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LARGE-VOLUME INJECTION OF SAMPLES DISSOLVED IN A NON-ELUTING SOLVENT

APPLICATION TO THE DETERMINATION OF ANTIPYRINE USING NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The usefulness of injection of a large volume of biological sample dissolved in a non-eluting solvent similar to the mobile phase has been demonstrated in reversed-phase high-performance liquid chromatography (HPLC) to increase the effective sensitivity of HPLC methods for drug-level determinations. In order to extend this concept to normal-phase HPLC, standards of antipyrine and phenacetin were dissolved in a series of solvents less eluting (or eluotropic) than the mobile phase (chloroform–methanol, 97.5–2.5), and a large volume (500 μl) of these solutions was injected on to the column (LiChrosorb Si 60, 5 μm, 15 cm × 4.6 mm I.D.). Injection in carbon tetrachloride or *n*-pentane–methanol (97.5:2.5) maintained the width at half height of the peaks at their minimum, but the relationship previously reported for reversed-phase HPLC was not satisfied. On the other hand, the repetitive injection of large volumes of carbon tetrach loride disturbed the chromatographic equilibria. Therefore, to maintain the maximum of efficiency, selectivity and sensitivity, repetitive injection of large volumes of samples could only be done in *n*-pentane–methanol (97.5:2.5).

These results extend the possibilities of large volume injection to increase both the sensitivity and the rate of sample analysis. However, it is more difficult to apply this proceudre in normal-phase than in reversed-phase HPLC.

INTRODUCTION

The injection of a large volume of solute dissolved in a non-eluting solvent similar to the mobile phase has been demonstrated to be an easy means of increasing the sensitivity and the rate of throughput of sample for some drug determinations in biological fluids using reversed-phase high-performance liquid chromatography (HPLC)[1–5].

If the solute was injected in a non-eluting solvent (*i.e.* a weak eluent which, if used as mobile phase on the chromatographic column, would lead to very long or infinite retention times), the variance of the chromatographic peak was independent of

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the injected volume and an increase of retention volume equal to the injected volume was observed[1,2]. Consequently, all the volume available for injection could be injected without any extra-column band-broadening due to the injected volume, and the "overall sensitivity" was maintained at the maximum that could be expected with the instrument and the chromatographic conditions used. If the non-eluting solvent used to inject the solute was similar to the mobile phase (*e.g.* a dilution of the mobile phase in its weakest component), repetitive injections of large volumes did not distrub the chromatographic equilibria and the retention times remained constant. Thus the automatic injector was straight forward and the rate of throughput of sample was increased. All these considerations concerned analyses carried on with reversed-phase HPLC. The application to normal-phase HPLC of large volumes of solute dissolved in non-eluting solvent was not investigated.

The aim of the present study is to extend this concept to normal-phase HPLC. Its feasibility was examined during the development of an analytical method for antipyrin in biological fluids. For this purpose, the investigation was divided into three parts: (1) the search for a non-eluting solvent as close as possible to the mobile phase; (2) the effect of increasing the volumes of solvent selected in part (1) on retention volumes, and band broadening or peak heights; (3) the study of the influence of repetitive injections of some of these solvents on chromatographic behaviour.

EQUIPMENT

The separation was carried out either on a Micromeritics 7000 B liquid chromatograph with manual injection valve (volume of loop, 1000 μl), UV–visible spectrophotometer and Perkin-Elmer 56 recorder, or on a laboratory-built automated liquid chromatograph comprising the following system: Micromeritics 725 Automatic Injector (with 1 500 -μl loop), LDC Constametric II G pump, LDC Monitor III UV spectrophotometer and Perkin–Elmer Sigma 10 Chromatographic Data Station. In order to avoid variations of chromatographic parameters due to temperature variations, the column was thermostatically controlled by a water jacket (Touzard & Matignon, Vitry-sur-Seine, France) and a circulating water bath FE2 (Haake, Karlsruhe, G.F.R.).

SOLVENTS AND STANDARDS

Chloroform and carbon tetrachloride, reagent grade, and methanol, UV spectroscopic grade, were purchased from Prolabo (Paris, France) and *n*-pentane Uvasol from Merck (Darmstadt, G.F.R.). Antipyrine was purchased from Aldrich Europe (Beerse, Belgium) and phenacetin, used as an internal standard, from Sigma (St. Louis, MO, U.S.A.). The chemical structures of antipyrine and phenacetin are shown in Fig. 1.

CHROMATOGRAPHIC CONDITIONS

The chromatographic system consisted of a mobile phase of chloroform–methanol (97.5:2.5) percolating at a flow-rate of 1.00 ml min^{-1} through a stainless-steel column (15 cm × 4.6 mm I.D.) packed in the laboratory[6] with LiChrosorb Si 60 (5

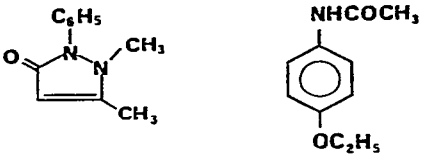

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Fig. 1. Chemical structures of (I) antipyrine and (II) phenacetin.

μm). The detector wavelength was set at 275 nm, which corresponded to the largest difference between antipyrine and mobile phase absorptions. The column temperature was maintained at 23°C. In all the studies, constant amounts of 5 μg of antipyrine and of 10 μg of phenacetin were injected on to the column at each injection. A typical chromatogram is shown in Fig. 2.

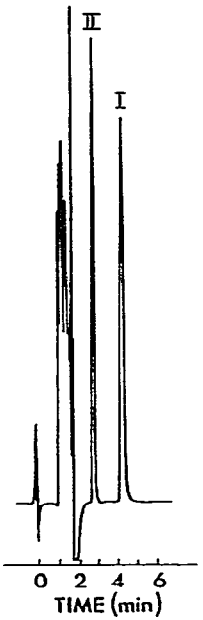

Fig. 2. Typical chromatogram of (I) antipyrine (5 μg) and (II) phenacetin (10 μg) after injection in 500 μl of *n*-pentane–methanol (97.5:2.5).

SEARCH FOR A NON-ELUTING SOLVENT

In a previous study[1] the non-eluting solvent was experimentally determined as a dilution of the mobile phase in its weakest component in order to maintain the chromatographic equilibria inside the column. With injection in such a non-eluting solvent, the retention volume V_R increased with the injected volume, V_{inj}, according to eqn. 1[2,3].

$$V_R = V_R^0 + V_{inj} \quad (1)$$

where V_R^0 is the retention volume corresponding to a small injection volume ($< 10\ \mu l$). The contribution of the injection to peak broadening was independent of V_{inj}. As a consequence, the width at half height of the peak, $b_{0.5}$, and also h_p, the height of the peak (for a given amount of solute injected) were independent of V_{inj}.

In contrast, as demonstrated elsewhere[2,7], not only V_R, but also $b_{0.5}$ and consequently h_p, varied with V_{inj} when a given amount of solute was injected dissolved in the mobile phase according to the following equations:

$$V_R = V_R^0 + \frac{V_{inj}}{2} \tag{2}$$

$$b_{0.5}^2 = 8 \ln 2 \left(\frac{V_{inj}^2}{K^2} + \Sigma\sigma^2 \right) \tag{3}$$

$$\left(\frac{h_p^0}{h_p} \right)^2 = \frac{V_{inj}^2}{K^2\ \Sigma\sigma^2} + 1 \tag{4}$$

where $\Sigma\sigma^2$ is the sum of the variances of the contributions of all the parameters (apart from injection volume) to band broadening, K is a constant depending on the injection device[5] and h_p^0 is the height of the peak corresponding to a small injection volume ($< 10\ \mu l$).

As a consequence, if the eluotropic strength of the solvent used for injection decreased (the amount of solute and the volume which were injected being constant), V_R would increase by $\frac{1}{2}\ V_{inj}$ from injection in the eluent to injection in a non-eluting solvent. Simultaneously, $b_{0.5}$ would decrease and h_p would increase. When the non-eluting status was achieved, V_R, $b_{0.5}$ and h_p would stay constant.

Experimental

In order to cover a large range of eluotropic strength the composition of the injection solvent was progressively varied from the mobile phase (or a mixture close to mobile phase) to a considerably weaker mixture, according to the following procedure.

(1) The mobile phase (MP) was progressively diluted in its weakest component (chloroform) to make a mixture X MP + $(1 - X)CHCl_3$.

(2) Chloroform, the weakest component of the mobile phase, was progressively dissolved in a still weaker solvent (carbon tetrachloride) to make a mixture X $CHCl_3$ + $(1 - X)CCl_4$.

(3) The mobile phase was progressively diluted in a weaker solvent containing the same proportion of methanol (*n*-pentane–methanol, 97.5:2.5) to make a mixture X MP + $(1 - X)$pentane–methanol.

These three mixtures corresponded to the three different possibilities of decreasing the eluotropic strength. In each case X was decreased from 1 to 0. For each value of X, constant amounts of antipyrine and phenacetin were injected, dissolved in 500 μl of the corresponding solvents.

Results and discussion

Variations of V_R and $b_{0.5}$ with X for injections made in mobile phase/chloroform and chloroform–carbon tetrachloride are displayed in the same figure (Fig. 3a for V_R and Fig. 3b for $b_{0.5}$) in order to emphasize the continuous decrease of eluotropic strength in the injection solvent.

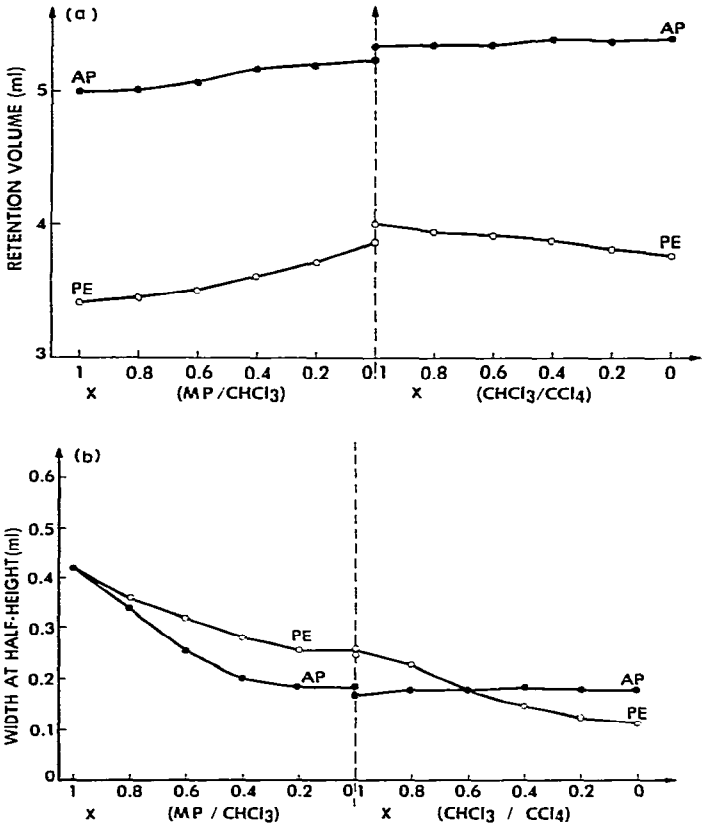

Fig. 3. (a) Influence of the composition of injection solvent: variation of retention volumes (mobile phase: chloroform–methanol, 97.5:2.5). (b) Influence of the composition of injection solvent: variation of width at half height (mobile phase: chloroform–methanol, 97.5:2.5). AP = antipyrine; PE = phenacetin.

Variations of V_R and $b_{0.5}$ with X for injections made in mobile phase *n*-pentane–methanol are displayed respectively in Figs. 4a and 4b.

As injection solvent was modified from mobile phase to chloroform, so the retention volumes of antipyrine and phenacetin increased regularly from, respectively, 4.97 to 5.21 ml (ΔV_R = 0.24 ml) and 3.42 to 3.85 ml (ΔV_R = 0.43 ml). Simultaneously the width at half height decreased from 0.42 to 0.18 ml for antipyrine and from 0.42 to 0.25 ml for phenacetin (Fig. 5a, d).

The progressive variation of injected solvent from chloroform to carbon tetrachloride had no effect on either the retention volume or the width at half height for antipyrine. These results indicate that pure chloroform seems to be a non-eluting solvent for antipyrine. However, for phenacetin the retention volume and the width

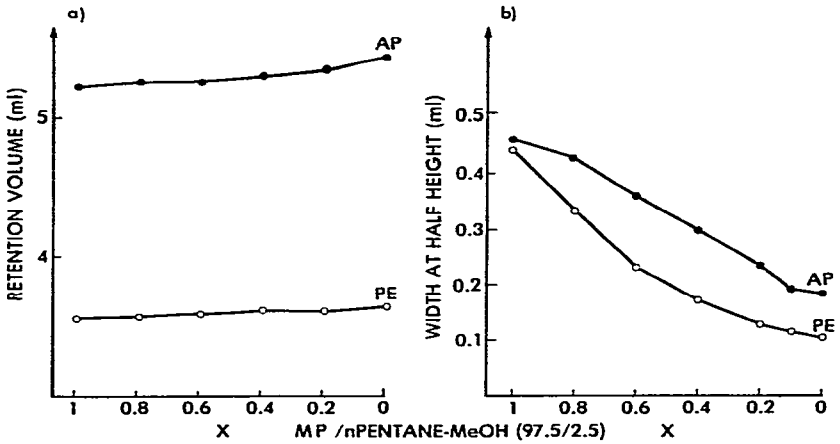

Fig. 4. Influence of the composition of injection solvent: variations of (a) retention volumes, (b) width at half height (mobile phase: chloroform–methanol, 97.5:2.5). MeOH = Methanol; AP = antipyrine; PE = phenacetin.

at half height decreased from 4.00 to 3.76 ml (ΔV_R = 0.24 ml) and from 0.255 to 0.114 ml, respectively (Fig. 5a, c).

The progressive modification of injection solvent from mobile phase to *n*-pentane–methanol led to an increase in retention volume from 5.2 to 5.42 ml (ΔV_R = 0.22 ml) for antipyrine and from 3.53 to 3.64 ml (ΔV_R = 0.11 ml) for phenacetin. Simultaneously, the widths at half height decreased from 0.26 to 0.182 ml for antipyrine and from 0.44 to 0.105 ml for phenacetin (Fig. 5a–c).

The experimental results obtained for injection of antipyrine dissolved in chloroform or in *n*-pentane–methanol agree quite closely with theory. The variations of V_R (0.23 and 0.22 ml, respectively) were close to $\frac{1}{2} V_{inj}$ (0.25 ml), and the variations of $b_{0.5}$ (0.241 and 0.278, respectively) were of the same order of magnitude as the theoretical value (0.340 ml) obtained from eqn. 3, assuming V_{inj} = 0.5 ml and K = 3.6 (plug injection).

This close agreement between experimental and theoretical values did not exist for phenacetin because the variation of V_R was considerably larger than expected when injected in chloroform (0.43 ml instead of 0.25 ml) and considerably smaller (0.09 ml) when injected in *n*-pentane–methanol. Moreover, the retention volume decreased when injection solvent was varied from chloroform to carbon tetrachloride. Nevertheless, the variations of $b_{0.5}$ for injection from mobile phase to carbon tetrachloride or *n*-pentane–methanol (0.311 and 0.335 ml, respectively) are in good agreement with the theoretical value.

The ratios h_p^0/h_p for antipyrine and phenacetin, however, when injected dissolved in carbon tetrachloride or *n*-pentane–methanol (Fig. 6a and b) were equal to 1.

These results clearly indicated that no non-eluting solvent, as defined elsewhere[1,2] *i.e.* similar to the mobile phase, could be determined in this study. As the "overall sensitivity" of the chromatographic separation was maintained when carbon tetrachloride or *n*-pentane–methanol was used for injection, these two solvents were investigated further.

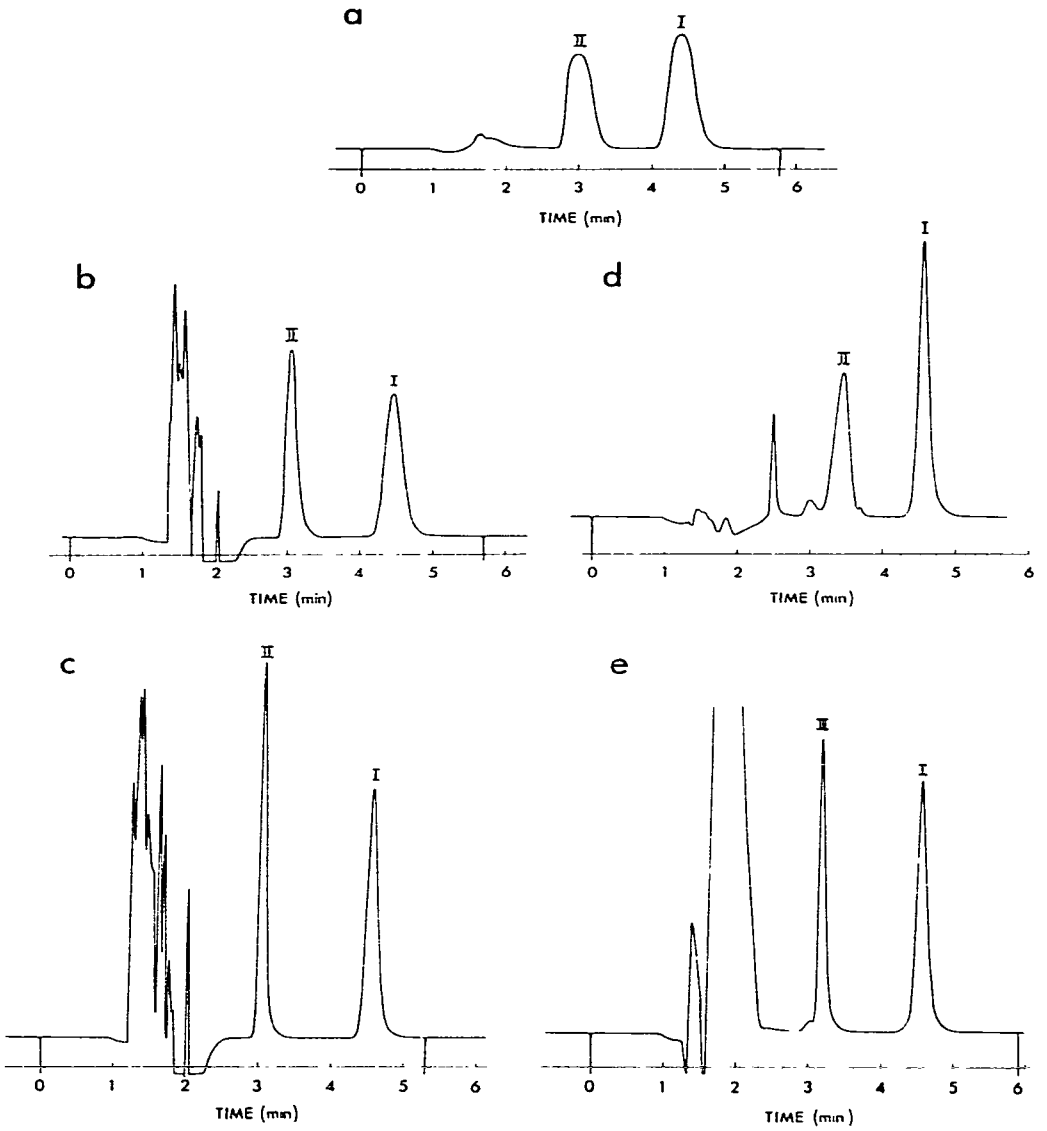

Fig. 5. Influence of the composition of injection solvent. Typical chromatograms of (I) antipyrine (5 μg) and (II) phenacetin (10 μg) after injection in 500 μl of (a) mobile phase (chloroform–methanol, 97.5:2.5) (X = 1), (b) mobile phase/(*n*-pentane–methanol, 97.5:2.5) (50:50) (X = 0.5), (c) *n*-pentane–methanol (97.5:2.5) (X = 0), (d) chloroform and (e) carbon tetrachloride.

INFLUENCE OF INJECTED VOLUME ON RETENTION TIME, WIDTH AT HALF HEIGHT AND PEAK HEIGHT

Experimental

The solvents selected, carbon tetrachloride and *n*-pentane–methanol, are not exactly similar to the mobile phase (chloroform–methanol), and consequently their

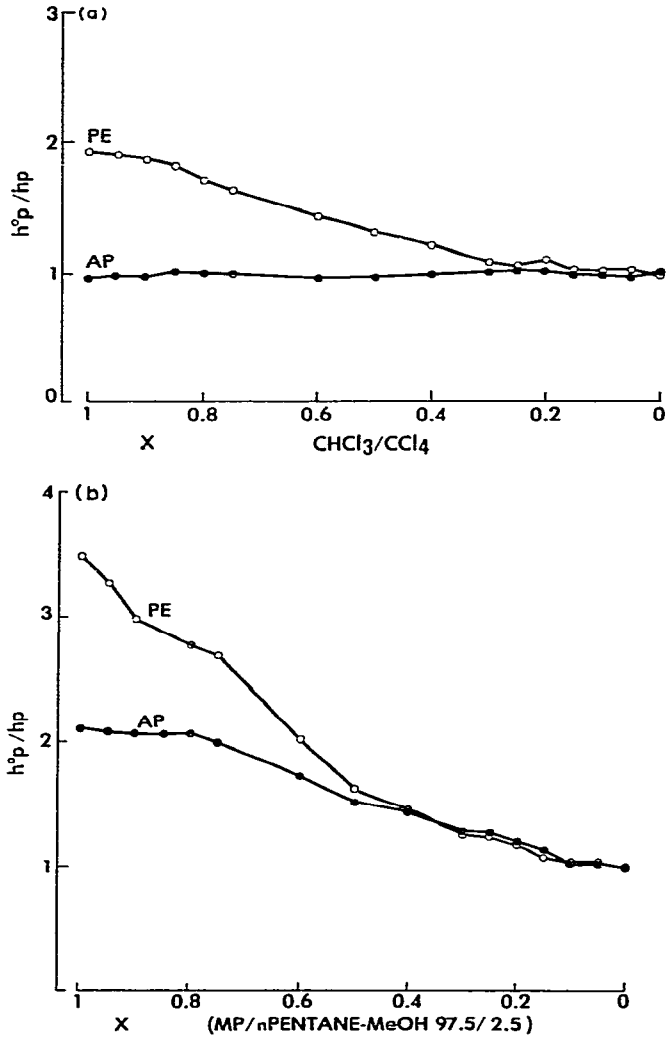

Fig. 6. Influence of the composition of injection solvent: variation of the overall sensitivity after injection of 500 µl. (a) Chloroform–carbon tetrachloride; (b) mobile phase/*n*-pentane–methanol (97.5:2.5). AP = antipyrine; PE = phenacetin.

influence on chromatographic behaviour was investigated by injecting constant amounts of antipyrine and phenacetin in increasing volumes of carbon tetrachloride or of *n*-pentane–methanol.

Results and discussion

The variation of retention volume ΔV_R with the injected volume is displayed in Fig. 7a and b. The retention volumes increased linearly and their variation ΔV_R appeared to be proportional to V_{inj}. For injections in carbon tetrachloride the slope seemed independent of the injected solute: 0.92 for antipyrine and 0.89 for phenac-

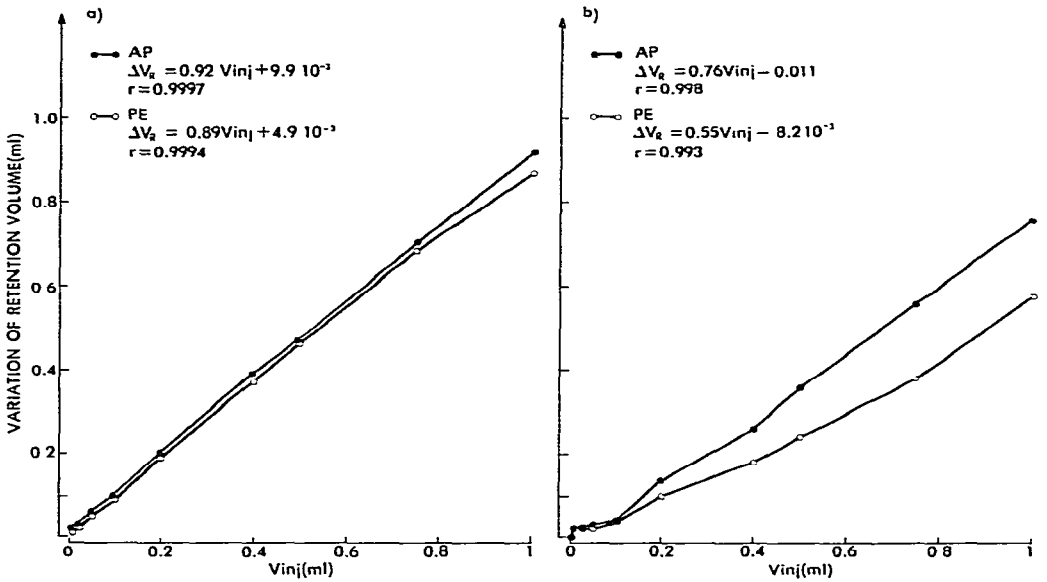

Fig. 7. Influence of the injected volume on the retention volumes after injection of solutes dissolved in (a) carbon tetrachloride, (b) *n*-pentane–methanol (97.5:2.5). AP = antipyrine; PE = phenacetin.

etin; for injections in *n*-pentane–methanol, the slope seemed related to the solute 0.76 for antipyrine and 0.55 for phenacetin.

Whatever the injection solvent and the injected volume, the values of $b_{0.5}$ for antipyrine remained almost constant as it is shown in Figs. 8 and 9. For phenacetin, the values of $b_{0.5}$ (Figs. 8 and 9) appeared independent of V_{inj} when injected in carbon tetrachloride; when injected in *n*-pentane–methanol, they remained constant as long as V_{inj} was less than 500 μl, but increased drastically when V_{inj} became larger. As a consequence, when $b_{0.5}$ increased the ratio h_p^0/h_p decreased.

These results clearly demonstrate that the studied injection solvent does not act either as an eluent or as a non-eluting solvent. The slope of the linear relation between ΔV_R and V_{inj} ranged from 0.5 (characteristic of injection in mobile phase) to 1 (characteristic in a non-eluting solvent similar to the mobile phase). Moreover, this slope seemed to be related to the injection volume, to the injection solvent and to the solute.

It is evident that for the chromatographic conditions used in this study, carbon tetrachloride and *n*-pentane–methanol cannot be considered as real non-eluting solvents. They were not similar to the mobile phase, and the observed variation from the theoretical equations could probably be explained by this difference.

Nevertheless, when used for injections of less than 500 μl, these solvents could be used for injection of solute without noticeable band-broadening, if repetition of such an injection did not modify the chromatographic behaviour.

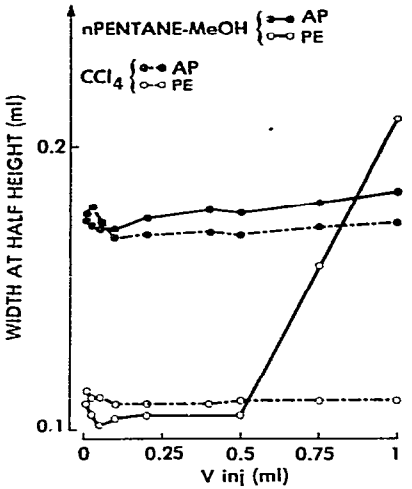

Fig. 8. Influence of the injected volume on the width at half height of peaks after injection of solutes dissolved in carbon tetrachloride (— — —) and in *n*-pentane–methanol (———). MeOH = Methanol; AP = antipyrine; PE = phenacetin.

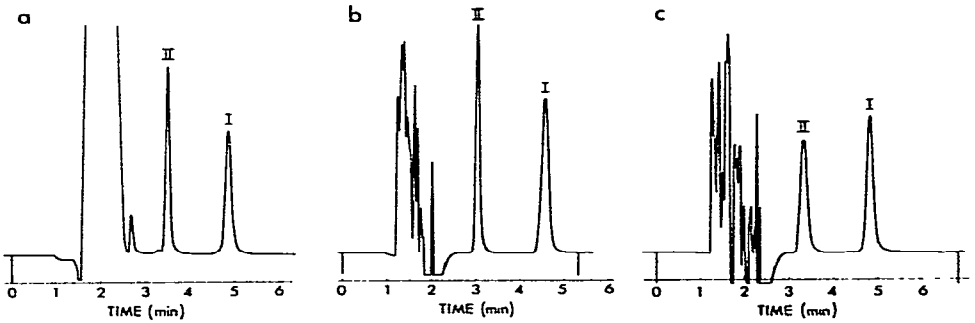

Fig. 9. Typical chromatograms of (I) antipyrine (5 μg) and (II) phenacetin (10 μg) after injection in (a) 1000 μl carbon tetrachloride, (b) 500 μl *n*-pentane–methanol (97.5:2.5), (c) 1000 μl *n*-pentane–methanol (97.5:2.5).

INFLUENCE OF REPETITIVE INJECTION OF LARGE VOLUMES OF APPARENT NON-ELUTING SOLVENTS ON CHROMATOGRAPHIC BEHAVIOUR

It has already been shown that the injection of a large volume of a solvent different from the mobile phase can modify the equilibria existing in the column[1] in reversed-phase HPLC. This possibility must be checked in normal-phase HPLC, for which the chromatographic equilibria take longer to reach.

Experimental

In order to investigate their effects on chromatographic behaviour, repeated injections of standards dissolved in 500 μl of carbon tetrachloride or of *n*-pentane–

methanol were made following the general schedule described below:

n_1 injections were made at time intervals $1\Delta T = 7$ min
n_2 injections, every $2\Delta T = 14$ min
n_3 injections, every $3\Delta T = 21$ min
n_4 injections, every $4\Delta T = 28$ min
n_1 injections, every $1\Delta T = 7$ min.

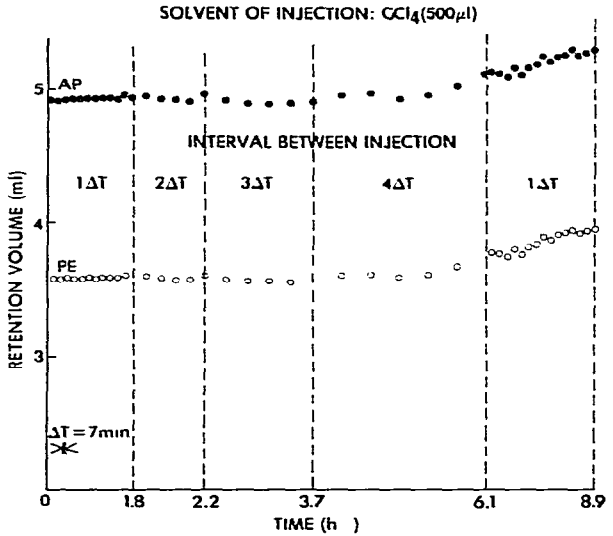

Fig. 10. Injection of solute dissolved in carbon tetrachloride, a solvent less eluting than mobile phase: effect of the repeated injections on retention volumes.

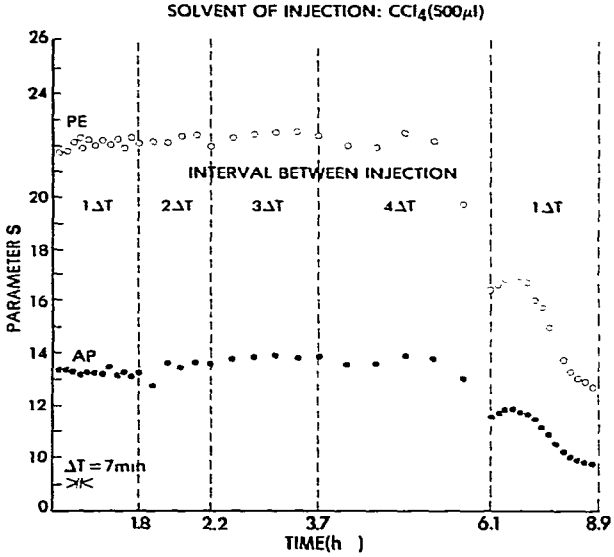

Fig. 11. Injection of solute dissolved in carbon tetrachloride, a solvent less eluting than mobile phase: effect of the repeated injections on the "overall sensitivity".

TABLE I

INFLUENCE OF REPEATED INJECTIONS ON CHROMATOGRAPHIC BEHAVIOUR: EXPERIMENTAL PARAMETERS, MEAN RETENTION VOLUME AND MEAN OVERALL SENSITIVITY (S)

S.D. = Standard deviation; C.V. = coefficient of variation.

Injection solvent	$n_{\Delta T}$	n_i	*Phenacetin*		*Antipyrine*	
			$\bar{V}_R \pm$ *S.D. (C.V.) (ml)*	$\bar{S} \pm$ *S.D. (C.V.)*	$\bar{V}_R \pm$ *S.D. (C.V.) (ml)*	$\bar{S} \pm$ *S.D. (C.V.)*
Carbon tetrachloride	1	12	3.59 ± 0.008 (0.2%)	22.1 ± 0.19 (0.9%)	4.93 ± 0.009 (0.2%)	13.3 ± 0.09 (0.6%)
	2	5	3.59 ± 0.018 (0.5%)	22.2 ± 0.18 (0.8%)	4.94 ± 0.024 (0.5%)	13.6 ± 0.09 (0.6%)
	3	5	3.56 ± 0.007 (0.2%)	22.4 ± 0.10 (0.5%)	4.90 ± 0.008 (0.2%)	13.8 ± 0.06 (0.4%)
	4	6	3.64 ± 0.070 (1.9%)	21.7 ± 1.10 (5.1%)	4.99 ± 0.070 (1.4%)	13.2 ± 0.87 (6.6%)
	1	15	3.85 ± 0.070 (1.8%)	14.8 ± 1.90 (13.0%)	5.19 ± 0.070 (1.4%)	10 ± 2.50 (25.0%)
n-pentane–methanol	1	15	3.52 ± 0.020 (0.6%)	22.0 ± 1.50 (7.0%)	5.23 ± 0.025 (0.5%)	13.2 ± 0.14 (1.1%)
	2	3	3.50 ± 0.007 (0.2%)	22.6 ± 0.06 (0.3%)	5.20 ± 0.008 (0.15%)	13.2 ± 0.02 (0.6%)
	3	5	3.51 ± 0.005 (0.2%)	22.9 ± 0.10 (0.5%)	5.20 ± 0.004 (0.08%)	13.3 ± 0.07 (0.5%)
	4	9	3.52 ± 0.008 (0.2%)	22.6 ± 0.25 (1.1%)	5.22 ± 0.016 (0.3%)	13.1 ± 0.08 (0.6%)
	1	19	3.53 ± 0.009 (0.3%)	22.6 ± 0.33 (1.5%)	5.23 ± 0.013 (0.3%)	12.8 ± 0.12 (0.9%)

In each series ΔT was set on the automatic injector as the time between two consecutive injections to make each injection at ΔT. In order to make the injections at intervals of 2, 3 and $4\Delta T$, one, two or three vials filled with mobile phase were placed on the rack between two vials containing standard solutions. The values of n_i are given in Table I. The injections were made over 9.9 h for injection in *n*-pentane–methanol and over 8.9 h for injection in carbon tetrachloride.

Results and discussion

The mean values of the retention volumes of antipyrine and phenacetin are shown in Table I, together with the corresponding values of S, defined as the ratio between the height and the area of the peak. This parameter was introduced in order to eliminate the influence of the injected amount on the "overall sensitivity" of the chromatographic analysis.

The retention volume and the parameter S for antipyrine and phenacetin injected in solution in *n*-pentane–methanol appeared to be independent both of the interval between two consecutive injections and of the number of injections. However, the use of carbon tetrachloride as injection solvent was unsatisfactory, as is shown in Figs. 10 and 11. Retention volumes and the parameter S remained constant during a certain period of time and suddenly varied in a non-reproducible way. When the sequence of injections in carbon tetrachloride was duplicated, the variation of V_R and S occurred sooner in the second experiment than in the first. These variations, which were not related to any parameter, could probably be explained by a disequilibrium in the column owing to the repetitive suppression of the most polar solvent, methanol. Such variations were not observed for injection in *n*-pentane–methanol.

CONCLUSION

This study did not make it possible to define a concept of non-eluting solvent applicable to normal-phase HPLC, as has already been done for reversed-phase HPLC. Different hypotheses could be put forward to explain this failure, but none of the results from this study indicates which is the most likely. However, large volumes (500 µl) of a less eluting solvent, *n*-pentane–methanol, in place of the mobile phase, chloroform–methanol, in the same proportion were used to inject solute in a normal-phase column without band broadening or loss of sensitivity. This injection system was used in routine analysis without trouble for up to 100 injections of biological extracts on the same column.

This experience demonstrates the usefulness for normal-phase HPLC of injecting solute dissolved in a large volume of a solvent less eluting than the mobile phase. Unfortunately, unlike reversed-phase HPLC, no theoretical considerations can help in the choice of the injection solvent, which must be determined experimentally.

These results show that it is possible to increase both the sensitivity and the rate of sample analysis by large volume injection.

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