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Evaluation of the performance of capillary liquid chromatography–fast atom bombardment mass spectrometry systems with precolumn addition of glycerol as a viscous matrix

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ABSTRACT

A series of experiments were conducted in order to evaluate the boundaries within which precolumn addition of viscous matrices can be systematically used in capillary LC–fast atom bombardment MS systems and to evaluate the broadening caused by components of the systems such as the column, the transfer capillary tube and the probe interface. The effect of the addition of a viscous matrix on the capillary system was studied by monitoring important chromatographic parameters such as capacity factors, selectivity, number of theoretical plates, peak width and resolution. The results obtained indicate that glycerol contents in the mobile phase higher than 5% have a deleterious effect on most chromatographic indicators. The overall effect of the presence of glycerol on the chromatographic system can be rationalized in terms of the modification of the analyte distribution between the mobile and stationary phases and changes in the kinetics of the system created by an increase in the viscosity of the mobile phase. The main contribution to band broadening in the system can be attributed to the interface and is related to the formation of a liquid droplet at the tip of the probe. Other contributions such as broadening in the column and in the transfer capillary affect the total variance of the chromatographic system but to a smaller extent.

INTRODUCTION

Fast atom bombardment mass spectrometry (FAB-MS) and liquid secondary ion mass spectrometry (LSIMS) are commonly used to generate mass spectra from polar, thermally labile and involatile compounds. Each technique involves the bombardment of an analyte dissolved in a viscous organic matrix by high energy (3–10 keV) fast-moving neutral species (FAB) or by a high-energy Cs^+ ion beam guided to a static sample [1,2]. These techniques have extended the scope of their initial applications by the introduction of different continuous-flow devices allowing the dynamic introduction of sample [3–

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6]. The advent of dynamic flow systems has not only increased the power of the techniques but has also allowed their interfacing with liquid chromatography (LC–FAB-MS). However, the need for low flow-rates (2–7 μl/min) and for the presence of a non-volatile matrix in the LC–FAB-MS systems have generated problems in the routine application of LC coupled to dynamic FAB-MS. To date, few stategies have been used to solve the constraints encountered in LC–FAB-MS analyses. The strategies developed initially have favored postcolumn splitting of the chromatographic effluent along with postcolumn addition of a matrix in conventional and microbore columns [7,8]. Recent strategies, however, have mostly been concerned with the direct coupling of the liquid capillary column with dynamic introduction systems [3,5,9–15]. In separation schemes using capillary columns, the analyst may use postcolumn addition of the viscous matrix in order to allow the independent optimization of the LC conditions [9]. However, in some instances such as when using open-tubular liquid chromatography coupled to FAB-MS, postcolumn addition is necessary as the efficient operation of the mass spectrometer requires that a make up liquid be added to the chromatographic effluent (≤100 nl/min). In other situations, uncommon matrices may be necessary in order to produce good-quality mass spectra. These matrices may react with the stationary phase or metal parts of the chromatographic system [16] and in such instances postcolumn addition is a useful mitigating alternative.

Precolumn addition of the viscous matrix is the second option that can be used in order to introduce the matrix in LC–FAB-MS systems. However, the intrusion of a polar and viscous matrix in the chromatographic process necessarly introduces changes in the distribution of the analyte between the mobile and stationary phases. Also, the diffusive processes taking place inside and outside the column will usually be altered, as previously observed in conventional LC–FAB-MS systems [16–18], since the introduction of a viscous matrix in the mobile phase will change its physical properties. The primary objective of this study was to investigate and to quantify the perturbation in the chromatographic process induced by the precolumn addition of glycerol to the mobile phase of packed capillary liquid chromatographic systems. A second objective was to assess the extent of broadening induced by the presence of the FAB matrix in a frit-FAB system.

EXPERIMENTAL

Instrumentation

The liquid chromatographic system consisted of a Carlo-Erba Phoenix-20 pump connected to a Valco Model C14W 60-nl injector. Detection was achieved with an Isco uLC-10 variable-wavelength detector set at 280 nm. The chromatographic column used consisted of a laboratory-made capillary column (Spherisorb ODS-2, d_p = 5 μm, 225 mm × 0.250 mm I.D.). The temperature was maintained at 25°C by a temperature equalization chamber provided with the detector. Experiments involving continuous-flow FAB (CF-FAB) were performed on a VG Autospec-Q mass spectrometer using the dynamic LSIMS probe. This probe consists of a fused-silica transfer tube of 50 or 75 μm terminated by a stainless-steel frit of 2 mm diameter.

Chemicals

The peptides met-enkephalin and leu-enkephalin were obtained from Sigma (St. Louis, MO, USA). Glass-distilled glycerol (>99%) and trifluoroacetic acid were purchased from Aldrich (Milwaukee, WI, USA). All compounds were used without further purification and the mobile phase were prepared using HPLC-grade acetonitrile and distilled, deionized water obtained with a Milli-Q purification system (Millipore, Bedford, MA, USA).

Preparation of mobiles phases

The eluents were carefully prepared by mixing appropriate volumes of distilled, deionized water and organic modifiers. The mobile phase used for peptide analysis contained fixed proportions of trifluoroacetic acid (TFA) and acetonitrile

(ACN) and the proportion of water was adjusted to complement the volume of glycerol (GLY) in the solution (ACN–H_2O–GLY–TFA 30:70 – x:x:0.1). Sufficient amounts of mixture were prepared in order to ensure that all experiments would be conducted with the same mobile phase. In all instances, the solvents were filtered (0.45-μm filter) and degassed prior to use.

Chromatographic measurements

All chromatographic experiments were carried out at 25°C after the chromatographic system had been equilibrated for at least 45 min. Before the experiments were conducted in the presence of glycerol, the capillary column was tested to evaluate its efficiency by injection of a standard solution of amylbenzene ($k' = 5$) eluted with acetonitrile–water (75:25, v/v). Precise values for the volumetric flow-rates were measured for each experiment. The retention of sodium nitrate was taken as the dead volume (t_m) and the average linear velocity was calculated using the length of the chromatographic column. The number of theoretical plates (N) was estimated from the widths at half-height of the peaks. The Van Deemter plots were generated by measuring the theoretical plate height (H) with linear velocities over the range 0.02–4 mm/s.

Band broadening measurements

The evaluation of the variance related the broadening generated by the continous-flow introduction probe coupled to the mass spectrometric system was performed by indirect UV experiments and direct MS experiments. The variance associated with the capillary transfer tube found in the dynamic FAB interface was determined by replacing the column in the UV system by capillary tubes with the same inner diameter (50 or 75 μm) but with different lengths [19,20]. By plotting the total variance obtained from the width at the base W_b ($\sigma_t^2 = (W_b/4)^2$] *versus* the length of the capillary tube on a graph and by extrapolating the line obtained to the intercept on the ordinate, the instrumental variance originating from injector and detector can be estimated. The subtraction of the estimated instrumental variance from the total variance measured for a 1-m long capillary tube allows the determination of the broadening generated by the capillary transfer tube of the dynamic FAB-MS interface. Replacing the column in the system and doing the same subtraction for the extra-column contribution allows the determination of typical values for the broadening generated by a capillary column in presence of a FAB matrix in the mobile phase. The broadening produced at the tip of the dynamic introduction probe was evaluated by injecting a solution of met-enkephalin into the interface of to the mass spectrometer. The variance observed minus the previous variance evaluated for the capillary transfer tube allows the quantification of the broadening at the probe tip.

RESULTS AND DISCUSSION

It is important to characterize the influence of the presence of a FAB matrix in the mobile phase of the capillary liquid chromatographic system since this type of chromatography is becoming the standard in LC–FAB-MS analysis. In order to gain a descriptive and comprehensive view of the effect of the precolumn addition of a FAB matrix in packed capillary liquid chromatography coupled to dynamic FAB-MS systems, a series of experiments that should allow the determination of the effects induced on the key chromatographic indicators can be conducted.

The effect on the retention properties of some peptides induced by an increasing concentration of viscous matrix in the mobile phase can be seen in Figs. 1 and 2. Fig. 1 shows the changes in the capacity factors induced by variation of the glycerol content in the mobile phase between 0 and 10%. It can be seen that the capacity factors decrease with increase in the concentration of the matrix in the chromatographic eluent. This decrease, of the order of 40% for met-enkephalin and 46% for leu-enkephalin, is significant as the glycerol content is raised from 0 to 10%. This decrease in the capacity factors implies that the addition of a FAB matrix can cause severe compression of the retention times of the analytes. However, examination of Fig. 1 reveals that the acceleration of the elution becomes

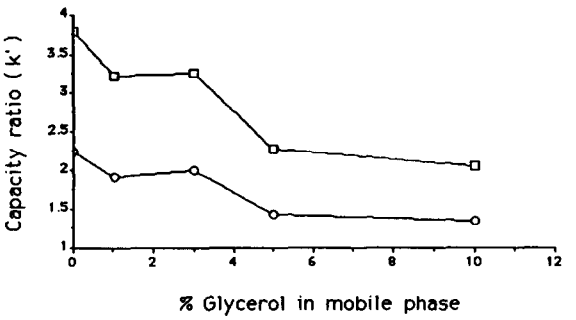

Fig. 1. Effect of concentration of glycerol in the mobile phase on the capacity factor (k'). ○ = Met-enkephalin; □ = leu-enkephalin.

significant only when more than 3% of matrix is added to the mobile phase. The decrease in the capacity factors that has been observed is greater than that previously observed in conventional chromatography in the presence of glycerol or thioglycerol [16,18]. Possible interactions at the fused-silica wall of the capillary column and the differences between the phase ratios of the capillary and the conventional columns may be involved in the differences noted. Further experiments are needed, however, in order to elucidate the causes of that effect. The addition of the FAB matrix to the liquid vector induces other changes in the separation of the analytes. As shown in Fig. 2, by plotting the variation in selectivity against the glycerol content in the mobile phase, the increased presence of glycerol decreases the selectivity for the pair met-enkephalin and leu-enkephalin. This decrease is not drastic but it varies regularly, passing from 1.69 with no matrix to 1.53 with 10% matrix added to the mobile phase. Such changes in selectivity indicate that the retention of compounds with higher capacity factors are more affected by the presence of glycerol.

The efficiency of the column is another parameter that it is important to characterize because it is an indicator of the potential of the chromatographic system to separate complex mixtures. In the presence of glycerol the efficiency must change as the addition of a viscous matrix to the chromatographic eluent perturbs most diffusive processes that control the kinetics of the chromatographic process. The effect on the number of theoretical plates (N) of increasing amounts of glycerol added to the mobile phase can be seen in Fig. 3, which shows the decrease in the normalized theoretical plate number (N_x/N_0) with increase in glycerol content in the capillary chromatographic system. Normalization of the number of theoritical plates at a specific matrix content (N_x) to the number of theoritical plates measured in absence of matrix (N_0) allows the quantification of the decrease in efficiency of the column in the presence of glycerol. The reduction is of the order of 33% and 38% for met-enkephalin and leu-enkephalin, respectively, with 10% of added matrix. As in Fig. 2, small changes are seen with the first 3% of added matrix and the decrease is faster at higher matrix contents. This decrease in efficiency comes from the broadening of the elution band. Fig. 4 shows the behavior of the normalized peak width ($w_{1/2}/t_r$) with glycerol content. The curves show that

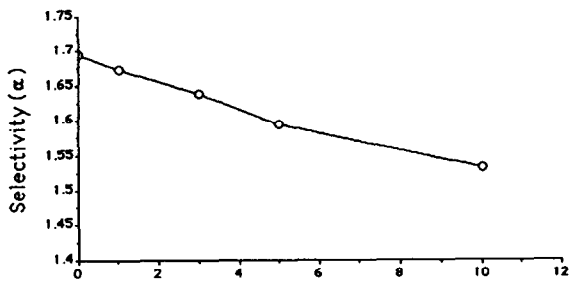

Fig. 2. Effect of concentration of glycerol in the mobile phase on the selectivity (α). ○ = Met-enkephalin; □ = leu-enkephalin.

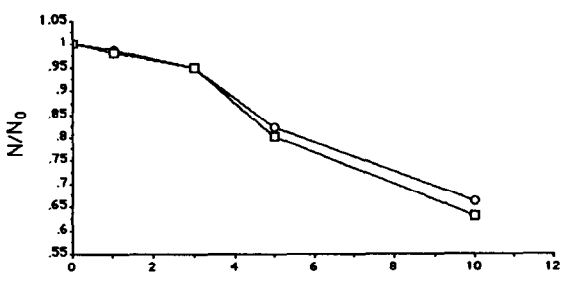

Fig. 3. Effect of concentration of glycerol in the mobile phase on the normalized number of theoretical plates (N_x/N_0). ○ = Met-enkephalin; □ = leu-enkephalin.

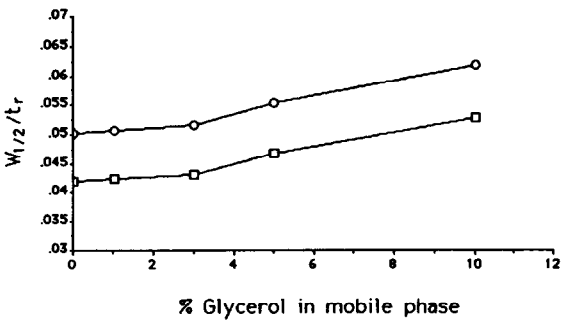

Fig. 4. Effect of concentration of glycerol in the mobile phase on the normalized peak width ($w_{1/2}/t_r$). ○ = Met-enkephalin; □ = leu-enkephalin.

broadening increases in the presence of glycerol. The band spreading increases by 25% at 10% of added matrix for the two peptides analysed. Hence, the decrease in efficiency observed for the leu-enkephalin (38%) *versus* met-enkephalin (33%) originates from both sources: the increase in broadening and the greater decrease in the retention time of leu-enkephalin. The net effect of the differential decrease in retention and increase in the peak width for met-enkephalin and leu-enkephalin can be seen in Fig. 5. The data in the figure, which shows the changes in resolution in the presence of glycerol, demonstrate that the addition of a FAB matrix to the mobile phase significantly decreases the resolution of the pair of peptides.

In order to characterize the effect of the presence of glycerol on the kinetics of the capillary system, Van Deemter plots at different matrix contents present in the mobile phase have

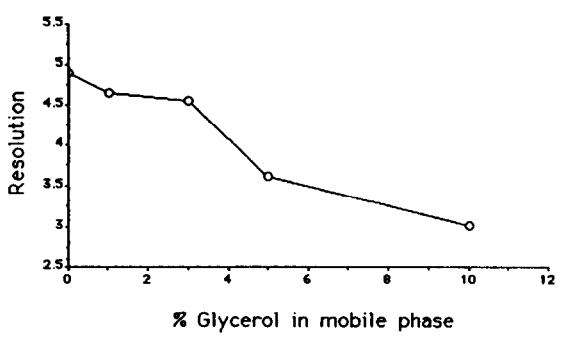

Fig. 5. Effect of concentration of glycerol in the mobile phase on the resolution (R_s). ○ = Met-enkephalin; □ = leu-enkephalin.

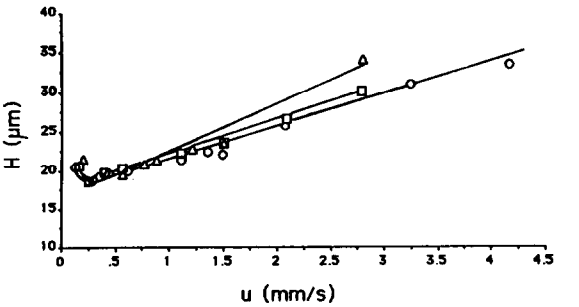

Fig. 6. Van Deemter plots for met-enkephalin at various glycerol concentrations in the mobile phase: ○ = 0%; □ = 3%; △ = 5%.

been generated. Fig. 6 shows the height equivalent to a theoretical plate in relation to the average linear velocity of the mobile phase in the capillary column. It can be seen that the addition of a FAB matrix such as glycerol mainly affects the mass transfer (right-hand side) processes involved in the chromatographic equilibrium. The similarity of the curves obtained at 0% and 3% of matrix added to the liquid vector is an indication that negligible or very small perturbations are caused by the presence of glycerol at low levels in the mobile phase. These observations explain why only small perturbations have been measured in the capacity factor, selectivity, efficiency, normalized peak width and resolution with less than a 3% matrix content in the mobile phase. Our observations on the Van Deemter plots are also in agreement with reported comments indicating that analyses with 1% matrix added to different mobile phases induce almost no change in chromatographic performance during LC–FAB-MS analysis [10–13]. The decrease in efficiency noted at high glycerol concentrations is related to less efficient mass transfer in the capillary column. As indicated by previous studies [16,17], this is caused by an increase in the viscosity of the mobile phase which produces a lower diffusivity of the solute, thus inducing more band spreading in the system.

In order to identify the factors responsible for band broadening, the variances associated with each component of the system were examined. Table I gives the measured variances associated with each component of the LC–FAB-MS system in the presence of a FAB matrix such as

TABLE I

MEASUREMENT OF THE BROADENING CAUSED BY THE COMPONENTS IN THE LC–FAB-MS SYSTEM

Glycerol (%)	σ^2_{col} (μl^2)	σ^2_{tu} (μl^2)						σ^2_d (μl^2)[a]
		50 μm I.D.			75 μm I.D.			
		2.5 μl/min	4.0 μl/min	5.0 μl/min	2.5 μl/min	4.0 μl/min	5.0 μl/min	
0	0.148	0.0083	0.0115	0.0137	0.0179	0.0259	0.0352	–
1	0.141	0.0086	0.0120	0.0141	0.0194	0.0264	0.0375	0.170
3	0.151	0.0090	0.0127	0.0147	0.0204	0.0301	0.0397	0.207
5	0.177	0.0095	0.0134	0.0152	0.0224	0.0323	0.0422	0.256
7	0.238	0.0101	0.0146	0.0158	0.0237	0.0344	0.0421	–

[a] Frit-FAB.

the variance associated with the chromatographic column (σ^2_{col}), the transfer tube (σ^2_{tu}) and the droplet (σ^2_d). The variance associated with the chromatographic system (σ^2_{CHR}) was calculated using eqn. 1, where H is the height equivalent to a theoretical plate, t_r is the retention time and L is the length of the column:

$$\sigma^2_{CHR} = \frac{Ht_r^2}{L} \quad (1)$$

The total variance of the chromatographic system (σ^2_{CHR}) can be expressed as the sum of the broadening occuring in the column (σ^2_{col}) and by components external to the column (σ^2_{ex}):

$$\sigma^2_{CHR} = \sigma^2_{col} + \sigma^2_{ex} \quad (2)$$

The subtraction from σ^2_{CHR} of the variances associated with the injector and the detector will result in the variance associated with the column. The results indicate that the variance associated with the column is between 0.148 and 0.238 μl^2 when the glycerol content in the mobile phase varies from 0 to 7% and therefore its contribution to the total variance is relatively important.

The data in Table I indicate that the variances associated with the probe tip are between 0.170 and 0.256 μl^2 when the glycerol content is varied from 1 to 5%. These variances are responsible for a dispersion of 1.6–2.0 μl for the frit-FAB interface used in this study. The widths of these bands, however, are smaller than those which can be observed in conventional CF-FAB interfaces, which are of the order of 2.5 μl [4,17]. It thus appears that variances associated with the interface are considerably more important than those associated with the chromatographic column.

Variances associated with transfer capillary tubes of 50 or 75 μm I.D. are between 0.0083 and 0.0421 μl^2, as indicated by the data of Table I. These are functions of the flow-rate as indicated by the Taylor–Golay equation (eqn. 3) [21], which allows the theoretical estimation of the variances associated with capillary tubing:

$$\sigma^2_{tu} = \frac{\pi r^4 LF}{24 D_m} \quad (3)$$

where F is the flow-rate and D_m is the diffusion coefficient. Comparison of the variances associated with the transfer capillaries (Table I) reveals that variances associated with tubing of 75 μm I.D. are systematically 15% of the variance associated with the probe tip at a flow-rate of 5 μl/min and a glycerol content of 5%. With a flow-rate of 2.5 μl/min and a 5% content of glycerol the same variance is around 4%. Hence the dispersion produced by this component appears to be relatively small and its contribution should not significantly affect the total variance of the system.

From the data presented, it can be concluded that the major contribution to broadening comes from the probe tip. However, broadening produced by the chromatographic column has to be considered relatively important under the present conditions as it accounts for almost 50% of the broadening induced by the probe tip. Both of these effects will obviously result in a decrease in the chromatographic resolution for the overall system. It is possible to reduce the total dispersion in the system by using a low concentration of glycerol in the system, but it must be realized that these contributions will still be important even at glycerol contents of the order of 1% (*ca.* 0.33 μl^2).

Thus it can be seen from Table I that the contribution of the interface is mainly due to the formation of a liquid droplet at the end of the probe tip. The dispersion occurring at the tip is relatively difficult to evaluate as there are many phenomena occurring in the droplet that forms at the end of the probe tip (evaporation, diffusion, sputtering, mixing, etc.). Two approaches can be used to estimate the dispersion of the system. One approach is to concentrate on the droplet itself and consider it as a connecting tube and the variance associated with it can be obtained from the Taylor–Golay relationship (eqn. 3). The other approach is to consider the droplet as a mixing chamber, in which case the variance associated with it can be obtained from the equation [22]:

$$\sigma^2_{drop} = V^2_{drop} = \pi^2 r^4_{tc} L^2_{tc} \quad (4)$$

TABLE III

ESTIMATED VARIANCE ASSOCIATED WITH A MIXING CHAMBER

Diameter × thickness (mm × mm)	σ^2_d (μl^2)
2 × 0.050	0.025
2 × 0.045	0.020
2 × 0.040	0.016
2 × 0.035	0.012
2 × 0.030	0.009

where r_{tc} is the radius of the droplet and L_{tc} is its length.

By assuming that the composition of the liquid phase is constant within the droplet, the variance can be estimated. The estimated values for the variance associated with the droplet that can be obtained using each model described above are given in Tables II and III. The comparison of the measured variance (Table I) with the estimated value (Tables II and III) shows that the first approach, "the connecting tube approach", seems to be a more suitable model that fits in with the data. The results obtained indicate that the variance would correspond to a film height of the order of 35–40 μm. Therefore, a decrease in the radius of the frit should lead to a significant decrease in the broadening due to the formation of the liquid droplet, as can be seen from eqn. 3 where the broadening is directly proportional to r^4. Further experiments are needed, however, in order to confirm that the formation of the liquid

TABLE II

ESTIMATED VARIANCE CAUSED BY A CAPILLARY TRANSFER TUBE

Diameter × thickness (mm × mm)	σ^2_d (μl^2)				
	0% glycerol	1% glycerol	3% glycerol	5% glycerol	10% glycerol
2 × 0.050	0.262	0.264	0.276	0.285	0.336
2 × 0.045	0.236	0.238	0.248	0.256	0.302
2 × 0.040	0.210	0.211	0.221	0.228	0.268
2 × 0.035	0.184	0.185	0.193	0.199	0.235
2 × 0.030	0.157	0.158	0.166	0.171	0.201

droplet can be estimated using this model, which gives a very good approximation of the variance associated with the formation of the droplet.

CONCLUSIONS

As previously observed for conventional LC–FAB-MS, the precolumn addition of a viscous matrix such as glycerol can also significantly alter the chromatographic conditions in capillary LC–FAB-MS systems. In general terms, the negative effect of precolumn addition of glycerol on the chromatographic indicators are comparable in conventional and in capillary LC–FAB-MS systems. The source of chromatographic broadening can be attributed mainly to changes in the diffusivity of the analytes induced by an increase in the viscosity of the mobile phase. The variance associated with the interface can be related mainly to effects occurring at the tip of the probe, as the variance with the dead volume of the transfer capillary tube is found to be small. However, as has been demonstrated, the dispersion induced in the chromatographic column by the presence of a matrix cannot be neglected as it is of the same order of magnitude as that induced by the probe tip.

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