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EFFECT OF ORGANIC SOLVENTS IN SAMPLE SOLUTIONS AND INJECTION VOLUMES ON CHROMATOGRAPHIC PEAK PROFILES OF ANALYTES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The effects of organic solvents in sample solutions on chromatographic peak profiles, assessed by retention time, peak height and peak width, were different for acetronitrile than for methanol in reversed-phased high-performance liquid chromatography. Two benzimidazole carbamate degradation products of the fungicide benomyl were studied: methyl-2-benzimidazole carbamate (MBC) and 3-butyl-2,4-dioxo[1,2-*a*]-*s*-triazinobenzimidazole (STB). The overall effect was more noticeable with STB than with MBC. Peak splitting was observed only with STB when larger volumes of solvents were injected. Peak broadening was observed even with 10-μl injections in some instances. In general, as the percentages of organic solvents were increased in sample solvents, greater deterioration of the peak profiles was observed. This, however, was not always so with STB.

INTRODUCTION

Extensive studies of the importance of the use of appropriate mobile phase solvents in reversed-phase high-performance liquid chromatography (RP-HPLC) have been carried out. Some of these are closely related to the topics discussed in this paper, including selectivity, retention properties and peak shapes[1–4].

The importance of the effect of solvent compositions in which analytes are dissolved has received much less attention than that of mobile phase composition, but it is gradually gaining recognition. For example, unexpected peaks produced as a result of an inappropriate choice of sample solvent were called ghost peaks[5] or system peaks[6]. Williams *et al.*[7] found peak broadening of the analytes when the percentage of organic solvent in the sample solution was increased. Peak distortion including peak splitting has also been observed by other workers[8,9]. Chiba and Singh[10] stated

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that the resolution of two compounds that they tested was strongly influenced by the percentage of organic solvent, pH and buffer strength of sample solutions. Perlman and Kirschbaum observed that the extent of the effect of sample solvents was dependent on the compound to be analyzed[11]. Recently, Hoffman *et al.*[12] reported on the distortion and multiplication of peaks when the sample solution was significantly stronger than the mobile phase. It is clear from the above that further studies are required in order to understand fully the complicated effects associated with solvent composition in order to perform efficient RP-HPLC analyses.

In this work we used two specific benzimidazole carbamate degradation products of the fungicide benomyl: methyl-2-benzimidazole carbamate (MBC) and 3-butyl-2,4-dioxo[1,2-*a*]-*s*-triazinobenzimidazole (STB) (Fig. 1). Chiba and Singh[10] developed a method for determining these two compounds individually in water. During the course of the method development, they noticed the importance of the composition of the sample solutions.

We report here some findings of our systematic studies and discuss the extent of the effect of the type and percentage of organic solvents in sample solutions on the retention times and peak profiles of the two benzimidazole compounds in relation to the injection volumes of samples.

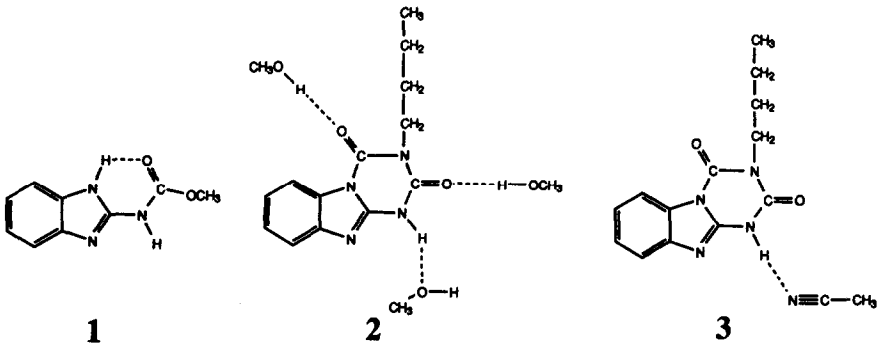

Fig. 1. Structures of MBC (1) and STB (2 and 3), and hydrogen bonding and solute–solvent interactions for MBC and STB. 1, MBC intramolecular hydrogen bonding; 2, STB–methanol intermolecular hydrogen bonding; 3, STB–acetonitrile intermolecular hydrogen bonding.

EXPERIMENTAL

Material used

MBC and STB were of analytical-reagent grade. The solvents used were HPLC-grade methanol and acetonitrile from Caledon Labs. (Georgetown, Ontario, Canada). Disodium hydrogenphosphate and potassium dihydrogenphosphate, used in buffer solutions, were of analytical-reagent grade from BDH (Poole, U.K.).

Preparation of sample solutions

The basic sample solution contained 5 μg/ml each of STB and MBC, and the composition of solution was acetonitrile–methanol–0.07 *M* phosphate buffer (pH 7.0)–water (5:5:10:80, v/v). Stock solutions of STB and MBC (both at 100 μg/ml)

were initially prepared in acetonitrile and methanol, respectively. The use of individual solvents was necessary because these compounds did not dissolve well in other solvents.

To study the influence of acetonitrile, sample solutions were prepared by using concentrations of acetonitrile of 5, 25, 35 and 50%. Similarly, the influence of methanol was studied at concentrations of 5, 25, 35 and 50%.

In addition to the above, three concentrations of STB and MBC standard solutions were prepared, at 1.0, 0.5 and 0.25 μg/ml. The composition of the solvents used for these solutions was identical with that of the above 5 μg/ml standard.

HPLC analysis

The HPLC unit used was a Hewlett-Packard HP-1090 LC system equipped with an HP-79835A solvent-delivery system, an HP-79846A autoinjection module with programmable injection capability in the volume range 1–250 μl using a syringe-loop injector, an HP-1040A diode-array detector and an HP-85B personal computing system.

The analytical column used was a Regis Hi-Chrom reversible column of 5-μm Spherisorb ODS (15 cm × 4.6 mm I.D.), which was preceded by a precolumn (5 cm × 4.6 mm I.D.) dry packed with Co PELL ODS, 37–40 μm (Whatman).

The mobile phase used throughout the study was acetonitrile–water–buffer solution (35:55:10, v/v), run isocratically at a flow-rate of 1 ml/min.

Sample solutions prepared as above were injected by the autoinjector with a computerized variable-volume injector using injection volumes of 10, 50, 100 and 200 μl for each sample.

The detector wavelength used was 280 nm throughout, and absorbance spectra were recorded from 240 to 340 nm at the apex of each chromatographic peak whenever required.

The results of individual chromatographic runs were evaluated by analyzing retention time, peak height, peak width and peak area. Peak-height and peak-area counts at higher injection volumes (50, 100 and 200 μl *vs.* 10 μl) were divided by corresponding factors of volume increases (5, 10 and 20 for 50-, 100- and 200-μl injections, respectively) so that all the data obtained could be directly compared.

RESULTS

Influence on retention time

As the concentration of acetonitrile in the sample solutions was increased from 5 to 50%, the retention times of STB and MBC decreased. This reduction in retention time became greater at higher injection volumes, and was more pronounced for STB than MBC. For example, for a 200-μl injection, the retention times of STB and MBC decreased by 11.9% and 6.9%, respectively, as the concentration of acetonitrile in the sample solutions was increased from 5 to 50%.

An interesting observation was made with methanol. When its concentration was increased from 5 to 50%, the retention time of STB increased but that of MBC decreased. This trend was more pronounced at large injection volumes. For example, for STB, increases in retention time of 0.7, 2.6, 4.5 and 6.3% were observed when 10, 50, 100 and 200 μl of samples, respectively, were injected. In contrast, for MBC,

reductions in retention time of 0, 0.6, 1.2 and 3.0%, respectively, were observed under the same conditions.

Peak height

An increase in the concentration of acetonitrile from 5 to 50% resulted in a reduction in peak height for both STB and MBC (Fig. 2). Once again, the reduction was greater at larger injection volumes, particularly for STB. For a 200-μl injection, the peak height of STB was reduced as much as 76.8% on increasing the concentration of acetonitrile in the sample solutions from 5 to 50%. For 100- and 200-μl injections at 50% acetonitrile, STB was eluted in two and three well defined peaks, respectively. Confirmation that the multiple peaks were due to STB was obtained by a UV scan of each peak. Even with the 10-μl injection the reduction was fairly substantial (26.3%). Similarly, the reductions were 56.4% and 66.4% for 50- and 100-μl injections, respectively. Corresponding reductions for MBC were 10.0, 33.0, 47.0 and 64.9% with 10-, 50-, 100- and 200-μl injections, respectively (Fig. 2).

The effect of the methanol concentration in the sample solution on the peak height of STB was distinctively different from that of acetonitrile. A similar finding was reported by Hoffman *et al.*[12]. As a general trend, the peak height of STB increased as the concentration of methanol increased. This increase, however, was

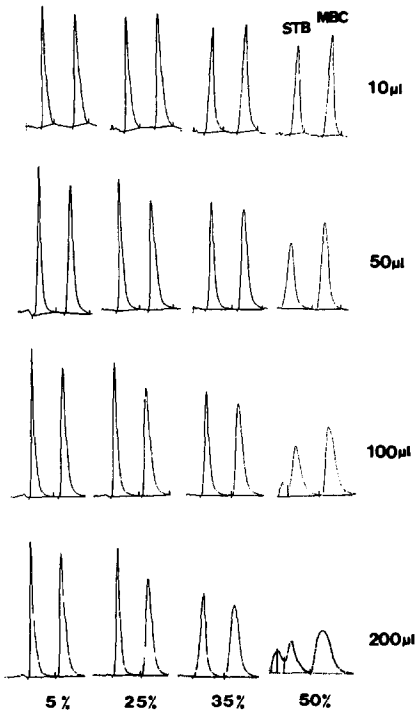

Fig. 2. Chromatographic profiles of STB and MBC when injected in sample solutions with various concentrations of acetonitrile (5, 25, 35 and 50%). The remainder of the sample solution consisted of 5% methanol, 10% 0.07 *M* phosphate buffer and water. The mobile phase solution consisted of acetonitrile, water and 0.07 *M* phosphate buffer at 35, 55 and 10%, respectively. Injection volumes were 10, 50, 100 and 200 μl, respectively.

dependent on the injection volume and the amount of analyte injected. This is clearly indicated in Table I. When the total mass of analyte injected was kept low at 50 ng by injecting 10, 50, 100 and 200 μl of 5.0, 1.0, 0.5 and 0.25 μg/ml of sample solutions, respectively, the peak height increased as the concentration of methanol was increased from 5 to 50% at all injection volumes except at 50% and 200 μl [Table I, (a)]. In contrast, when the amount of analyte injected was increased by increasing the injection volume using the same concentration of analyte (5 μg/ml), the peak-height increase was observed only with 10- and 50-μl injections. At larger injection volumes, peak splitting and peak-height reductions were frequently observed [Table I, (b)]. These results indicate that an optimum solvent composition exists that will give a substantially higher peak height for STB.

With MBC, the peak height decreased systematically (Table I), as was the case with acetonitrile.

TABLE I

PEAK HEIGHTS OF STB AND MBC IN HPLC ANALYSES WHEN THE CONCENTRATION OF METHANOL IN SAMPLE SOLUTIONS WAS INCREASED FROM 5 TO 50% AT FOUR INJECTION VOLUMES BY INJECTING CONSTANT AND INCREASING MASS OF ANALYTES

Injection volume (μl)	*Methanol (%)*	*(a) Injections at constant analyte mass using different concentrations of analytes*			*(b) Injections at increasing analyte mass using a constant concentration of analytes*		
		Analyte concentration (μg/ml)	*Peak height. (mAU)*[a]		*Analyte concentration (μg/ml)*	*Peak height. (mAU)*	
			STB	*MBC*		*STB*	*MBC*
10	5	5.0	7.06	6.42	5.0	7.06	6.42
	25	5.0	7.25	6.27	5.0	7.25	6.27
	35	5.0	7.15	6.21	5.0	7.15	6.21
	50	5.0	7.26	6.11	5.0	7.26	6.11
50	5	1.0	7.25	7.78	5.0	6.53	7.11
	25	1.0	7.95	7.13	5.0	7.80	6.95
	35	1.0	8.58	6.69	5.0	7.92	6.86
	50	1.0	9.19	7.06	5.0	8.20	6.77
100	5	0.5	7.73	8.09	5.0	7.16	6.73
	25	0.5	7.59	7.59	5.0	5.36[b] 5.87[b]	6.61
	35	0.5	8.38	7.03	5.0	6.35	6.39
	50	0.5	8.93	6.86	5.0	6.48	6.09
200	5	0.25	10.79	10.63	5.0	6.96	6.31
	25	0.25	11.84	9.99	5.0	4.65[b] 3.52[b]	5.23
	35	0.25	12.41	9.04	5.0	3.58[b] 4.19[b]	5.15
	50	0.25	9.25	7.42	5.0	2.25[b] 4.15[b]	4.69

[a] Each value represents the average of six analyses and the average coefficient of variation of these results was 0.85%.

[b] Split peaks.

Peak width

An increase in acetonitrile concentration of the sample solutions resulted in peak broadening for both STB and MBC at all four injection volumes (Fig. 2). Although this trend was greater with larger injection volumes, it was obvious even with 10-μl injections. When the concentration of acetonitrile in the sample solution was 5%, no increase in peak width was observed, even with 200-μl injections. At 35% acetonitrile, equivalent to the concentration of acetonitrile used in the mobile phase, the peak shapes were substantially worse than those with 5% and 25% acetonitrile solutions.

The influence of the methanol concentration of the sample solutions on peak broadening was substantially smaller than that of acetonitrile for both STB and MBC.

Peak area

All the peak-area count readings, when corrected for solute mass, were constant throughout the experiment, regardless of the solvent composition of sample solutions.

DISCUSSION

Effect of sample composition

The main objective of this study was to determine the extent of the effect on peak profiles of changing sample solvent compositions. We tried to find possible reasons for this influence, and to suggest possible ways to optimize sample compositions. For this purpose we examined the effect of methanol and acetonitrile, which were essential for dissolving MBC and STB, respectively.

At present the recommended procedure for RP-HPLC analyses is to inject the smallest volume possible (less than 10 μl if possible) and to dissolve the sample in the mobile phase in order to minimize baseline disturbances[13]. In many instances this rule is not adhered to because it is impractical for routine analysis, or is simply ignored.

Effect of acetonitrile in the sample solution. The influence of acetonitrile on retention time, peak height and peak width for STB and MBC was substantially stronger than that of methanol. The observed reduction in retention time and peak height and the increase in peak width with increase in acetonitrile concentration can be attributed to the increase in the eluting strength of the sample solution. The results suggest that a difference in the acetonitrile concentration of only 10 or 20% between samples could introduce significant errors into the quantitative results if peak heights are used in calculations. The study also demonstrated that variation of the mobile phase composition in the sample solution does not necessarily lead to the most efficient separation. For this reason, the solvents used in sample preparation should be aqueous and contain the minimum percentage of organic solvent necessary for complete dissolution of analytes.

At high injection volumes (100 and 200 μl) and high acetonitrile concentrations, split peaks were often observed for STB but not for MBC. When peak splitting occurs, erroneous interpretation of the results could be obtained regardless of whether peak height or area is used for determination, if one is not aware that an extra peak is due to peak splitting of the analyte.

The results also indicated that the peak heights of the two analytes were affected to different extents by variation of the sample solvent composition. This observation has important implications for quantitative analyses by peak heights even if an internal standard is used, as the peak-height responses may not be the same for the analytes and the standards if the solvent compositions for analytes and standards are different. The results of this study are consistent with the observations by several other groups who studied different types of compounds[7–9,12].

Perlman and Kirschbaum[11] reported that significant reductions in peak heights occurred when the injection solvent used was less polar, but this occurrence was observed only with the analytes capable of forming intromolecular hydrogen bonds. Our study disagrees with this suggestion as reductions in peak heights occurred not only with a compound capable of forming intramolecular hydrogen bonds (MBC) (Fig. 1, 1) but also with a compound that is not (STB, which forms intermolecular hydrogen bonds instead, 2 and 3). Berridge[14] and Chan and Yeung[15] also disputed this suggestion.

Effect of methanol concentration in the sample solution. The effect of increasing methanol concentration was very similar to that of acetonitrile for MBC but was different for STB (Table I). The retention time and peak height of MBC decreased and the peak width increased with increase in methanol concentration. The behaviour of MBC could be atrributed primarily to the increase in the eluting strength of the sample solvent.

The influence of methanol on the STB peak profile was different to what we expected. Both the retention times and peak heights increased with increase in methanol concentration (Table I). We assume that the major reason for such a different response of STB can be attributed to its molecular structure, but further studies are required.

Effect of injection volume

The reason for studying injection volumes was that suppliers of columns with microparticulate packings of 5 μm or smaller recommend the use of injection volumes of less than 10 μl for optimum chromatographic results[13]. In practice, however, this advice is often not observed and volumes of 50 and 100 μl are routinely used, mainly because the sample concentrations are low or there are some technical limitations during the sample preparation. It was therefore of interest to see the influence of injection volume on the peak profiles.

The effect of injection volume was evident from this study as shown in Fig. 2. It was largely dependent on the sample solvents. For example, even with a 10-μl injection, peak deterioration was observed if the elution strength of the sample solution was stronger than that of mobile phase. In contrast, a 200-μl injection was acceptable when the elution strength of the sample solution was weaker than that of the mobile phase. However, the magnitude of the effect was different for STB and MBC.

CONCLUSION

Peak profiles of different analytes may be influenced in different ways by the type and percentage of organic solvents used in sample solutions. This is especially true when methanol is used, and in some instances it may be possible to optimize the

peak profiles of specific analytes by varying the percentage of methanol in the sample solutions. Under any circumstances, solvent compositions should be kept constant for both samples and standards.

The use of the mobile phase as the sample solvent does not necessarily result in best chromatograms. In general, peak profiles can be substantially improved and injection volumes up to 200 μl can be acceptable if the eluting power of sample solutions is substantially less than that of the mobile phase.

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