Note

Dead volume determination in reversed-phase liquid chromatography

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A problem often encountered in reversed-phase liquid chromatography (RP-LC) is that of an accurate determination of the dead volume of the column (V_M). In spite of its frequent use, no generally established method is available for an objective determination of this parameter. In fact, a variety of substances have been tested, but the relationship of their retention volumes to the dead volume is questionable. Water and ^2H_2O have been employed[1–4], but these cannot be detected by the photometric detector and also suffer from other drawbacks[5]. Of organic non-ionisable compounds, acetone[6] and dimethylformamide[7] have been used in mobile phases with a phosphate buffer. Of ionisable substances, sodium benzenesulphonate[8], sodium nitrate[9,10]; and sodium chloride[11] have been employed.

The requirements imposed on a substance to be applied to the determination of the V_M value are rather stringent: it must not be retarded by the stationary phase yet must not pass through the column more quickly than the mobile phase, and these properties must be preserved even if the content of the organic modifier or the pH is varied. Moreover, the substance of choice must be easily detectable, *e.g.* by a photometric detector working at 254 nm.

EXPERIMENTAL

Two commercial stainless-steel columns (Laboratory Works, Prague, Czechoslovakia) were used: one, 125 × 6 mm I.D., was packed with spherical 28–30 μm particles of Separon SE (a styrene–ethyleneglycoldimethacrylate copolymer, exclusion limit 300,000); the other, 250 × 4 mm I.D., was packed with spherical 10 ± 2 μm particles of Separon Si C-18-VSK (silica gel with octadecyl functional groups).

The eluent was delivered by means of a high-pressure linear proportioner (Development Workshop, Czechoslovak Academy of Sciences, Prague, Czechoslovakia).

The equipment was fitted with a UVD 5 photometric detector set at 254 nm (Development Workshop, Czechoslovak Academy of Sciences) and a refractometric detector (Laboratory Works). The retention times were visualized on a minigrator (Spectra-Physics).

Methanol–water mixtures with different proportions of the components served as the mobile phase; its flow-rate, checked by means of a microburet inserted after

the detector, was 0.20 ml/min. The pH was adjusted by addition of either sodium hydroxide or sulphuric acid.

RESULTS AND DISCUSSION

As mentioned above, the following requirements must be taken into account when seeking a suitable substance for the determination of the dead volume in RP-LC: on the one hand, the interaction of the compound of choice with the stationary phase must be as weak as possible, while on the other hand, its affinity for the mobile phase must be very high

The first possibility examined was that of determining the dead volume by extrapolating the retention volumes of hydroxybenzenes containing five or six hydroxy groups in a molecule. The measurements, which were carried out on the mono-, di- and trihydroxybenzenes, resulted in the following conclusion: the retention volumes of members of hydroxybenzene series obey the exponential relationship

$$V_R = V_B' \cdot c^n + V_M \qquad (1)$$

where V_B' is the reduced retention volume of benzene, $c < 1.0$ is a constant (different for symmetrical and unsymmetrical hydroxybenzenes) allowing for the effect of the hydroxy groups, n is the number of hydroxy groups in a molecule, and V_M is the dead volume of the column. This relationship can be extrapolated for hexahydroxybenzene, the molecule of which should have so low an affinity for the stationary phase that it would pass through the column at a rate identical with that of the mobile phase. Examining this dependence using hydroquinone and phloroglucinol and mobile phases of various compositions, we found the above exponentials to intersect close to (for Separon SE) or directly at (for Separon Si C_{18}) the point corresponding to phloroglucinol (Figs. 1a and 2a). This implies that the retention volume of phloroglucinol is invariant with respect to the methanol concentration in the mobile phase. When hydroquinone and phloroglucinol were replaced with pyrocatechol and pyrogallol, respectively, slightly different curves were obtained. Pyrogallol, the unsymmetrically substituted trihydroxybenzene, does not exhibit a steady retention volume independent of the mobile phase composition (Figs. 1b and 2b).

These results suggest that phloroglucinol may serve well for the determination of the dead volume for the systems here examined; therefore, this substance was compared with other compounds commonly used in RP-LC, such as sodium benzenesulphonate, dimethylformamide, sodium nitrite, sodium nitrate, and acetone, all of which absorb at 254 nm. However, because their absorptivities differ widely, the amounts injected were chosen so as to achieve comparable detector responses for all of them. To make the graphical representation clear, the masses injected are plotted in the logarithmic scale. Fig. 3 shows the dependence of the retention volumes of the substances tested on the amounts applied. As a consequence of the occurrence of Donnan's potential, the ionisable substances are seen to be eluted from the column in a shorter time if a smaller amount is injected, irrespective of the eluent composition. For some of them the elution is so rapid that they leave the column in a volume smaller than one-half of the total column volume. Similar behaviour was observed when a basic eluent (pH 11.0) was used (Fig. 4a). This tendency is less apparent for
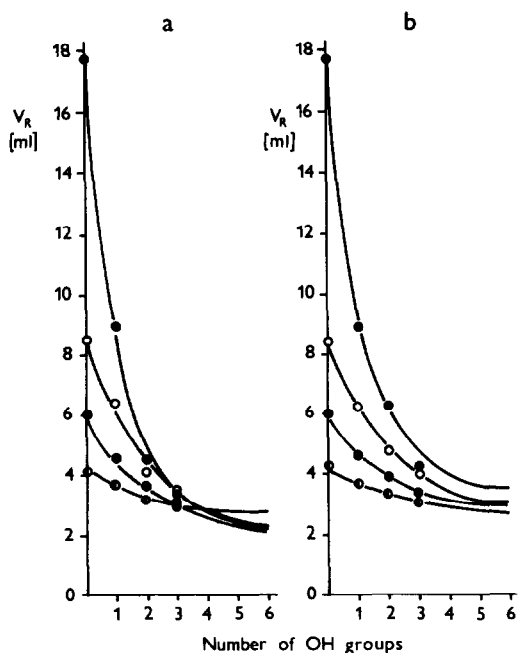
Fig. 1. Plots of benzene and hydroxybenzene retention volumes against the number of hydroxy groups in the solute molecule for various methanol–water mixtures. The curves were calculated according to eqn. 1. Column, 125 × 6 mm I.D. Separon SE. (a) Benzene, phenol, hydroquinone, phloroglucinol; (b) benzene, phenol, pyrocatechol, pyrogallol. Key: ○, 100% methanol; ⊗, 90%; ○, 80%; ●, 70%.
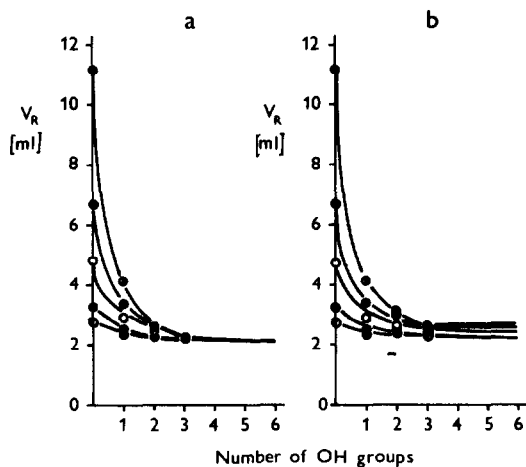

Fig. 2. Plots of benzene and hydroxybenzene retention volumes against the number of hydroxy groups in the solute molecule for various methanol–water mixtures. The curves were calculated according to eqn. 1. Column 250 × 5 mm I.D. Separon Si C-18-VSK. (a) Benzene, phenol, hydroquinone, phloroglucinol; (b) benzene, phenol, pyrocatechol, pyrogallol. Key: ○, 100% methanol; ⊗, 90%; ○, 80%; ●, 70%; ⊙, 60%.
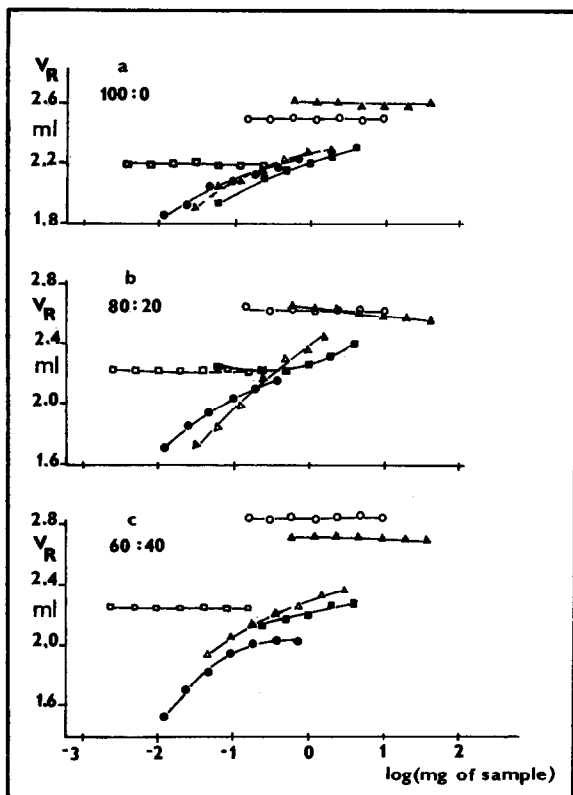
Fig. 3. Plots of phloroglucinol (□), sodium benzenesulphonate (●), N,N-dimethylformamide (▲), acetone (○), NaNO$_3$ (■) and NaNO$_2$ (△) retention volumes against the amount of sample injected. Mobile phase, methanol–water in ratios (a) 100:0, (b) 80:20, (c) 60:40; column, 250 × 5 mm I.D. Separon Si C-18-VSK.

an acid eluent (pH 4.0). The retention volume of sodium nitrite even increases appreciably (Fig. 4b); this is probably due to liberation of nitrous acid, which then passes through the column more slowly than the salt. Two peaks thus emerge, the first of which diminishes with decreasing concentration of sodium nitrite. Not meeting the fundamental demand, viz. that of a constant retention volume independent of the amount injected, these substances are evidently unsuitable for the determination of V_M.

For non-ionisable substances, such as by acetone and dimethylformamide, the retention volumes are found to be independent of the concentration. However, both these substances interact with the stationary phase because the retardation becomes more pronounced as the content of water in the mobile phase is increased, particularly for acetone, and the retention volume is ca. 83% of the total column volume. This value is intolerably high, though roughly constant with varying pH.

For phloroglucinol, on the other hand, the retention volumes in mobile phases of different composition are nearly identical, even at pH 4, and correspond to 70–72% of the total column volume, which agrees well with the predicted value. More-
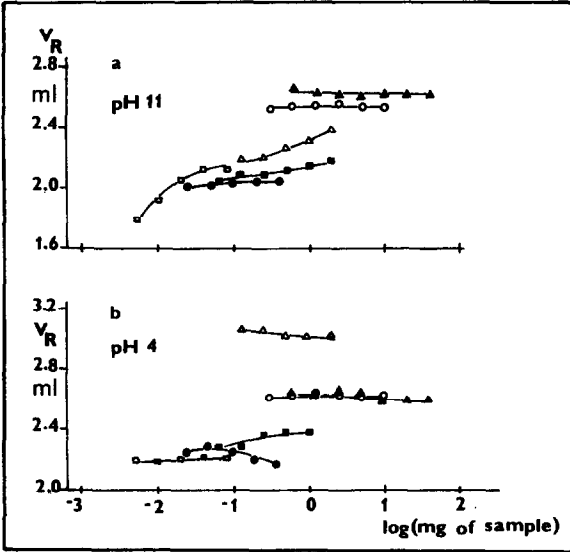
Fig. 4. Plots of phloroglucinol (□), sodium benzenesulphonate (●), N,N-dimethylformamide (▲), acetone (○), $NaNO_3$ (■) and $NaNO_2$ (△) retention volumes against the quality of injected samples. Mobile phase, methanol–water (80:20) at (a) pH 11.0, (b) pH 4.0; column, 250 × 5 mm I.D. Separon Si C-18-VSK.

over, the retention volume does not vary with the amount injected, and owing to the high absorptivity at 254 nm the concentration applied can be the lowest of all the substances tested.

The retention behaviour of phloroglucinol was also compared with that of water. For this purpose, the refractometric detector was used, showing a high response to the injection of several microlitres of water. The water injected formed with methanol a mixture with a refractive index higher than those of pure water and of pure methanol. A volume contraction took place, causing an increase in the density and also in the viscosity of the mixture. The refractive index varied as shown by the
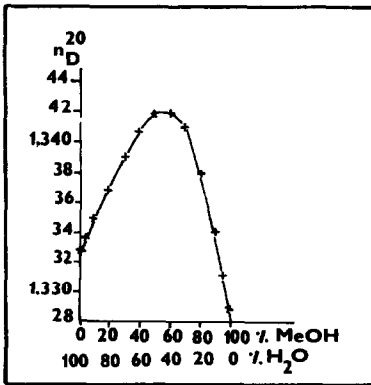

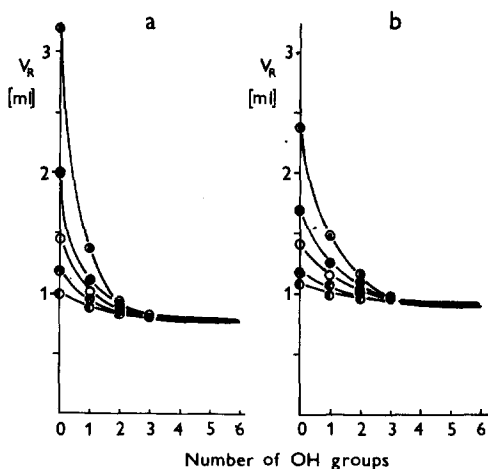Fig. 5. Plot of refractive index against methanol–water ratio (v/v).

Fig. 6. Plots of the retention volumes of benzene, phenol, hydroquinone and phloroglucinol against the number of hydroxy groups in the solute molecule for various methanol–water mixures. The curves were calculated according to eqn. 1. Columns, 15 × 0.34 cm I.D. (a) LiChrosorb RP-18, (b) LiChrosorb RP-8. Key: ◑, 100% methanol; ⊗, 90%; ○, 80% ●, 70%; ◉, 60%.

curve in Fig. 5. As a result, the retention of water was somewhat higher than that of phloroglucinol, and the peak of water appeared after that of phloroglucinol.

In order to verify the results another two stationary phases were tested: Li-Chrosorb RP-18 and LiChrosorb RP-8 (Merck, Darmstad, F.R.G.). Both sorbents were packed in glass columns, 15 × 0.34 cm I.D., and the measurements were carried out in the same manner as for Separon SE and Separon Si C-18-VSK. The results are given in the Fig. 6a and b. The retention volume of phloroglucinol does not depend on the mobile phase composition in′the range examined and agrees very well with the expected dead volume.

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