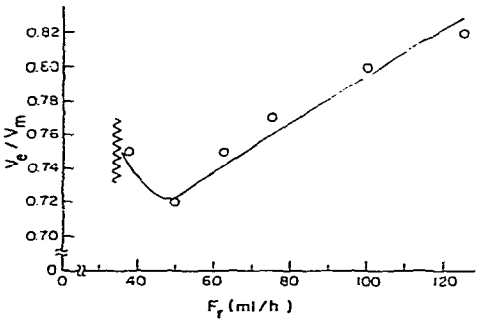


Fig. 7. Calibration curve for a column of 500 ft. × 0.02 in. I.D. Mobile phase, 1% ethylene glycol in water; flow-rate, 125 ml/h.

Fig. 8. The effect of column diameter on a calibration curve. Column A is 300 ft. × 0.015 in. I.D.; Column B is 500 ft. × 0.02 in. I.D. Mobile phase, 1% ethylene glycol in water.



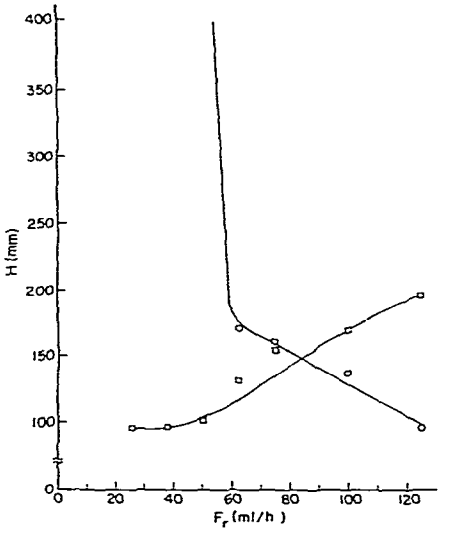
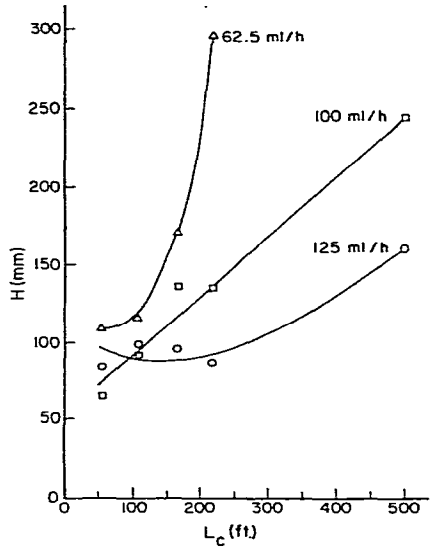


Fig. 9. The effect of flow-rate on V_e/V_m for a 10-μm silica particle. Column, 300 ft. × 0.015 in. I.D. packed with 10-μm LiChrospher; mobile phase, 1% ethylene glycol in water.

Fig. 10. The effect of flow-rate on plate height for a 10-μm silica particle (○) and glycyl-L-tyrosine (□). Column, 165 ft. × 0.02 in. I.D.; mobile phase, 1% ethylene glycol in water.

system was examined. The resolution of this system and the recovery of particles was much lower than expected. With particles over 1 μm in size it was often difficult to even observe a discernable chromatographic peak. A study of plate height *versus* column length shown in Fig. 11 indicates that plate height increases with both increased column length and decreased mobile phase velocity. The most probable explanation for this phenomenon is increased particle interaction with the column



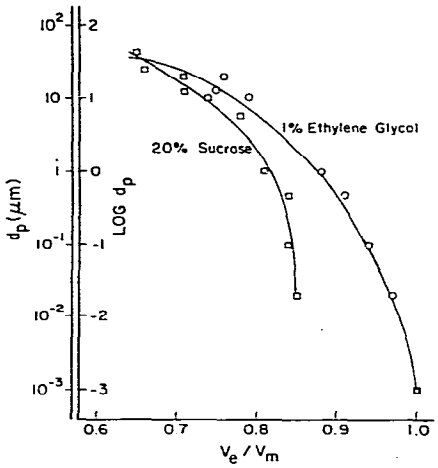
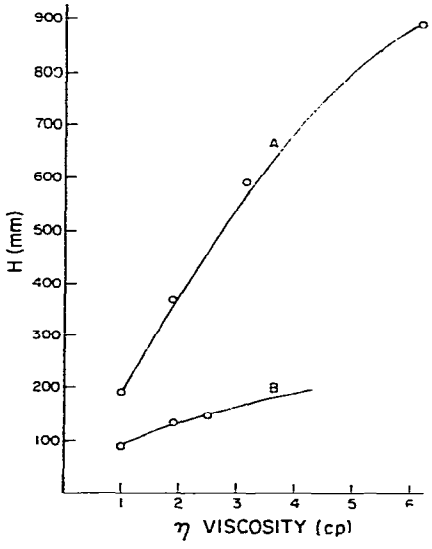


Fig. 11. The effect of column length on plate height at different flow-rates. Column, 0.02 in. I.D.; mobile phase, 1% ethylene glycol in water; solute, 10-μm silica.

Fig. 12. Calibration curves in mobile phases of different viscosities. Column, 165 ft. × 0.02 in. I.D.; flow-rate, 112 ml/h.

walls as the surface area is increased and the separation forces are decreased. The differences observed in Fig. 11 with latex particles again show the strong influence of mobile phase velocity in capillary hydrodynamic chromatography.



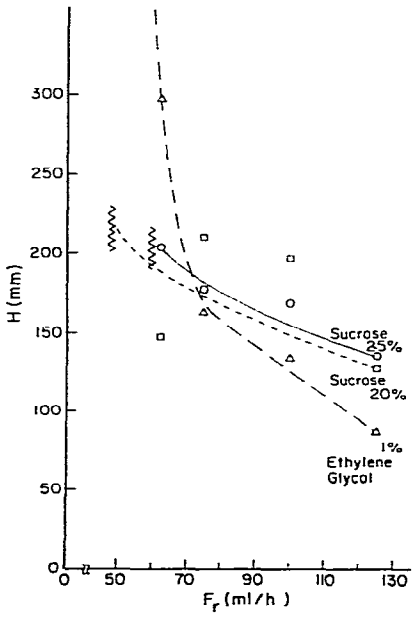


Fig. 13. The effect of viscosity on plate height for 10-μm silica (A) and glycyl-L-tyrosine (B). Column, 220 ft. × 0.02 in. I.D.; mobile phase, sucrose in water; flow-rate, 125 ml/h.

Fig. 14. The effect of flow-rate on plate height at different viscosities. Column, 220 ft. × 0.02 in. I.D.; solute, 10-μm silica.

Mobile phase viscosity also plays an important role in the separation of particles by open tubular hydrodynamic chromatography. A mobile phase with high viscosity displaces the particle size calibration curve as seen in Fig. 12. It will be noted that the V_e/V_m ratio of small particles is substantially displaced while the elution volumes of large particles are almost the same. These data suggest that particles smaller than 1 μm are separated by a different mechanism than those which are larger. The separation mechanism for small particles is viscosity dependent while that for large particles is viscosity independent.

Plate height is also affected by viscosity as seen in Fig. 13. Higher mobile phase velocities are needed to separate particles in viscous liquids. Column efficiency again increases with increasing mobile phase velocity as seen in Fig. 14. Fig. 13 also illustrates the deleterious effects of high-viscosity solvents on the column efficiencies of a small dissolved solute in the same system.

DISCUSSION

Transport of an aqueous suspension of particles through tubes has been examined by a number of investigators[5–15]. They have generally observed that when rigid spheres are transported in a Poiseuille flow through a straight, cylindrical tube they undergo radial displacement and move along trajectories which asymptotically approach straight lines parallel to the tube axis at a fixed distance between the latter and the wall. Perhaps the first observation of the segregation of particles in a capillary was when Poiseuille[5] noticed that there were areas free of red cells near the walls of capillaries in the circulatory system. Taylor[6] later confirmed this phenomenon and also observed a particle free region at the center of tubes in which particles are being transported by flowing liquid.

A detailed experimental analysis of particle segregation in laminar flow systems was undertaken by Segré and Silberberg[7–9] in the early 1960s. As a result of these studies they proposed the term "tubular pinch" to describe this focusing of particles into an annular ring. They showed that a neutrally bouyant sphere being transported through a tube by Poiseuille flow is subject to radial forces that carry it to an equilibrium position at approximately 0.6 tube radii from the tube. The origin of the force causing this radial displacement or focusing is in the inertia of the moving liquid.

Examination of literature concerning the "tubular pinch effect" suggests that the radial equilibrium position of a particle in Poiseuille flow is a function of both mobile phase velocity and the ratio a/R, where a is particle radius and R is the tube radius[15]. By combining the data of several other groups, Walz and Grün[15] were able to show that particles assume equilibrium positions nearer to the tube wall when the liquid velocity is increased in a tube. This paper also indicates that radial position varies inversely with particle radius.

It should be noted here that the particles and tube radii used in the studies reported in this paper are much smaller than those used in the "tubular pinch effect" studies. As a consequence, it cannot be assumed that all of the data derived from these macrosystems apply to the separation of microparticulates in capillaries. However, any particle that is radially segregated in a capillary should have an elution behavior that is predicted by the equations for laminar flow.

The velocity of a radially segregated particle will be the same as the liquid

velocity at that position (Fig. 15). This velocity may be obtained from the equation

$$v_z = \frac{(P_0 - P)\, R^2}{4\eta L}\left[1 - \left(\frac{r}{R}\right)^2\right], \tag{1}$$

where v_z is the liquid velocity at a given radial position, $P_0 - P$ is the pressure drop across the column, R is the tube radius, r is the radial position, η represents liquid

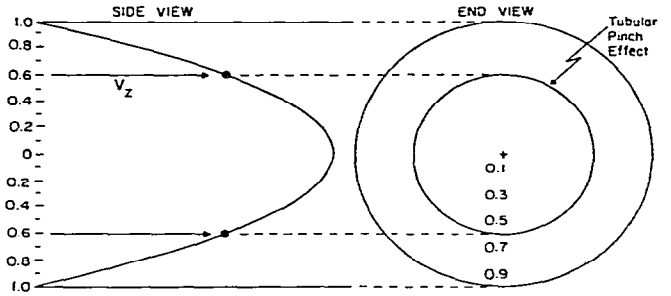


Fig. 15. Profile of laminar flow and the annular rings produced.

viscosity, and L is tube length. The experimentally observed relationship between particle diameter and velocity in a capillary may be seen in Fig. 16. From this figure the equation

$$\log d_p = 0.0669\, t + C = 0.0669\, \frac{L}{v} + C \tag{2}$$

where d_p is particle diameter, t is time, and v is particle velocity (may be derived). The velocity terms in eqns. 1 and 2 are equal and the two equations may be combined to show a relationship between particle diameter and radial position:

$$\frac{r}{R} = \left[1 - \frac{0.267\, \eta L^2}{(P_0 - P)\, R^2\, (9.64 - \log d_p)}\right]^{1/2} \tag{3}$$

Eqn. 3 predicts that small particles are also located at a radial position between 0.6 and 0.7 tube radii and that large particles focus in an annulus near the tube axis as indicated in Fig. 15. These theoretical values agree very well with those experimentally determined by Segré and Silberberg[9] in their macroparticulate studies. Although there is insufficient evidence to conclude that particle resolution in capillary hydrodynamic chromatography is the result of the "tubular pinch effect", it is at least implied.

Walz and Grün[15] noted in "tubular pinch" systems that the annular ring size for a given size of particles increased with fluid velocity. If it is assumed that the "tubular pinch" model applies to capillaries, then the variation in V_e/V_m at increased velocities noted in Fig. 9 would result from an expansion in the annular ring and be identical to the results of Walz and Grün cited above. When the mobile phase velocity decreases to a certain minimum level, there is apparently insufficient force to focus particles into an annular ring and the chromatographic peaks disappear. The existence of a minimum focusing velocity is further substantiated in Fig. 10.

Fig. 10 also shows that the mechanism for band spreading with dissolved molecules and particles is different. Interestingly the relationship between band spreading and mobile phase velocity is reversed for the two species. Since the mech-

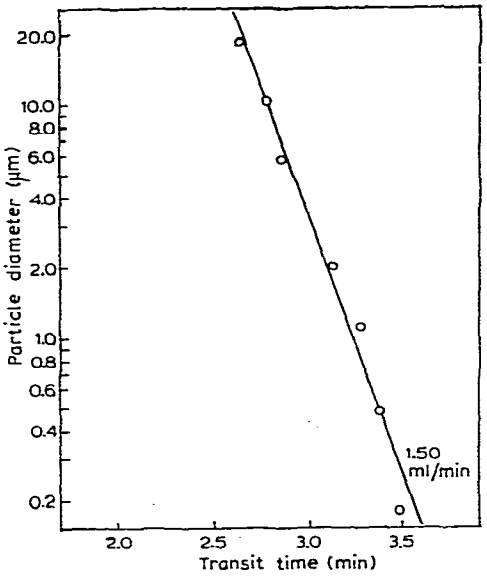


Fig. 16. The effect of particle diameter on transit time. Column, 200 m × 0.381 mm I.D.; mobile phase, methanol; flow-rate 1.5 ml/min.

anism for band spreading is different, the separation mechanism may also be different. Additional indications of a multiple mechanism separation were found in Figs. 7 and 8. A careful examination of both figures shows a slight break in the curve at a particle diameter of approximately 1 μm. Combination of these data with the pronounced effect of viscosity changes on particles less than 1 μm in diameter (Fig. 12) strongly suggests that particles of roughly 1 μm or greater are separating by a different mechanism than those under 1 μm.

We would speculate that the resolution of large particles is occurring by the "tubular pinch effect" while small particles are being retained by a stagnant layer of liquid at the tube wall. The "tubular pinch" separation is new and unique to capillaries while the wall effect is the same as the model described by Small[4] for the separation of particles in a packed bed.

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