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COMPUTER SIMULATION OF ABNORMAL HIGH-PERFORMANCE LIQUID CHROMATOGRAMS CAUSED BY SOLVENTS

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SUMMARY

The effects of solvents used to dissolve test samples on the peaks observed in high-performance liquid chromatography were studied in normal phase, reversed-phase and also ion-pair reversed-phase systems. It was found that various patterns of distortions and peak splitting occur depending on the solvent and the chromatographic system involved. Computer simulation of the abnormal chromatograms was achieved by means of a program based on a model in which the retention ratio changes as elution proceeds due to the effect of the solvent on the column.

INTRODUCTION

A relatively unexplored and poorly understood phenomenon in high-performance liquid chromatography (HPLC) is the peak distortions caused by the solvent used for injecting the test samples into the chromatograph. Unlike gas chromatography (GC), where the solvents do not affect the stationary phase material to any significant extent and are quickly swept away by the carrier gas, the solvents used in HPLC for injecting samples can temporarily modify the stationary phase by taking part in the liquid–liquid equilibrium or the liquid–solid equilibrium in the column. As a result, these solvents can significantly influence the elution of the compounds of interest.

A number of cases of such peak distortions or peak splitting caused by solvents have been documented[1–4]. These were mostly attributed to the solvent being "stronger" than the mobile phase, *i.e.*, being able to elute the compounds of interest faster than the mobile phase is able to do. However, an alternative explanation is that the multiple peaks are caused by adsorption on the column[1]. This explanation has been considered less likely[3]. To explain the peak splitting in an ion-pair reversed-phase system, a computer calculation based on the interplay of two distinct retention mechanisms (ion pairing and ion exchange) has recently been published[5].

In this paper, we describe a computer simulation of the solvent effect in HPLC by means of an elution model where a segment (or "slug") of solvent having a different elution strength from that of the mobile phase is allowed to pass through the column and eluted according to the retention ratios involved. This approach is applicable to the various modes of HPLC.

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EXPERIMENTAL

The chromatograms were obtained on a Hewlett-Packard 1084 B high-pressure liquid chromatograph equipped with an autosampler and injector, a variable wavelength UV detector and an integrator. The volume injected was 30 μl and the solute concentration was of the order of 1 mg/ml. The silica column used was a 250 × 4 mm Whatman Partisil 5-μm prepacked column. The reversed-phase columns used were 250 × 4 mm RP-8 and RP-18 LiChrosorb 10-μm columns from E. Merck.

A Perkin-Elmer 3600 Data Station was used for computing and a Perkin-Elmer 660 printer/plotter was used to plot the simulated chromatograms.

COMPUTER PROGRAMMING

Consider the injected solvent as a cylindrical segment ("slug") bounded at both ends by the mobile phase. This "slug" carries in it the compound or solute to be chromatographed.

On a particular column, the retention ratios of the solute in the injected solvent and in the mobile phase can be different and are represented by P and P_0 respectively. [The retention ratio is the fraction of the solute in the solvent or mobile phase; it is related to the capacity ratio, k', by the formula $P = 1/(1 + k')$][6]. For the purpose of computation, the "slug" is divided into ten "slices" and the solute is assumed to be evenly distributed at the ten leading boundaries of the "slices". This "slug" is then allowed to run into the column and the chromatography of the solute is monitored by following the movement of the ten leading boundaries of the ten "slices" along the column.

In order to follow the progress of the elution, a smaller unit of measurement of the longitudinal distance along the column is defined: this is a "sub-slice" which is equal to one-tenth the length of a "slice". With this unit, the column can be measured longitudinally from the column inlet and any point along the column can be determined from its "sub-slice" number (see Fig. 1i). Hence, the "slug" has a length of 100 "sub-slices" and it moves down the column by a distance of 10 "sub-slices" a time.

At the column inlet, when a "slice" is being run into the column, the new longitudinal coordinate, n', of its leading boundary is given by

$$n' = 10P$$

where P is the retention ratio of the solute in the injected solvent. However, once in the column, the boundary is eluted by the mobile phase or solvent behind it (Fig. 1i and ii). As the "slice" moves one "slice" forward, the new longitudinal coordinate, n', of its leading boundary is given by

$$n' = n + (n - n_0)\, P_i + [10 - (n - n_0)]\, P_{i+1}$$

where n = old coordinate of the boundary being eluted (as measured from the column inlet in "sub-slice" units), n_0 = lower coordinate of the slice, P_i = retention ratio of the solute in the solvent or mobile phase in the "slice" and P_{i+1} = retention ratio of the solute in the solvent or mobile phase being moved into the "slice".

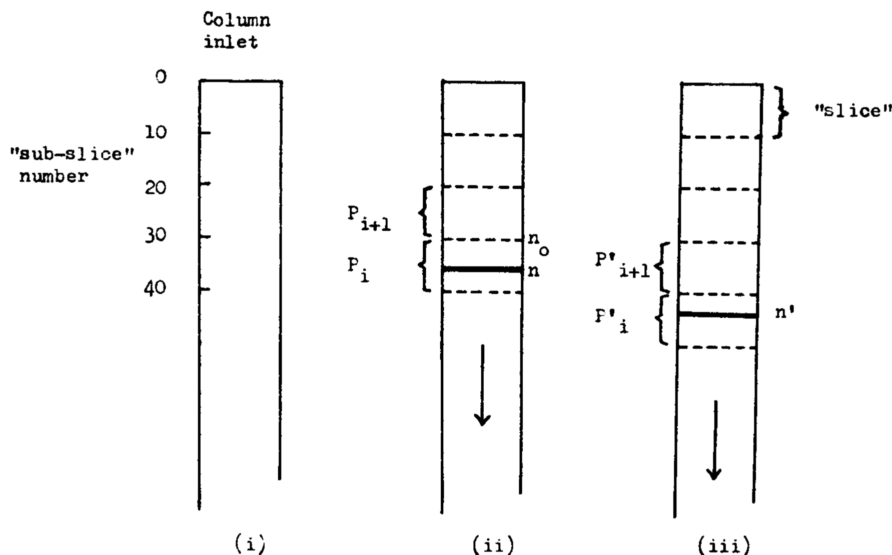

Fig. 1. Schematic diagram of the simulated elution. Dotted lines delineate the "slices" of the injected "slug"; thick horizontal lines represent the boundary containing solute. (i) Column longitudinal coordinates in terms of "sub-slices". (ii) Coordinate of a boundary to be moved: n = coordinate of the boundary; n_0 = lower coordinate of "slice"; P_i = retention ratio in the "slice"; P_{i+1} = retention ratio in the following "slice". (iii) New coordinate, n' of the boundary just moved.

As elution proceeds the ten boundaries containing the solutes from the original "slug" injected are moved along the column at various rates according to the mobile phase in their immediate vicinities. As a result, the boundaries are no longer evenly spaced at 10 "sub-slices" but become closer or further apart depending on the solvent or mobile phase that moved across them. It should be noted that "slice" and "sub-slice" are fixed units of measurement and are not changed throughout the elution. Only the separations between the boundaries (in "sub-slice" units) are changed by the elution. By this process, the "slug" of solvent is moved further and further ahead of the boundaries which are retained as the solutes they carry are retained. The concentration of solute at each boundary also remains unchanged in the elution. This process is repeated many times as the "slug" is moved down the column by steps of 10 "sub-slices" each time.

After a sufficient length of elution for the sample solvent to move well ahead of all the boundaries, the chromatogram is constructed by a two-step process. First, the solute at each boundary is allowed to spread backward evenly to fill the gap between the boundary and the boundary eluting after it, *i.e.*

$$I_i = I_0/x$$

where I_i = intensity (solute content) at each of the "sub-slices" between the boundary under consideration and that eluting after it, I_0 = intensity (solute content) at the boundary under consideration and x = number of "sub-slices" between the boundary under consideration and that eluting after it. Secondly, the "sub-slices" are re-

grouped in tens into new "slices" and the final chromatogram is obtained from these re-grouped "slices" by plotting the combined group intensity of each "slice" against the "slice" number. This is the simulated chromatogram.

In the process of developing the program, it was found necessary to introduce a factor to take account of the fact that the column may be perturbed or temporarily modified by the solvent that passes through it. This factor, B, allows the retention ratio of a "slice", P'_{i+1}, to be determined by that in the solvent that is moving into the "slice", P_{i+1}, as well as by that in the solvent that is being replaced, P_i:

$$P'_{i+1} = BP_{i+1} + (1 - B) P_i$$

effectively this means that some sample solvent is retained on the column to the extent that it affects the retention capacity of the column with respect to the solute.

Factor B can vary from 1.0 to 0.0. At a value of 1.0, no "memory effect" is operating in the system and the retention ratio depends solely on the solvent moving into the "slice". In such a case, the simulated chromatogram essentially shows a broad peak with a retention ratio near to that of the solute in the system's mobile phase. With lower B values, the peak splits into two distinct peaks, and as B becomes smaller, the later eluting peak becomes weaker compared to the earlier eluting peak. It would seem that a smaller B value indicates a greater degree of perturbation by the injected solvent of the liquid–liquid equilibrium or the liquid–solid equilibrium in the column. The perturbation sets in as the injected solvent segment passes through the column and part of this solvent is adsorbed onto the column. This produces an adsorbed layer which has a significantly altered retention capacity from that of the column. On elution, the adsorbed layer is gradually stripped by the mobile phase until, eventually, the column returns to its original state. The stronger the interaction between the injected solvent and the column, the smaller is the value of B and the longer it will take for the column to recover from the perturbation. Thus B is characteristic of a particular system comprising the solvent, mobile phase and column and it affects the elution because the solvent acts as a variable modifier of the chromatographic systems, *i.e.*, it can be eluted either as a compact band or as a broad and grossly tailing band. However, there is no simple relationship between B and the capacity ratio of the solvent in the mobile phase used because the large amount of solvent involved represents a situation of overloading in chromatography.

Thus, the computer program "LCP1" was developed. The input parameters for this program are as follows: P = retention ratio of the solute in the injected solvent; P_0 = retention ratio of the solute in the mobile phase; B = factor determining the "memory effect" of the injected solvent on the column and F = total equivalent number of "slices" in the experiment.

The run time of the program depends on the total number of "slices" that the final chromatogram is to be made up of, *i.e.*, on the extent of simulated elution. For F = 50, the simulated chromatogram is made up of five times the length of the injected segment and the run time is 8 min. For F = 100, it is about 30 min. As the elution proceeds, the earlier eluting peak tends to be reduced in intensity as it is spread out further.

It may be noted that simulated chromatograms produced in this way do not fully match the actual HPLC chromatograms because the elutions have not been

carried out long enough for the peaks to broadened in accordance with the limitation of the column efficiency. To achieve full matching would require much larger computing capacity and time and also the incorporation of a suitable band-broadening factor. This was not pursued because the simulated chromatograms obtained by LCP1 were already capable of showing peak-distortion trends and were adequate for an understanding of the phenomenon.

RESULTS AND DISCUSSION

Normal phase system

For a normal phase silica column, injection of the test sample in a solvent of stronger eluting power than the mobile phase can cause the chromatographic peak to split into two well resolved peaks. Fig. 2 shows this effect for decamethrin, a synthetic pyrethroid insecticide, on a silica column with a mixture of hexane–dichloromethane–ethanol as the mobile phase. It is seen that about 4/5 of the decamethrin were eluted at 1.61 min ($k' = 0.40$) while only 1/5 was eluted at 1.94 min ($k' = 0.68$) when dichloromethane was used as the solvent. The peak at 1.61 min was eliminated when samples dissolved in hexane or the mobile phase were injected.

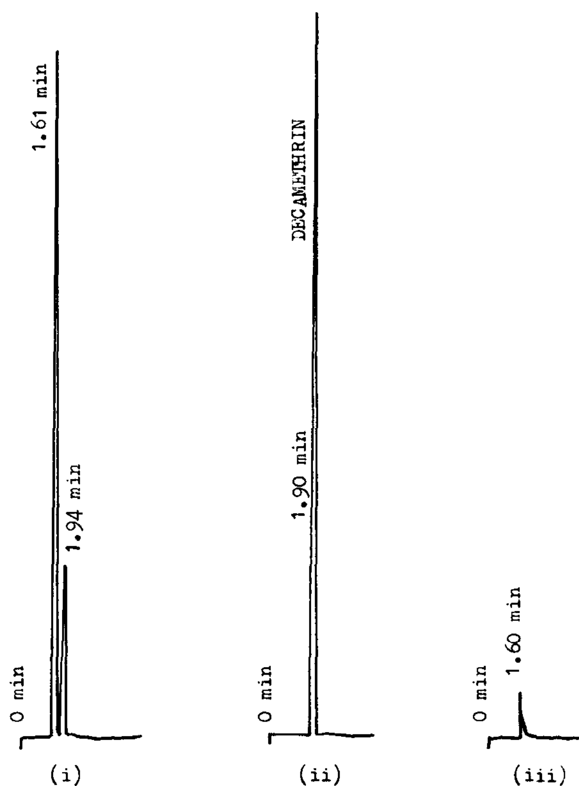

Fig. 2. Chromatograms of decamethrin dissolved in (i) dichloromethane and (ii) mobile phase. Silica column with a mobile phase of hexane–dichloromethane–ethanol (120:9:1.5) at 2.0 ml/min. (iii) Blank chromatogram obtained by injecting dichloromethane. Detection at 254 nm.

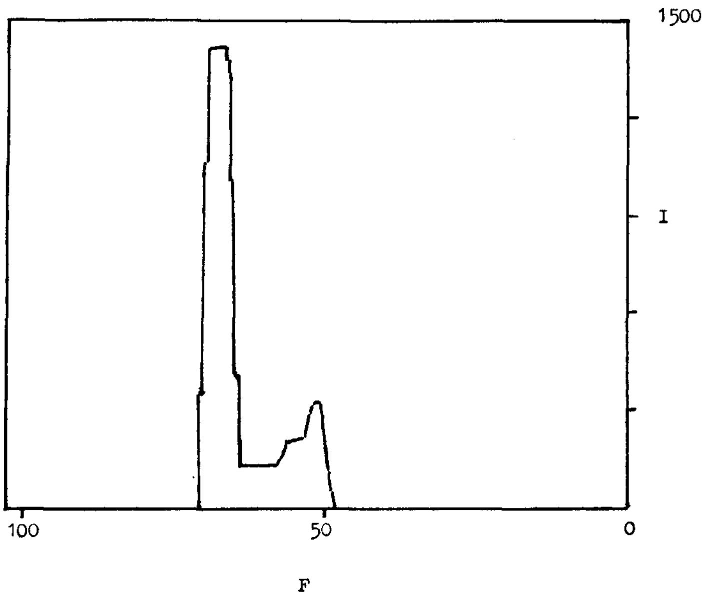

Fig. 3. Simulated chromatogram obtained with the computer program LCP1 and using $P = 0.70$, $P_0 = 0.50$, $B = 0.75$ and $F = 100$ (see text for notations).

Blank determinations showed that 1.60 min is the retention time of dichloromethane in this system. Hence, it is clear that the earlier eluting peak is an artifact caused by the use of an unsuitable solvent for injecting the sample. It may be noted that this peak contains decamethrin as well as dichloromethane, but because the corresponding peak of dichloromethane in the blank determination was small, the area of this peak can be taken to represent the decamethrin content.

Computer simulation of this chromatogram was achieved using suitable parameters ($P = 0.70$, $P_0 = 0.50$, $B = 0.75$) and is shown in Fig. 3. Two distinct peaks can be seen with similar intensity ratio to that in Fig. 2, *i.e.*, the earlier eluting peak is much more intense than the later one. The computer simulation also showed that with $B = 0.75$, the earlier eluting peak has a retention ratio of 0.683 after a run of ten times the length of the injected segment. This means that when the peak finally emerges from the column its retention ratio will still be close to that in the injected solvent (0.70) and would produce a chromatogram similar to that observed as shown in Fig. 2.

With a different mobile phase, hexane–tetrahydrofuran, a different type of distorted chromatogram was obtained, Fig. 4. Injecting the sample dissolved in tetrahydrofuran also caused the peak to split, but, contrary to Fig. 2, the earlier eluting peak in this case was weaker than the later one. A higher value of B was required to simulate this chromatogram, Fig. 5 ($P = 0.50$, $P_0 = 0.30$, $B = 0.90$). In this case, with $B = 0.90$, the retention ratio of the earlier eluting peak decreased very rapidly on elution and it is clear that when it eventually emerges from the column it will have a retention ratio closer to that in the mobile phase (0.30). This would closely resemble

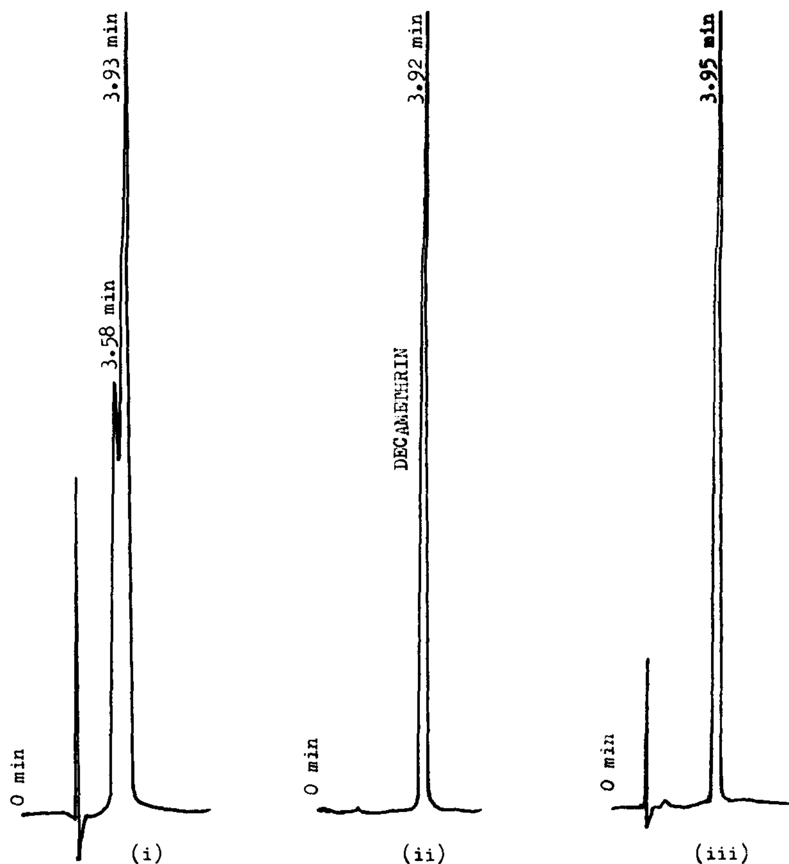

Fig. 4. Chromatograms of decamethrin dissolved in (i) tetrahydrofuran; (ii) mobile phase and (iii) hexane. Silica column with a mobile phase of hexane–tetrahydrofuran (90:10) at 1.8 ml/min. Detection at 278 nm.

the situation in Fig. 4. The broader peak width in Fig. 5 is also consistent with the width of the doublet peak in Fig. 4.

From the two cases described above, it would seem that injecting a "stronger" solvent would cause an additional peak to appear before the "normal" peak. This additional peak may be more or less intense than the "normal" peak depending on whether factor B is small or large.

The effect of the solvent, however, is not always as predictable as that shown in the above case. Multiple peaks can occur in certain systems, especially when the solvent is not a component of the mobile phase and can act as a significant modifier of the mobile phase. We have observed this in a system where decamethrin dissolved in dichloromethane was injected into a silica column and eluted with hexane–ethanol (998:2). Up to four additional peaks were found before the "normal" peak. Computer-simulated chromatograms with multiple peaks can be produced by the use of certain parameters, but good matching cannot be achieved probably because the situation is more complex than that assumed in this computer simulation.

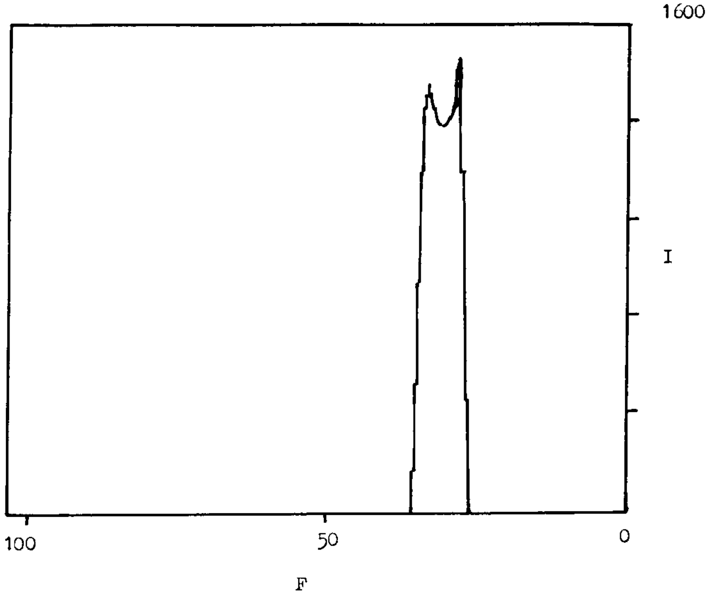

Fig. 5. Simulated chromatogram obtained with the computer program LCP1 and using $P = 0.50$, $P_0 = 0.30$, $B = 0.90$ and $F = 100$ (see text for notations).

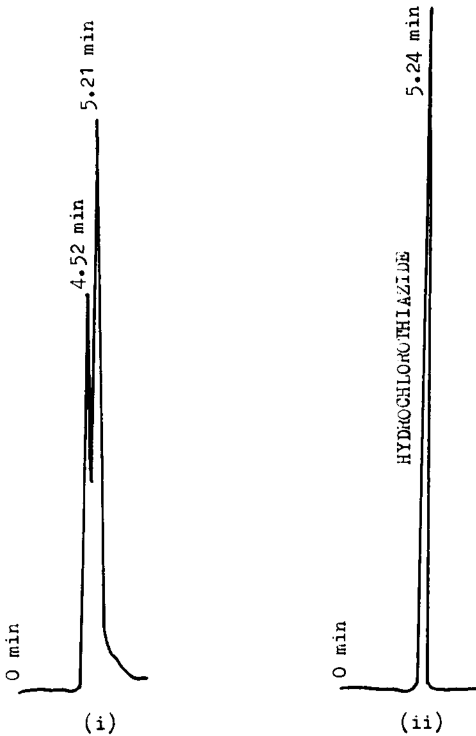

Fig. 6. Chromatograms of hydrochlorothiazide dissolved in (i) acetonitrile and (ii) mobile phase. RP-18 column with a mobile phase of acetonitrile–water–acetic acid (18:81:1) at 1.0 ml/min. Detection at 275 nm.

Reversed-phase system

With a reversed-phase column, similar peak distortions were also observed. The chromatograms for hydrochlorothiazide chromatographed on an RP-18 column using a mobile phase of acetonitrile–water–acetic acid is shown in Fig. 6. In this system, hydrochlorothiazide dissolved in the mobile phase was eluted as a sharp peak at 5.24 min. When dissolved in acetonitrile (a "strong" solvent in the reversed-phase system), the peak splits into two. The second peak at 5.21 min corresponds to the "normal" peak but is much broader with tailing. This is similar to the situation in Fig. 4 and is simulated also by Fig. 5.

Another reversed-phase system studied is the chromatography of caffeine and salicylamide on an RP-18 column with the same mobile phase as above. In this system the effects of acetonitrile, water and water–acetic acid as injection solvents were investigated. The resulting chromatograms are shown in Fig. 7. With the concentrations normalized to 1.0 mg/ml, the peak heights for the two compounds are as shown in Table I.

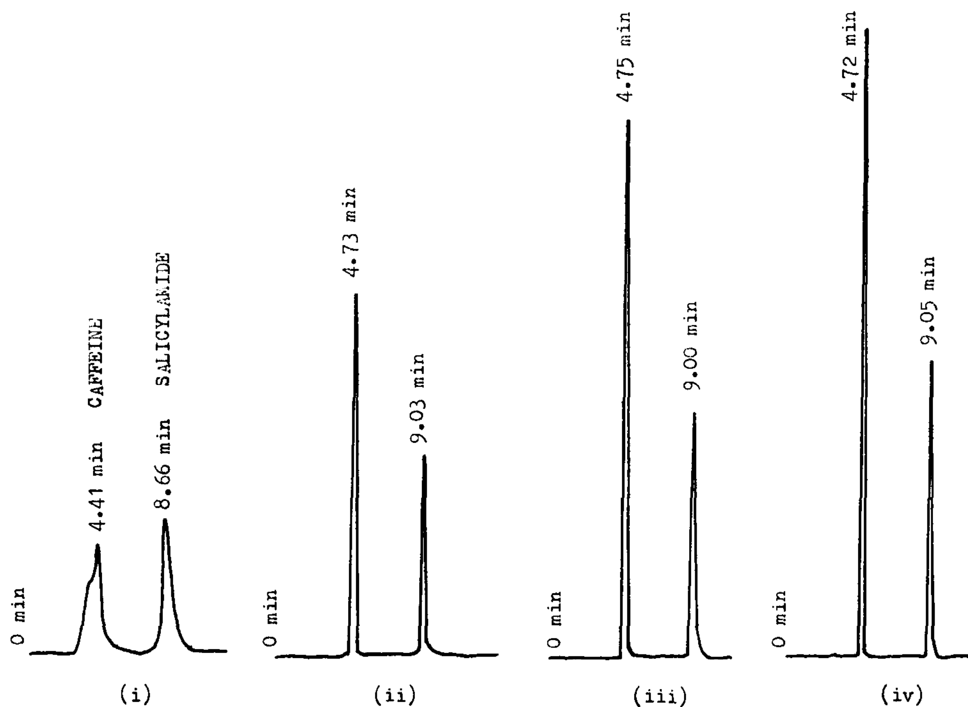

Fig. 7. Chromatograms of caffeine and salicylamide dissolved in (i) acetonitrile, (ii) mobile phase, (iii) water–acetic acid (81:1) and (iv) water. RP-18 column with a mobile phase of acetonitrile–water–acetic acid (18:81:1) at 1.0 ml/min. Detection at 275 nm.

TABLE I

VARIATION OF PEAK HEIGHT WITH SOLVENT IN RP-HPLC

Mobile phase: acetonitrile–water–acetic acid (18:81:1). Column: RP-18. Peak heights given are from determinations at lower concentrations and normalized to 1.0 mg/ml.

Solvent	*Peak height (cm) at 1.0 mg/ml*	
	Caffeine	*Salicylamide*
(1) Acetonitrile	70 (peak has shoulder)	13 (broad)
(2) Mobile phase	400	32
(3) Water–acetic acid (81:1)	570	40
(4) Water	680	45

It is therefore clear that the solvent can affect the chromatographic assay significantly, especially when peak heights are used for the calculation. A "stronger" solvent such as acetonitrile causes peak splitting and broadening, while a "weaker" solvent such as water causes the peak to become sharper and higher.

Both compounds were eluted faster when a "stronger" solvent was used for injecting the sample, *i.e.*, the retention time is reduced with a "stronger" solvent. The simulated chromatogram shown in Fig. 4 seems to be appropriate even in this case provided a higher degree of band broadening is applied. In Fig. 7i, the peak of caffeine has a shoulder at its leading edge as does that in Fig. 4. On the other hand, the broadened peak of salicylamide in Fig. 7i may indicate that the retention ratio of salicylamide in the solvent is quite close to that in the mobile phase so that its shoulder is not easily discernible.

Computer simulation with low values of P, representing solvents of low elution strength, showed that the chromatographic peaks become sharper and higher. This is in agreement with the results shown in Table I.

Ion-pair reversed-phase system

With ion-pair reversed-phase chromatography the distorted peak is slightly different in shape. Fig. 8 shows the chromatograms for nicotinamide on an RP-8 column eluted with a mixture of methanol and a phosphate buffer containing hexanesulphonic acid as the ion-pairing agent. It is seen that injecting nicotinamide in methanol caused the peak to split into an earlier and a later peak. This chromatogram is fairly similar to the simulated one given in Fig. 9 (P = 0.70, P_0 = 0.50, B = 0.90). However, the situation in ion-pair reversed-phase chromatography is probably more complex because the second peak in Fig. 8 is eluted later than expected (5.90 as against 5.50 min). This may be due to the effect that the injected solvent exerts on the interplay of the ion-pair and ion-exchange mechanisms in such a system[5]. Clearly, further study based on a more complex model incorporating this interconversion process is needed for a better simulation of the solvent effect in an ion-pair reversed-phase system.

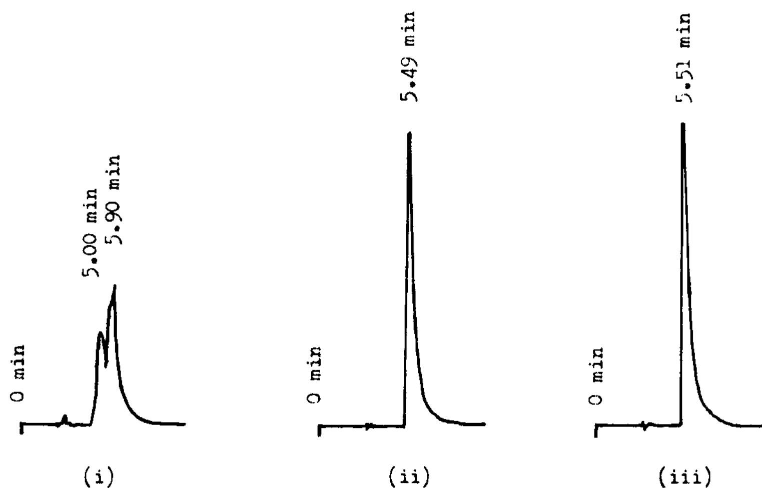

Fig. 8. Chromatograms of nicotinamide dissolved in (i) methanol, (ii) mobile phase and (iii) water. RP-8 column with a mobile phase of methanol–ion-pair buffer (0.4 ml ammonia + 0.95 g sodium hexanesulphonate + 1 l water adjusted with phosphoric acid to pH 2.8) (15:85). Flow-rate: 1.0 ml/min. Detection at 268 nm.

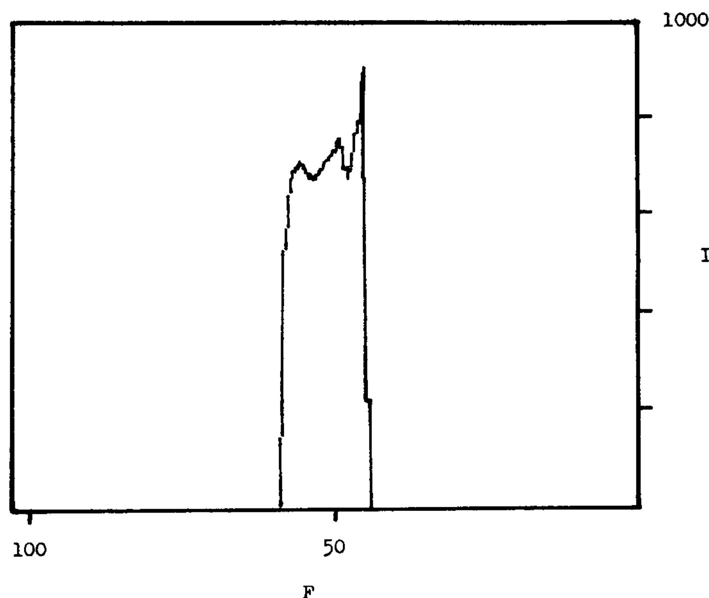

Fig. 9. Simulated chromatogram obtained with the computer program LCP1 and using P = 0.70, P_0 = 0.50, B = 0.90 and F = 100 (see text for notations).

CONCLUSIONS

The use of a suitable solvent for dissolving test samples for injection is a very important, but sometimes overlooked, aspect of high-performance liquid chromatography. Unsuitable solvents can cause serious peak distortions and even multiple peaks. The effect has been shown to be amenable to study by computer simulation. The results of the computer-simulated chromatography showed that the phenomenon is determined essentially by two factors: (1) the difference in retention ratios between the injected solvent and the mobile phase and (2) the changes in the retention capacity of the column due to the adsorption of the solvent on the column.

The computer simulation also showed that solvents which are "weaker" than the mobile phase tend to produce sharper peaks, while those which are "stronger" tend to produce broadened peaks or show multiple earlier eluting peaks. The situation in ion-pair reversed-phase chromatography seems to be more complex and may also involve a shift in the elution mechanisms.

Hence, in liquid chromatographic analyses, it is always good practice to use the mobile phase to dissolve or dilute the test substance. In the event that dissolution presents a problem and a different solvent needs to be used, it is important to ensure that the sample solution and the reference substance solution are closely matched in composition and that there is no significant solvent effect from the solvent used.

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