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INJECTION OF ELUITES IN SOLVENTS STRONGER THAN THE MOBILE PHASE IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A study was made of the distortion and multiplication of peaks that occur when an eluite is injected dissolved in a solvent that is significantly stronger than the mobile phase in reversed-phase liquid chromatography. The solvent strength and volume injected affected peak shape. Column length and diameter, particle size and type of reversed phase did not affect general peak shapes. A qualitative interpretation of the phenomenon is presented.

INTRODUCTION

Chromatographers have long practiced using the mobile phase as the injection solvent for the eluite in liquid chromatography. Many workers have observed abnormal chromatograms when solvents other than the mobile phase were used. Commonly multiple peaks appeared in the chromatogram of a single eluite.

Wu and Wittick[1] observed broad and tailing peaks for vitamins D_2 an B_{12} when the injection solvent was methanol and the mobile phase contained water in reversed-phase chromatography. Using the mobile phase for injection sharpened the peaks and reduced tailing. Tseng and Rogers[2] found with a C_{18} column *o*-, *m*- and *p*-dihydroxybenzenes gave single symmetric peaks when methanol was the injection solvent and the mobile phase. If water was used for injection, each isomer produced a split (double) peak. When water was the injection solvent and the mobile phase, single peaks were produced. When the injection solvent was methanol, the peak from each isomer had a leading shoulder. Kirschbaum *et al.*[3] found that, with an aqueous mobile phase containing methanol, hydrochlorothiazide gave a doublet peak when injected in methanol. However, it gave a single sharp peak using the mobile phase for injection. Tsimidou and Macrae[4] were able to eliminate a number of peak shoulders by reducing the injection volume of chloroform or changing to acetone for injection in triglyceride analysis. Yi *et al.*[5] observed multiplet peaks for purine compounds. Peak multiplicity was altered by altering column temperature and mobile phase methanol concentration. Low *et al.*[6] found peak splitting in the reversed-phase ion pair chromatography of sympathomimetic drugs. They suggested peak splitting was the

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result of competitive ion pairing and *in situ* ion exchange. Ng and Ng[8] simulated peak splitting with a microcomputer program based on a model of changing retention ratio as the injection solvent moved through the column leaving a memory effect in the stationary phase. Perlman and Kirschbaum[9] correlated peak shape with internal hydrogen bonding of the eluite, but Chan and Yeung[10] disagreed with this interpretation. Khachik *et al.*[11] found multiple peak formation in the reversed-phase chromatography of carotenoids. They believed the multiplicity to be dependent on the solubility of the carotenoid in the injection solvent and the mobile phase and on the interaction of the solvent and mobile phase as the injection solvent entered the column.

We wish to report a systematic study of injecting eluites dissolved in strong solvents in reversed-phase high-performance liquid chromatography (HPLC). We studied the peak shape that resulted when weak mobile phases were used. We investigated: (1) amino acids, whose charge was pH controlled; (2) electrically neutral eluites, phenylalkanols differing in the number of methylene groups; and (3) cimetidine, a compound that initially appeared insensitive to injection solvent effects. Column length and diameter, stationary phase type and particle size, injection solvent strength and volume were varied.

EXPERIMENTAL

The HPLC systems consisted of Waters M-6000 pumps; a Waters U6K, a Rheodyne 7125 or 7135 injector or a Waters WISP autoinjector; a Kratos 773 or Perkin-Elmer LC-55 variable-wavelength absorbance detector; and a Fisher Recordall 500, Houston 4511 recorder, or Waters 730 data module. A presaturation column packed with 50-μm silica was inserted between the pump and injector in some of the work. For some of the work, a guard column of 37-μm pellicular C_{18} packing was located between the injector and analytical column.

Columns were obtained from Alltech (Deerfield, IL, U.S.A.), Hamilton (Reno, NV, U.S.A.), Phenomenex (Rancho Palos Verdes, CA, U.S.A.) or packed with a Micromeritics slurry packer.

L-Phenylalanine, L-tryptophan, cimetidine, benzyl alcohol, 2-phenylethanol, 3-phenyl-1-propanol, 4-phenyl-1-butanol and 1-propanol were purchased from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile and methanol were from Burdick & Jackson or Chempure. Buffers were 5 m*M* sodium hydrogenphosphate or sodium dihydrogenphosphate and the pH was adjusted with 85% orthophosphoric acid or 50% sodium hydroxide.

Eluite stock solutions were stored at room temperature. The amino acids disappeared very slowly, but no decomposition products' peaks appeared in their chromatograms. A maximum disappearance of 26% occurred with tryptophan. Other eluites were stable in solution. In studying the effect of injection volume of phenylalkanols, injection solution concentrations were prepared to give a constant 50 ng injected. Except where otherwise noted, the amino acids and cimetidine were injected in 20-μl volumes, and the amount of amino acid was 0.91 μg and the amount of cimetidine was 1.0 μg. Composition of the injection solution and mobile phase are expressed in volume ratios. The detector wavelength used with phenylalkanols was 254 nm, and the wavelength used with amino acids and cimetidine was 210 nm. The flow-rate was 1.0 ml/min.

RESULTS AND DISCUSSION

In general, what was observed with a weak mobile phase when sufficient volume of a strong injection solvent was used for an eluite, was a range of peak distortions from a leading peak to a multiplicity of peaks. The peak obtained using the mobile phase for injection (referred to hereafter as peak a) was always present in the chromatogram as indicated by retention time, and any other peaks appeared at shorter retention times. Commonly one peak (peak b) appeared immediately preceeding peak a and another at the retention time of the injection solvent (peak c). Occasionally more than two peaks appeared prior to peak a.

Solvent strength

Fig. 1. illustrates the development of a double peak when benzyl alcohol was injected into a mobile phase of methanol–water (20:80) and the injection solvent was changed from water to acetonitrile–water solutions. The peak with a retention time (t_R) of 21.3 $\pm$ 0.1 min and a capacity factor (k') of 6.3, was present in all chromatograms. When the injection solvent composition reached 67% acetonitrile, significant leading was apparent. The leading region developed into a second peak with an injection solvent of neat acetonitrile.

Fig. 2 shows the changes in the peak of positively charged phenylalanine when it was injected into an acetonitrile–buffer (8:92) mobile phase from injection solvents of varying strength. Phenylalanine had a significantly smaller capacity factor than the benzyl alcohol previously discussed. A strong peak c developed as the injection solvent strength increased but a distinct peak b was not formed. With another more efficient column that gave a little larger k', however, a distinct peak b was resolved.

Figs. 1 and 2 illustrate the qualitative differences found in the chromatograms obtained when using injection solvents of strength greater than that of the mobile phase. Because peak a was always present in these chromatograms, its height was used as a quantitative measure of changes in the chromatograms.

Table I compares the heights of the phenylalanine and tryptophan peaks. Tryptophan gave leading peaks as the acetonitrile content of the injection solvent increased but no peak splitting. The phenylalanine peak, $k' = 0.9$, was more readily

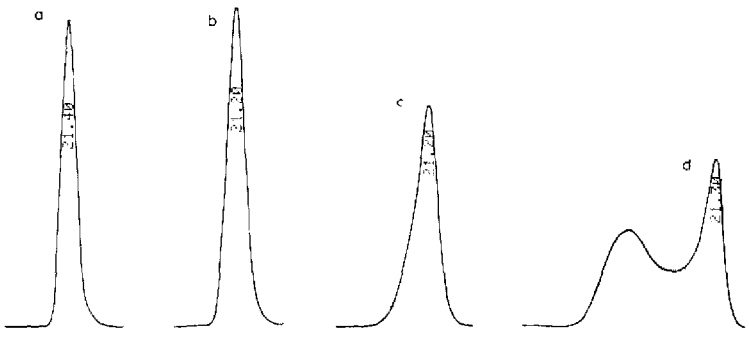

Fig. 1. Deterioration of the benzyl alcohol peak in a mobile phase of methanol–water (20:80). Injection solvent's acetonitrile percent in water: a = 0, b = 50, c = 67, d = 100. Injection volume: 70 μl. Benzyl alcohol concentration: varied.

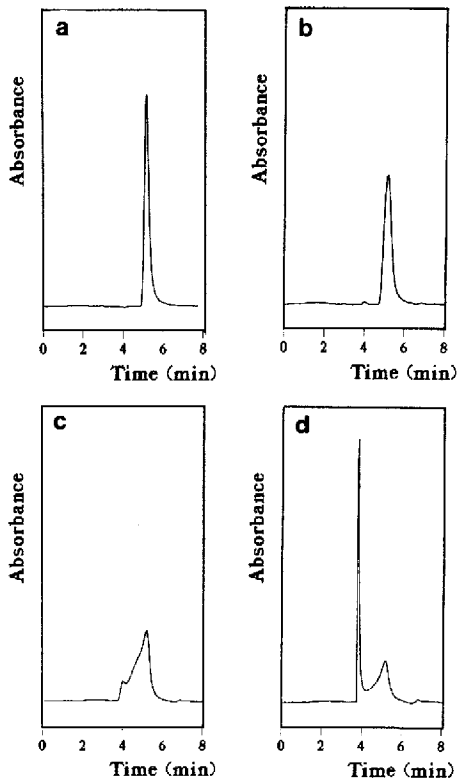

Fig. 2. Deterioration of the phenylalanine peak in a mobile phase of acetonitrile–buffer, pH = 3.5 (8:92). Injection solvent's acetonitrile percent in water: a = 0, b = 30, c = 50, d = 70. Injection volume: 20 μl. Phenylalanine concentration: 46 ng/μl.

TABLE I

HEIGHTS OF PEAK a FOR PHENYLALANINE AND TRYPTOPHAN

Mobile phase, acetonitrile–water (8:92); pH 3.5; column, 25 × 0.46 cm I.D. (10 μm, ODS).

Injection solvent % acetonitrile	*Peak height (h_p) (cm)*	
	Phenylalanine	*Tryptophan*
0		11.5
10	7.2	
20	7.0	11.7
30	6.2	
40	4.5	10.6
50	3.3	
60	1.2	9.7
70	1.0	
80	0.9	7.8
90	1.0	
100	0.6	4.9

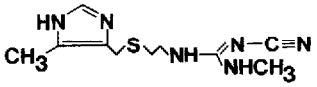

Fig. 3. Structure of cimetidine.

distorted than that of tryptophan, $k' = 2.7$. Cimetidine (Fig. 3), $k' = 6.5$, in the same volume of the same injection solvents as used for phenylalanine and tryptophan gave no peak distortion. Benzyl alcohol with a column and mobile phase that gave a k' of 1.1 as compared to 6.3 for the study shown in Fig. 1 gave multiple peaks when the injection solvent contained only 50% acentonitrile. Thus, the greater k' the less the tendency for the eluite peak to be distorted when the injection solvent was stronger than the mobile phase.

Table II presents peak height changes for phenylalanine and tryptophan when the organic component of the injection solvent was changed to 1-propanol. The tryptophan results were expected. The 1-propanol being a stronger solvent than acetonitrile caused a drop in peak height of over 30% when present at 40% in the injection solvent. By comparison (Table I) acetonitrile at that volume % level in the injection solvent caused less than a 20% drop in peak height. Furthermore, the tryptophan peak was split into a peak b and a. The splitting did not occur with acetonitrile.

The results with phenylalanine, however, were not expected. First, not shown in Table II, the phenylalanine peak never showed any signs of splitting, not even a shoulder. Second, the retention time of the peak decreased as the 1-propanol content increased. Third, the peak height rose initially as the 1-propanol content increased, and this rise was markedly higher than the drop in k' dictated. The results show that, with respect to sensitivity, injecting in 60% 1-propanol is more optimal than injecting

TABLE II

INJECTION OF PHENYLALANINE AND TRYPTOPHAN IN 1-PROPANOL–WATER SOLUTION

Mobile phase and column as in Table I

Injection solvent % 1-propanol	*Phenylalanine*[a]		*Tryptophan*	
	t_R *(min)*	h_p *(cm)*	t_R *(min)*	h_p *(cm)*
0	7.6	7.1		
10	7.2	7.4	15.7	10.6
20	7.1	8.2	15.7	9.9
30	7.0	9.0	15.7	8.6
40	7.0	10.1	15.7	7.0
50	6.9	11.6	15.5	6.3
60	6.8	11.7	15.7	4.6
70	6.7	11.3	15.6	4.0
80	6.5	11.1	15.8	3.5
90	6.4	9.9	15.7	3.2
100	6.4	9.5		

[a] $t_R = 6.4$, $h_p = 9.5$ for injection solvent of the mobile phase.

TABLE III

INJECTION OF NEAT STRONG SOLVENT AFTER TRYPTOPHAN INJECTION

All injections 20 μl. Mobile phase and column as in Table I.

Injection solvent % acetonitrile	*Initial injection*		*Tryptophan injection then 1-propanol*[a]		*Tryptophan injection then acetonitrile*[a]	
	t_R *(min)*	h_p *(cm)*	t_R *(min)*	h_p *(min)*	t_R *(min)*	h_p *(cm)*
0	11.9	14.4	10.4	7.7	11.4	11.4
20	12.2	14.8	10.6	7.1	11.4	10.7
50	11.9	11.8	10.5	7.2	11.6	10.0
70	11.7	8.6	10.0	6.5	11.6	8.3
100	11.0	5.4	9.7	4.5	11.2	4.3

[a] Injection of neat organic solvent followed the tryptophan injection 2 min later.

phenylalanine in the mobile phase. The pattern of peak height decrease with increasing solvent strength did not appear until levels of 1-propanol greater than 60% were reached. In all of our studies, these results with phenylalanine and 1-propanol were the only results that did not fit the pattern of peak deterioration with increasing solvent strength.

The purpose of the study shown in Table III was to determine if peak distortion would occur if neat organic solvent was injected after tryptophan had been injected into the column and had moved about 20% of the column length. Tryptophan injection in solvents of varying strength was followed 2 min later by an injection of neat acetonitrile or 1-propanol. The neat solvent injection caused peak leading, and this was indicated by a loss of peak height with both acetonitrile and 1-propanol. The neat solvent caused further peak leading even when the peak was already distorted because the tryptophan had been injected in a strong solvent. The effect of 1-propanol was greater than the acetonitrile effect in causing leading. Most significant in the results was that the leading caused by delayed injection of neat solvent was always smaller than the leading caused by injection in the neat solvent, *i.e.*, peak height (h_p) was always >5.4. We interpret this to mean the neat solvent band was diluted by the mobile phase before it reached the eluite band, and its effect was, therefore, diminished. This interpretation suggests that the cause of peak distortion when injecting in strong solvents acted very early in the column before the injection plug became too dilute.

To answer the question of whether the peak leading and the extra peaks formed might be the result of the appearance of new eluites, the sum of peak areas was measured in each chromatogram. With both phenylalanine and tryptophan, the total area remained constant when the amount of eluite injected was constant regardless of the shape of the peak(s). These results are consistent with those of others who showed by mass spectroscopy that the eluite was producing all of the split peaks[6]. Thus, new eluites were not being introduced by chemical or physical means, and then these producing asymmetry and multiple peaks.

Injection volume

Fig. 4 shows the increase of peak height as the injection volume of benzyl alcohol solutions increased. When the injection solvent was weak, 33% acetonitrile,

the peak height was proportional to injection volume. However, when the solvent contained 67% acetonitrile, the peaks were shorter than they should have been at injection volumes greater than 30 μl. When the injection solvent was neat acetonitrile, linearity, if it was present at all, ended at very low injection volumes. The peaks were shorter than they were with an injection solvent of 67% acetonitrile.

Cimetidine was injected in neat methanol with no peak distortion in a method for its determination in blood and plasma[12]. In our initial studies, cimetidine was unusual because it showed no peak distortion in going from a weak to a strong injection solvent using acetonitrile or 1-propanol. However, this initial work used injections of 20 μl or less. Fig. 5, in which cimetidine's peak height is shown as a function of injection volume shows that cimetidine's peak height was significantly lower when it was injected in a strong solvent provided the injection volume was large enough.

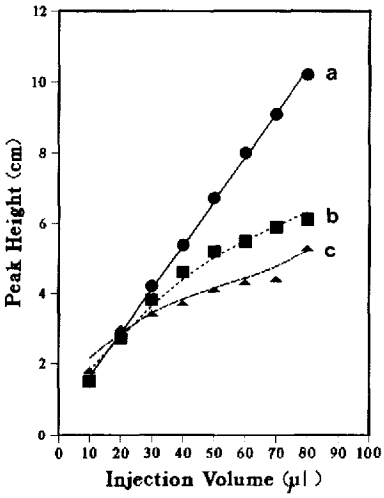

Fig. 4. Relationship between injection volume and peak height of benzyl alcohol injected into a mobile phase of methanol–water (20:80). Injection solvent's acetonitrile percent in water: a = 33, b = 67, c = 100.

In a more systematic study, the loss of peak height of a series of phenylalkanols was investigated. The loss of peak height was accompanied by peak leading as injection volume was increased and eventually the appearance of multiple peaks preceeding peak a. Table IV summarizes the results of phenylalkanol injection in acetonitrile–water (50:50) and Table V summarizes the results when the injection solvent was neat acetonitrile. In all cases peak height loss increased with increasing injection volume, and the losses were always greater when neat acetonitrile was the injection solvent. When either injection solvent was used, there appeared to be a slight tendency for the rate of peak height loss with injection volume to be greater, with lower molecular weight phenylalkanols.

Stationary phase

Tables VI and VII show the results of injecting 4-phenyl-1-butanol into a variety of columns using, respectively, 50% and neat acetonitrile. In all cases, peak leading

TABLE IV

INJECTION OF $C_6H_5(CH_2)_nOH$ IN VARYING VOLUMES OF 50% ACETONITRILE

Mobile phase, methanol–water (30:70); column, 8 × 0.46 cm I.D. (5 μm, methyl)

Injection volume (μl)	*h_p loss (%)*			
	n = 1	*n = 2*	*n = 3*	*n = 4*
2.0	0	0	0	0
4.0	8	11	8	9
8.0	12	17	17	14
16.0	33	36	31	26
32.0	62	61	58	53[a]
64.0		78	68	

[a] 73% loss with a 96-μl injection.

occurred as the volume increased and peak multiplicity appeared at the higher volumes. The loss of peak height was very similar from column to column even though the stationary phase changed. The linear velocity of the mobile phase also changed, despite the use of the same flow-rate (1.0 ml/min) throughout. Similar results between short and long columns suggest again that peak changes occurred at the beginning of the column. Consistent with other results, the loss of peak height was greater when the injection solvent was neat acetonitrile as compared to 50% acetonitrile. Despite the differences in columns, solvophobic sorption was common to all the chromatography indicated in Tables VI and VII. This similarity suggests that it is in the sorption process that peak distortion occurs.

Mobile phase

Provided sufficient injection volume was used, peak distortion occurred to the extent that there was a difference between the mobile phase strength and that of the injection solvent. Thus, when tryptophan dissolved in neat acetonitrile was injected into a mobile phase of acetonitrile–water (15:85) ($k' = 2.1$), the peak height loss was

TABLE V

INJECTION OF $C_6H_5(CH_2)_nOH$ IN VARYING VOLUMES OF NEAT ACETONITRILE

Same chromatographic conditions as in Table IV

Injection volume (μl)	*h_p loss (%)*			
	n = 1	*n = 2*	*n = 3*	*n = 4*
2.0	0	0	0	0
4.0	20	21	20	21
8.0	49	48	47	43
16.0	64	70	68	66
32.0	86	84	82	81
64.0		93	93	

TABLE VI

INJECTION OF 4-PHENYL-1-BUTANOL INTO DIFFERENT COLUMNS IN 50% ACETONITRILE

Column	h_p *loss (%)*				
	2.0 µl	*4.0 µl*	*8.0 µl*	*16.0 µl*	*32.0 µl*
10 × 0.46 cm I.D. 10 µm, SDVB[a]	0	9	17	29	59
15 × 0.42 cm I.D. 10 µm, SDVB	0	8	19	38	47
25 × 0.46 cm I.D. 10 µm, ODS	0	12	21	32	61
10 × 0.46 cm I.D. 5 µm, octyl	0	7	19	35	51
8 × 0.70 cm I.D. 3 µm, ODS	0	8	17	28	54
8 × 0.46 cm I.D. 5 µm, methyl	0	9	14	26	53

[a] Styrene–divinylbenzene copolymer or PRP-1.

48%. But, when the same injection was made into acetonitrile–water (35:65) ($k' = 0.4$) the loss was 12%.

In Table VIII is shown the effect of pH on the peak height of phenylalanine. At the pH values used, phenylalanine was electrically positive, net neutral, and negative. Regardless of charge, the peak underwent the changes observed with other eluites when the injection solvent strength was changed. At all three pH values, the magnitude of the peak height change was similar at a given solvent composition and the appearance of peak b occurred at the same solvent composition.

TABLE VII

INJECTION OF 4-PHENYL-1-BUTANOL INTO DIFFERENT COLUMNS IN NEAT ACETONITRILE

Column	h_p *loss (%)*				
	2.0 µl	*4.0 µl*	*8.0 µl*	*16.0 µl*	*32.0 µl*
10 × 0.46 cm I.D. 10 µm, SDVB	0	18	36	58	80
15 × 0.42 cm I.D. 10 µm, SDVB	0	24	40	64	73
25 × 0.46 cm I.D. 10 µm, ODS	0	28	31	66	88
10 × 0.46 cm I.D. 5 µm, octyl	0	19	38	53	79
8 × 0.70 cm I.D. 3 µm, ODS	0	18	29	54	71
8 × 0.46 cm I.D. 5 µm, methyl	0	21	43	66	81

TABLE VIII

EFFECT OF MOBILE PHASE pH ON PEAK HEIGHT OF PHENYLALANINE AT DIFFERING SOLVENT STRENGTHS

t_R(min): 5.8–6.1 (pH 3.5), 6.4–6.6 (pH 5.5), 5.9–6.1 (pH 7.6).

Injection solvent % acetonitrile	h_p *(cm)*		
	pH 3.5	*pH 5.5*	*pH 7.6*
0		8.0	
10	7.2	7.4	7.2
20	7.0	6.6	
30	6.2	6.2	4.8
50[a]	3.3	2.5	2.7
60	1.2	2.0	
70	1.0	1.8	
80	0.9	1.4	1.7
90	1.0	1.3	
100	0.6	1.0	1.3

[a] At each pH peak b appeared at 50% and higher levels of acetonitrile.

Interpretation

When an eluite is injected dissolved in a solvent of mobile phase composition, it is distributed initially at the top of the column in diminishing concentration because the head of the injection plug is depleted of eluite through sorption by the stationary phase as the plug moves into the column. But a symmetric peak quickly develops as the mobile phase moves the eluite along[13]. The initial concentration gradient is steeper for larger capacity factors. As the strength of the injection solvent is increased, the eluite concentration left at the very top of the column as the plug moves along is less but enough to produce a peak with a consistent retention time (peak a). The greater

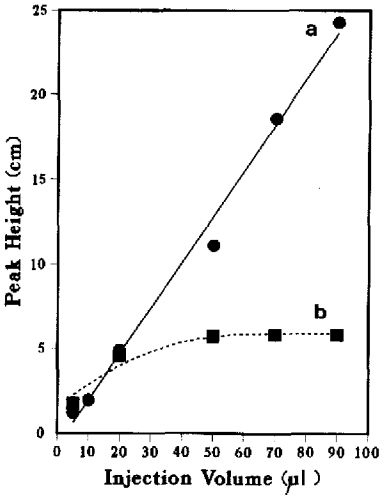

Fig. 5. Relationship between injection volume and peak height of cimetidine injected into a mobile phase of acetonitrile–buffer (15:85). Injection solvent: a = water, b = acetonitrile.

k', the higher the amount left at the top of the column. The remaining amount is carried further into the column by the solvent plug. This extension of eluite sorption produces a leading peak when the solvent is not too strong, but increasing the solvent strength extends the eluite sorption even further and a peak (b) is produced. With larger capacity factors, the amount sorbed at the column top is larger so that the solvent strength required to produce peak b must be greater. As the solvent strength becomes very great, the plug carries some of the eluite with it to the end of the column (peak c). The capacity factor must be small enough in this case to permit the plug to retain enough eluite to produce a peak. One could imagine a hypothetical case in which the solvent is so strong that the eluite is not retained and all of the eluite is found in peak c.

The injection volume effect is the result of rapid dilution of the solvent as it moves through the column. When the volume is small, rapid dilution of the injection solvent with the mobile phase puts the eluite in the same condition it would have been in had the injection solvent been mobile phase. As the volume for injection is increased, there is less dilution of the center of the plug. Thus, the solvent can carry the eluite away from peak a to produce peaks b and c as discussed above.

This view of the production of leading and multiple peaks requires no "memory" in the stationary phase[8]. Nor does it consider the hydrocarbon stationary phase modified significantly by the injection solvent.

Occasionally more than one peak appeared between peaks a and c. This may be the result of plug dilution in the pre-column hardware such as the injector. Or it may be that injection in strong solvents accentuates the effects of poor packing on peak shape, and the appearance of more than one peak between peaks a and c was the result of defective packing.

Using this view of the production of peak distortion and multiplicity, we are presently investigating this phenomenon by computer simulation.

We have not developed an interpretation of the peak height and plate number improvement through increasing the ratio of 1-propanol to water in the injection solvent for phenylalanine. The effect is reproducible, and the peak as described in Table II is taller when neat 1-propanol is used than when neat water is used. We believe the phenomenon is related to the relative rates of band movement of phenylalanine and 1-propanol.

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