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A STUDY OF MICRO-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. DEVELOPMENT OF TECHNIQUE FOR MINIATURIZATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

D. ISHII, K. ASAI, K. HIBI, T. JONOKUCHI and M. NAGAYA

Department of Applied Chemistry, Faculty of Engineering, Nagoya University, Chikusa-ku, Nagoya-shi (Japan)

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SUMMARY

Several technical problems such as the following have been solved in order to perform micro-high-performance liquid chromatography (MHPLC): (1) a method for packing a narrow column with stationary phase and selection of suitable column materials; (2) preparation of micro-flow cells suitable for a micro-column; (3) improvement of the detector system; (4) methods for the pressurized passage of mobile phase at low flow-rates and for injection of a micro-amount of sample solution; and (5) gradient elution methods suitable for micro-columns.

The theory of the spreading of sample components in narrow tubes for column connections is considered and examples of experiments using MHPLC are presented.

INTRODUCTION

The columns for most commercial high-performance liquid chromatographic (HPLC) instruments are too large to be used for analytical purposes alone, and many advantages should result from a reduction of the column cross-section. The relationship between column diameter and column efficiency was investigated by Wolf[1], who found that as the column diameter was reduced the column efficiency also decreased, and the practical minimum column diameter was about 2 mm. These phenomena have been sometimes said to result from "wall effects". However, we consider the phenomena to be the effects resulting from a relative increase in the extra-column dead volume, and that miniaturization of HPLC would be feasible by decreasing the extra-column dead volume.

EXPERIMENTAL

Preparation of micro-columns

PTFE tubing of 0.5 mm I.D. and 1.0 mm O.D. was selected as the main micro-

column material, as it is readily available commercially, chemically stable and easy to machine. This tubing has a cross-section about one-fifteenth of the *ca.* 2 mm I.D. tubing that is widely used in ordinary high-performance liquid chromatographs. Investigations were carried out mainly with columns 5–30 cm long.

The slurry packing technique was used for packing the stationary phase. A tube several times longer than that finally required was first prepared and the stationary phase was suspended in a suitable solvent as a slurry, which was placed in a small bottle. An air-tight syringe (*ca.* 250 μl) was connected with the tube and they were filled with the solvent that was used to prepare the slurry. Then the lower end of the tube was dipped into the slurry, the syringe was attached to a micro-feeder and the slurry was sucked up to the upper end of the tube by manual or electrical operation of the feeder. The lower end of the tube was plugged tightly with a small amount of quartz-wool (less than *ca.* 2 mm thick) so as to prevent the packing material from leaking out, and the micro-feeder was operated manually or electrically, thereby discharging the solvent. In this way, a dense packing of the stationary phase was achieved. This method easily gave a good packing ratio of 60:40 (particles:vacancies).

The condition of packing during the delivery of the solvent under pressure could be observed through the semi-transparent PTFE tube. If a loose packing condition was observed, the syringe was detached from the tube, filled with the solvent and re-connected with the tube to feed the solvent again under pressure.

The column thus packed was cut to the required length and a small plug of quartz-wool was placed on the packing at the upper part of the tube, leaving a space 1–2 cm long for connection of the mobile phase delivery tube.

The method described can be used to prepare any micro-column with a volume 1–2% of that of columns used in ordinary HPLC. The same method is applicable to packing a 0.25 mm I.D. tube with stationary phase.

It has often been stated that if the column diameter is decreased below *ca.* 2 mm, the HETP would increase, and this effect has been described as a "wall effect". However, the decreased "wall effect" for the PTFE tubing may be considered to result from the fact that as the PTFE tubing has a softer wall surface than that of stainless steel tubing, the stationary phase under pressure adheres to the wall surface with a resultant increase in surface coverage.

Preparation of micro-flow cell and improvement of spectrophotometric detector

An investigation was made of the necessary structure of micro-flow cells for UV absorption that are suitable for use with such micro-columns. In the initial stage, two parallel quartz plates and a PTFE spacer were used to form a Z-type cell, which was found to be unsuitable. In the method currently being used a quartz tube 0.3–1 mm I.D. is connected directly with the micro-column, as shown in Fig. 1, and the dead space at the joint is made as small as possible. This method permits the simple preparation of flow cells of various sizes by using quartz tubes of different inner diameters. The volume of such a micro-flow cell is about 0.1–0.3 μl.

A photometer or a UV/visible spectrophotometer (UVIDEC-1 or UVIDEC-100, from Japan Spectroscop. Co., Tokyo, Japan) can be used as the detector. The path length of a micro-flow cell is relatively short, which results in a low sensitivity, but this can be increased by using a light source with a higher output or a photomultiplier with a higher output and sensitivity. A high-sensitivity UV detector (SPD-1, from Shimadzu

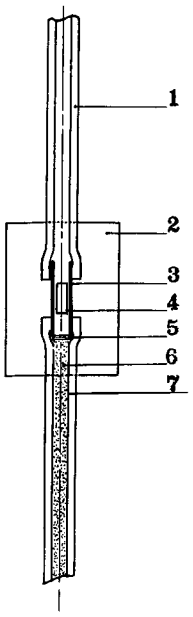


Fig. 1. Micro-flow cell. 1 = Outlet (PTFE tube); 2 = focused plate; 3 = micro-flow cell (quartz tube); 4 = optical window; 5 = quartz-wool; 6 = stationary phase; 7 = separation column.

Seisakusho, Kyoto, Japan) for HPLC is capable of giving good results even when connected directly with a micro-flow cell.

Methods for pressurized passage of mobile phase at low flow-rate and for injecting a micro-amount of sample solution

If the same linear flow-rate as that used in ordinary HPLC is used in micro-high-performance liquid chromatography (MHPLC), similar retention times are obtained. Thus, a flow-rate of *ca.* 10 μl/min is adequate with a 0.5 mm I.D. column in MHPLC. If one run of a chromatographic experiment is completed within 20 min with such a flow-rate, a total volume of about 200 μl of mobile phase will be required, which can be accommodated in a small syringe. As the diameter of the syringe plunger is reduced, less pushing power is required and the accuracy of the flow-rate is increased. An air-tight syringe of volume 50–250 μl was used to feed the mobile phase, fitted with a commercial micro-feeder that consisted of a small synchronous motor, gears and screws in order to push the plunger at a constant rate. Variable flow-rates could be obtained with this micro-feeder and these syringes. This system could also be used to charge the packing materials, as described under *Preparation of micro-columns*. The cross-section of the plunger of a 250-μl syringe is about 4 mm^2, so that, even if a pressure of 100 kg/cm^2 is required to feed the mobile phase, a plunger force of only 4 kg is needed. This estimate indicates that the pump for the pressurized feeding of mobile phase in MHPLC requires a very small force compared with that needed with large-bore columns for fractionation.

The sample volume to be injected must be reduced in proportion to the column size, and can be expected to be about 0.05–0.5 μl in MHPLC. The practical applicability of MHPLC depends on the exact injection of such a small volume of sample into

the column inlet without being spread or disturbed. In the initial stage of development, injection using a micro-syringe was tried. However, when the syringe needle was withdrawn after a sample injection the injected sample solution was spread and disturbed, with unfavourable effects, as the diameter of the micro-syringe needle was not negligible in comparison with the inner diameter of 0.5 mm of the micro-column. In MHPLC, the dead volume must be as small as possible, so that an on-column system for sample injection is preferable.

We therefore employed a new sampling method, in which the air tight syringe used for pressurized feeding of the mobiele phase, was available and this technique was successful. The method adopted is illustrated in Fig. 2. First, the mobile phase is sucked into the stainless-steel tube (*ca.* 0.3 mm I.D.) by operating the micro-feeder manually, its lower end is then dipped into the sample solution and the micro-feeder (equipped with a magnified micrometer) is slightly rotated manually so as to place a micro-amount of the sample solution into the tube. The tip of the stainless-steel tube is immediately dipped into a vessel containing mobile phase in order to wash the outside wall of the tube, and then a microamount of mobile phase is sucked in. Hence a sandwich sampling operation is applied (this sequence of washing and sucking the mobile phase can be omitted, when the sample solution is relatively diluted and the contamination from the outside wall of stainless tube gives no undesirable influences to chromatographic operation.) The stainlesssteel tube is then connected to the end of the micro-column and finally the microfeeder is electrically operated to effect a chromatographic elution.

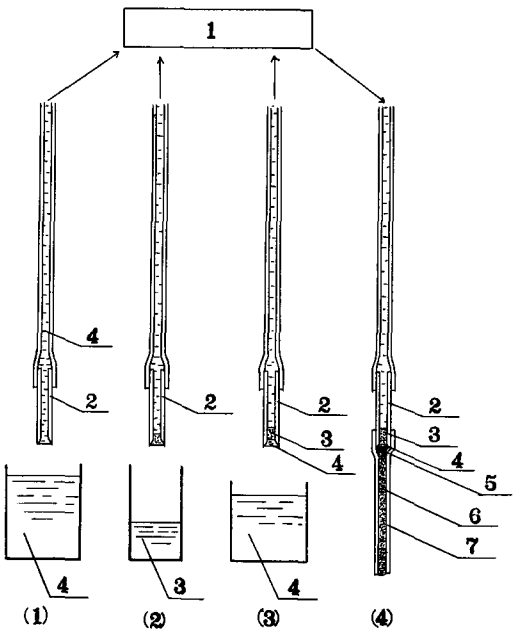


Fig. 2. Sample introduction. 1 = Micro-feeder; 2 = connection tube; 3 = sample solution; 4 = mobile phase; 5 = quartz-wool; 6 = packing material; 7 = micro-column. Operations: (1) suck up carrier liquid; (2) suck up sample solution; (3) suck up mobile phase; (4) connection.

Gradient elution

In HPLC, the gradient elution method is very useful as it allows the composition of the mobile phase to be varied continuously, and various gradient elution systems are commercially available. The gradient elution system is also suitable for MHPLC. Unlike ordinary HPLC, a total amount of only *ca.* 200 μl of mobile phase is required for one gradient elution chromatographic run in MHPLC. A continuous supply of such an amount of gradient mobile phase, although apparently difficult, can actually be achieved very easily, as follows. The gradient solution is sucked from a mixing vessel and stored in a fine tube (0.5 mm I.D. and *ca.* 1 m long), which is connected with a micro-column and, finally, the gradient solution is dispensed under pressure. The scheme of this method is shown in Fig. 3.

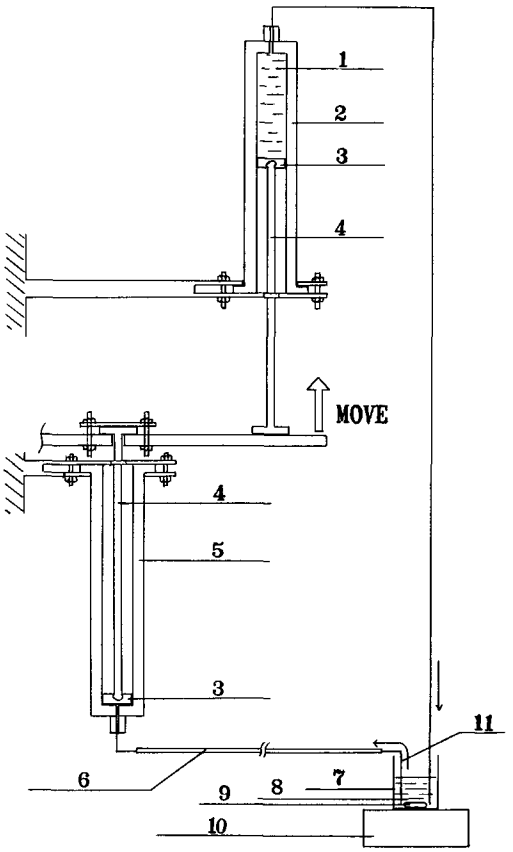


Fig. 3. System for preparation of gradient solution. 1 = Adding liquid; 2 = gas-tight micro-syringe; 3 = PTFE; 4 = syringe plunger; 5 = gas-tight micro-syringe; 6 = stock tube for gradient solution; 7 = mixing vessel; 8 = mixture; 9 = magnet; 10 = magnetic stirrer; 11 = connection tube.

RESULTS AND DISCUSSION

Examples of experiments using micro-high-performance liquid chromatography

In MHPLC, good baseline stability was obtained over a wide range of wavelengths and good resolution was achieved, as shown in Fig. 4. Fig. 5 shows the effect

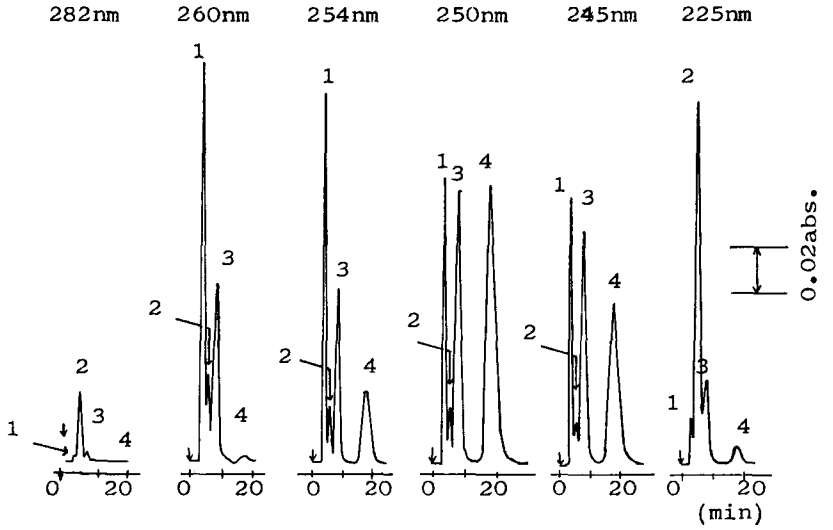


Fig. 4. Difference of peak response according to wavelength. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample: mixture of 2% of benzene, 0.02% of naphthalene, 0.02% of biphenyl and 0.004% of anthracene in methanol. Sample size: 0.05 μl. Column temperature: 15°. Column: 15 cm × 0.5 mm I.D. PTFE tube packed with Permaphase ODS (30 μm). Mobile phase: 60% methanol in water. Flow-rate: 8 μl/min.

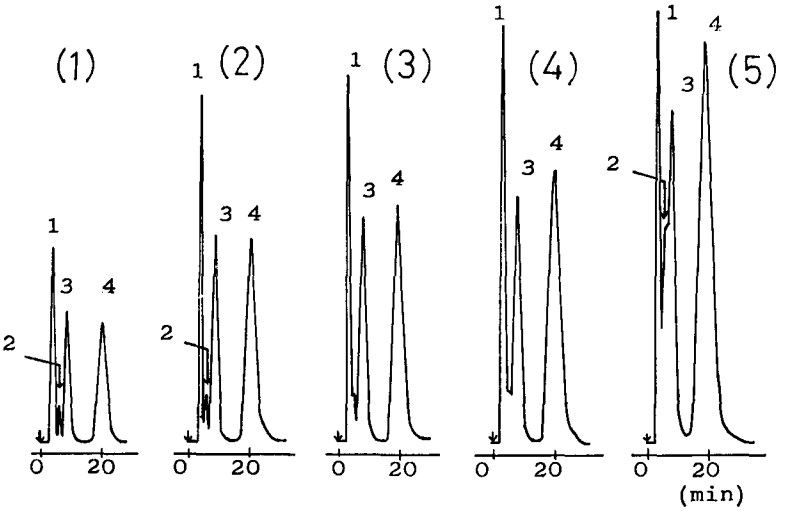


Fig. 5. Effect of sample loading on column efficiency. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample size: (1) 0.04 μl; (2) 0.08 μl; (3) 0.12 μl; (4) 0.17 μl; (5) 0.33 μl. Sample: mixture of 2% of benzene, 0.02% of naphthalene, 0.02% of biphenyl and 0.004% of anthracene in methanol. Column: 15 cm × 0.5 mm I.D. PTFE tube packed with Permaphase ODS (30 μm). Column temperature: 15°. Mobile phase: 60% methanol in water. Flow-rate: 8 μl/min. Wavelength: 250 nm.

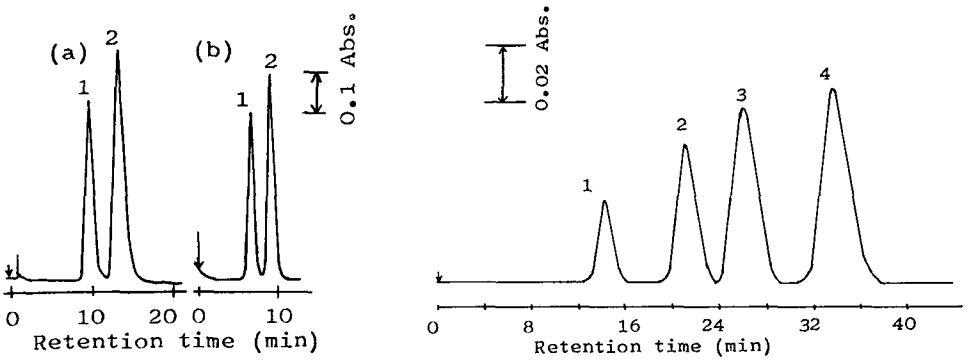


Fig 6 Typical separation on porous silica phase. Peaks: 1 = dioctyl phthalate; 2 = dibutyl phthalate. Sample size: 0.5 μl. Sample: mixture of 0.5% of dioctyl phthalate and 0.5% of dibutyl phthalate in *n*-hexane. Column: PTFE tube (8 cm × 0.5 mm I.D.) packed with silica beads (5 μm). Mobile phase: water-saturated dichloromethane. Flow-rate: (a) 3.2 μl/min; (b) 4.6 μl/min. Column temperature: ambient.

Fig. 7. Separation of aromatic hydrocarbons on Zorbax ODS. Peaks: 1 = naphthalene; 2 = biphenyl; 3 = fluorene; 4 = anthracene. Sample: mixture of 0.033% of naphthalene, 0.017% of biphenyl, 0.033% of fluorene and 0.0033% of anthracene in methanol. Sample size: 0.25 μl. Column temperature: 50.5°. Column: PTFE tube (12 cm × 0.5 mm I.D.) packed with Zorbax ODS. Mobile phase: 60% methanol in water. Flow-rate: 8 μl/min.

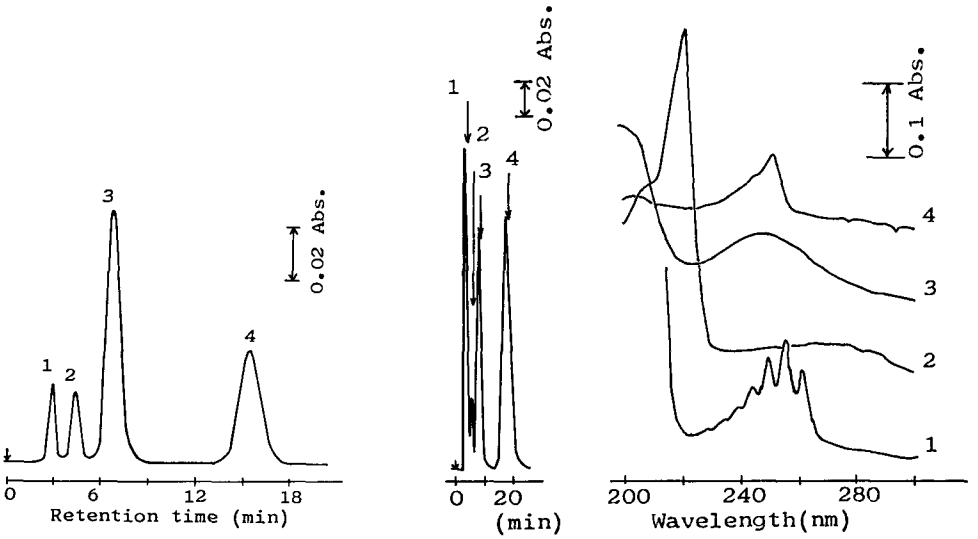


Fig. 8. Separation of aromatic hydrocarbons on a column of 0.25 mm I.D. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample: mixture of 0.5% of benzene, 0.02% of naphthalene, 0.02% of biphenyl and 0.004% of anthracene in methanol. Sample size: 0.06 μl. Column: PTFE tube (20 cm × 0.25 mm I.D.) packed with Permaphase ODS (30 μm). Mobile phase: 50% methanol in water. Flow-rate: 4 μl/min.

Fig. 9. Separation of aromatic compounds and measurement of UV spectra at the peak maximum. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample size: 0.05 μl. Sample: mixture of 2% of benzene, 0.02% of naphthalene, 0.02% of biphenyl and 0.04% of anthracene in methanol. Column: PTFE tube (15 cm × 0.5 mm I.D.) packed with Permaphase ODS. Column temperature: 15°. Mobile phase: 60% methanol in water. Flow-rate: 8 μl/min.

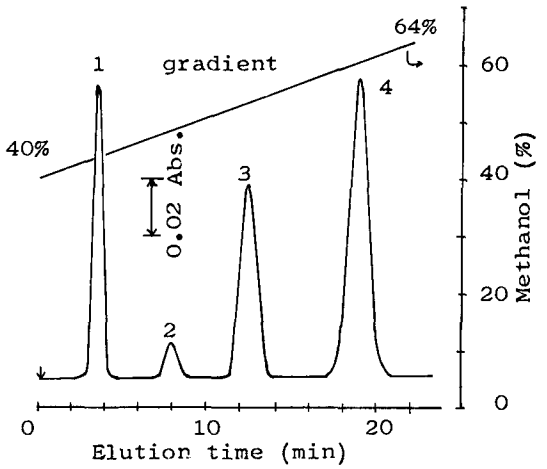


Fig. 10. Typical example of gradient elution. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample: mixture of benzene, naphthalene, biphenyl and anthracene in methanol. Sample size: 0.05 μl. Column: PTFE tube (15 cm × 0.5 mm I.D.) packed with Permaphase ODS (30 μm). Mobile phase: 40–60% methanol in water. Flow-rate: 8 μl/min.

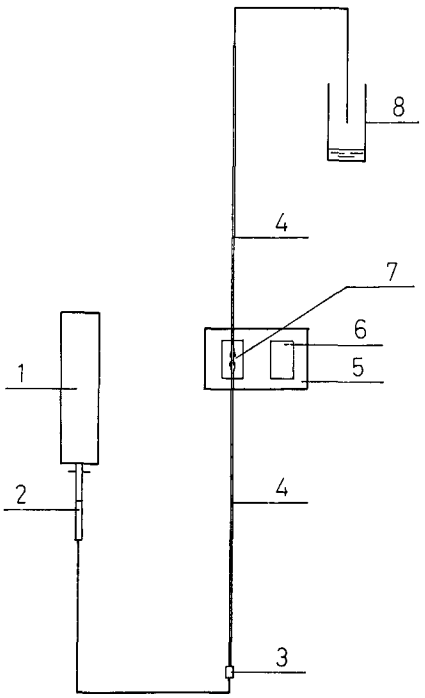


Fig. 11. Schematic diagram of apparatus for measuring the dispersion of a solute in a stream of solvent. 1 = Micro-feeder; 2 = gas-tight micro-syringe; 3 = sample inlet; 4 = PTFE tube; 5 = detector (UV spectrophotometer); 6 = reference cell; 7 = micro-flow cell; 8 = reservoir.

of sample loading on the column efficiency. It was found that the smaller was the sample size, the better was the resolution, and a sample size below 0.2 μl was preferable. Fig. 6 shows a typical separation of dioctyl and dibutyl phthalate on porous silica and Fig. 7 shows separation of aromatic hydrocarbons on a Zorbax ODS column. Fig. 8 shows a typical separation of aromatic hydrocarbons on a 0.25 mm I.D. column.

Spectra with good signal-to-noise ratios could be obtained at wavelengths from 200 to 300 nm using a micro-flow cell, and an example is shown in Fig. 9. Measurements of UV absorption spectra were made by temporarily stopping the pressurized delivery of mobile phase at a suitable position. This experiment showed that a mixture containing 1 μg of benzene, 10 ng of naphthalene, 10 ng of biphenyl and 2 ng of anthracene can be separated and that the measurement of UV absorption spectra is also possible together with component separation. Such separations, which are based on a rerversed-phase distribution provided by Permaphase ODS, give HETP values of 0.4–0.8 mm. The gradient elution method described under *Gradient elution* was applied to these mixed aromatic hydrocarbons and yielded a good separation (Fig. 10).

Dispersion of a solute in a stream of solvent through empty tubes

In a high-performance liquid chromatograph, at least two connecting tubes have generally been used, one at a point between the injection site and the column and the other at a point between the column and the detector. Even if the diameters of these tubes are small enough, the sample solute is dispersed in a stream of mobile phase through these tubes. In ordinary HPLC, the extra-column dispersion of solute in these connecting tubes may be estimated to be *ca.* 30–60 μl.

Such an extra-column dispersion in MHPLC may be less than 1 μl and it may be negligible, as "on-column" and "on-cell" systems are adopted in MHPLC. However, the peak dispersion in MHPLC is comparatively small and therefore dispersion

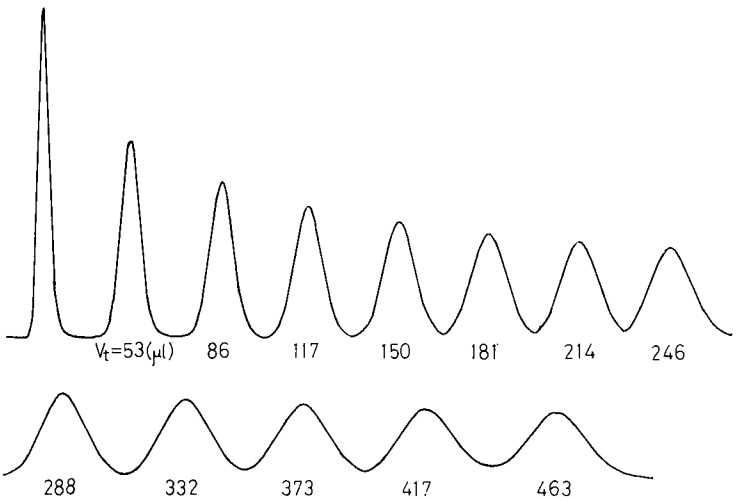


Fig. 12. Dispersion of a solute in a stream of solvent. V_t (μl) = amount of mobile phase required from injection until the peak maximum emerges. Column: 0.5 mm I.D. empty PTFE tube. Mobile phase: methanol. Flow-rate: 8 μl/min. Sample: monochlorobenzene. Sample size: 0.2 μl.

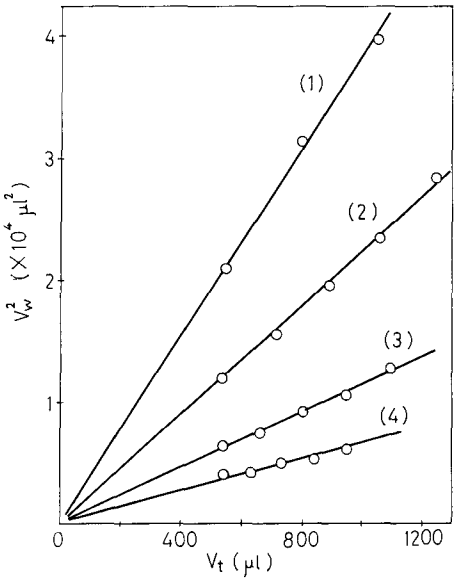
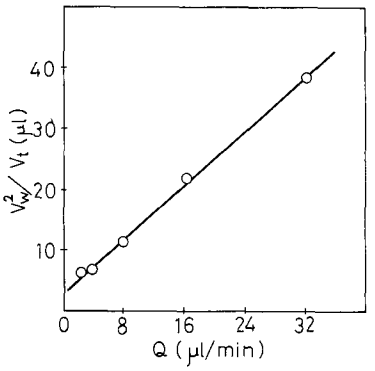


Fig. 13. Relationship between V_w^2 and V_t. V_w (μl) = dispersion of a solute in a stream of solvent; V_t (μl) = retention volume. Column: 70 cm × 1.0 mm I.D. PTFE tube. Mobile phase: methanol. Flow-rate: (1) 32 μl/min; (2) 16 μl/min; (3) 8 μl/min; (4) 4 μl/min. Sample: monochlorobenzene.

near the joints may be significant in comparison with the total peak dispersion, so this dispersion is considered below.

An apparatus for measuring this dispersion is shown in Fig. 11. Two empty tubes are connected to the front and rear of the micro-flow cell. Once the sample has been injected, the sample is detected repeatedly by changing the direction of flow of the mobile phase. The variation of the amount of mobile phase required until the peak



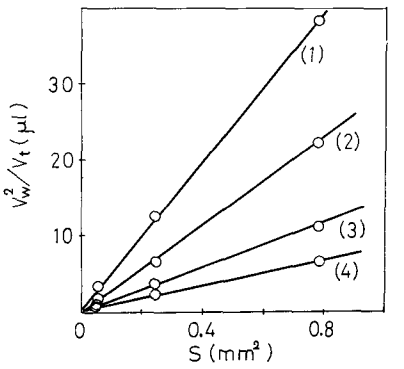


Fig. 14. Relationship between V_w^2/V_t and Q. V_w (μl) = dispersion of a solute in a stream of solvent; V_t (μl) = retention volume; Q (μl/min) = flow-rate of mobile phase. Column: 70 cm × 1.0 mm I.D. PTFE tube. Mobile phase: methanol. Sample: monochlorobenzene. Temperature: ambient.

Fig. 15. Relationship between V_w^2/V_t and S. V_w (μl) = dispersion of a solute in a stream of solvent; V_t (μl) = retention volume; S (mm^2) = cross-section of the column. Column: PTFE tube, I.D. 0.25, 0.5 and 1.0 mm. Mobile phase: methanol. Flow-rate: (1) 32 μl/min; (2) 16 μl/min; (3) 8 μl/min; (4) 4 μl/min. Temperature: ambient. Sample: monochlorobenzene.

TABLE I

DISPERSION OF A SOLUTE IN A STREAM OF SOLVENT

V_t (μl)	V_w (μl)	V_w^2/V_t (μl)
22.1	9.28	3.90
52.8	15.47	4.53
86.3	19.41	4.37
117.3	23.03	4.52
149.8	25.70	4.41
181.2	28.80	4.58
213.7	31.14	4.54
245.7	33.60	4.59
288.4	36.37	4.59
331.5	39.14	4.62
373.1	41.38	4.59
416.8	44.37	4.72
462.6	46.93	4.76
	Mean:	4.52

maximum is detected (V_t) was obtained by the above method. The inner diameter of the tubing was 0.25–1.0 mm and the volume of the micro-flow cell was about 1 μl. A commercial UV spectrophotometer was used as a detector. The results are shown in Figs. 12–15 and Table I.

The following equation can be derived from the results in Fig. 13:

$$V_w^2 = k_1 V_t \tag{1}$$

where V_w (μl) is the dispersion of a solute in a stream of solvent. Similarly, from the results in Fig. 14, we have

$$V_w^2 = k_2 Q \tag{2}$$

where Q (μl/min) is the flow-rate of the mobile phase. From the results in Fig. 15, we obtain

$$V_w^2 = k_3 S \tag{3}$$

where S (mm^2) is the cross-section of the tubing. Finally, from eqns. 1, 2 and 3, we find

$$V_w^2 = k V_t Q S \tag{4}$$

Taylor[2] dealt with the dispersion of a solute in a stream of solvent quantitatively, and Ouano[3] introduced an equation for calculating the coefficient of molecular diffusion:

$$D = 0.212 (Q/L) (V_t/V_w)^2 \tag{5}$$

which can be rewritten as

$$V_w^2 = \frac{0.212}{D} \cdot V_t Q S \tag{6}$$

where D is the coefficient of molecular diffusion and L is the length of the tubing.

It was found that the empirical eqn. 4 obtained by MHPLC agreed with eqn. 6. Further, we found that V_w^2/V_t (4.52 from Table I) agreed with the calculated value (4.49 from eqn. 5.)

The dispersion in the joints used in MHPLC is calculated to be less than 0.5 μl from eqn. 6, which is very small compared with the total peak dispersion (several microlitres in MHPLC) and could be neglected. Moreover, it is expected that the technique described will be useful for the simple measurement of D values.

CONCLUSION

It has been demonstrated that in HPLC the volume of the column can be reduced to 1–2% of its normal value without any deleterious effects on its performance. This reduction results not only in a great decrease in the amount of sample required but also in a great decrease in the consumption of mobile phase and expensive packings. Micro columns can be easily prepared by investigators themselves and may be applied to various separation by selecting the appropriate packing materials. and is expected to be useful when applied to preparatory experiments in order to select suitable conditions for fractionation by means of PHLC.

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