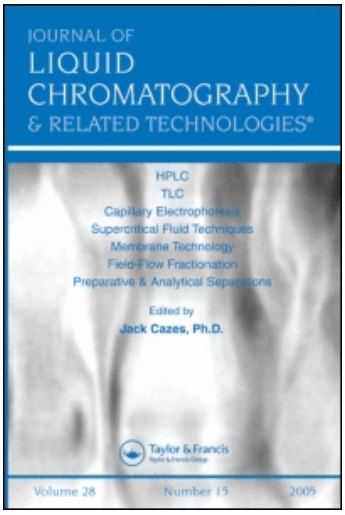

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A New Approach to Study Benzodiazepine Separation and the Differences Between a Methanol/Water and Acetonitrile/Water Mixture on Column Efficiency in Liquid Chromatography

Y. Guillaume[a]; E. J. Cavalli[a]; E. Peyrin[a]; C. Guinchard[a]
[a] Laboratoire de Chimie Analytique, Faculté de Médécine Pharmacie, Besançon Cedex, France

To cite this Article Guillaume, Y. , Cavalli, E. J. , Peyrin, E. and Guinchard, C.(1997) 'A New Approach to Study Benzodiazepine Separation and the Differences Between a Methanol/Water and Acetonitrile/Water Mixture on Column Efficiency in Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 20: 11, 1741 — 1756
To link to this Article: DOI: 10.1080/10826079708006329
URL: http://dx.doi.org/10.1080/10826079708006329

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1741-1756 (1997)

A NEW APPROACH TO STUDY BENZODIAZEPINE SEPARATION AND THE DIFFERENCES BETWEEN A METHANOL/WATER AND ACETONITRILE/WATER MIXTURE ON COLUMN EFFICIENCY IN LIQUID CHROMATOGRAPHY

Y. Guillaume,* E. J. Cavalli, E. Peyrin, C. Guinchard

Laboratoire de Chimie Analytique
Faculté de Médécine Pharmacie
Place Saint-Jacques 25030
Besançon Cedex, France

ABSTRACT

A chemometric methodology was used to study column efficiency and the separation of 10 benzodiazepines in reversed phase liquid chromatography. New simple mathematical models and the organic modifier (OM) organization of ACN in the water, explained differences on column efficiency observed when ACN is chosen instead of CH_3OH. A new response function, which takes into account the separation quality and the analysis time, was proposed for the separation optimization. The result, a mobile phase ACN/water (60/40)(V/V), with a flow rate = 1.00 mL/min and a column temperature = 47°C were optimum values for a rapid chromatographic separation.

INTRODUCTION

Several authors have developed chemometric methods for the study of column efficiency and separation optimization of compounds in reversed phase liquid chromatography (RPLC). The chromatographic response functions optimize, simultaneously, the separation quality and the analysis time.[1-5] Yau and Kirkland,[6] presented an improved algorithm for the characterization of band broadening of skewed (but well-resolved) peaks. Dondi and Gianferra[7] used sequential methods to optimize a mixture of natural secoiridoid compounds in HPLC. Chaminade et al[8] used the cubic spline interpolation algorithm for the selection of ternary mobile phase in HPLC. Larew and Olsen[9] proposed a comparison of theory based and empirical modelling for the prediction of chromatographic behavior in the ion pairing separation of benzodiazepines derived pharmaceutical compounds. Guillaume et al[10] described a rapid method suitable for routine analysis of a mixture of sulbactam and tazobactam in human serum. Guillaume and Guinchard[11] proposed a process to study, simultaneously, the effect of mobile phase composition, its flow rate and column temperature, on both column efficiency and the benzodiazepine separation in a methanol/water mixture. This paper discusses, using experimental data, improvements in peak efficiency for changing the organic modifier from methanol to acetonitrile. A new optimization process which obtains an efficient separation in a minimum analysis time is also proposed.

EXPERIMENTAL

Reagents

Acetonitrile was an analytically determined HPLC grade. Bromazepam, nitrazepam, flunitrazepam, clobazam, lorazepam, oxazepam, tofisopam, chlordiazepoxide, clorazepate dipotassic, and diazepam were obtained from Hoffmann La Roche (Basel, Switzerland). Naphtalene obtained from Merck (Nogent-sur-Marne, France) was used to determine the column efficiency. These were diluted to a concentration of 10-80 mg/mL.

Apparatus

The HPLC system consisted of a Waters HPLC pump (Saint Quentin, Yvelines, France), an Interchim Rheodyne injection valve Model 7125 (Montlucon, France) fitted with a 20 µL sample loop, a Merck L4000 variable

wavelength UV spectrophotometer detector, and a Merck D2500 chromato-integrator (Nogent-sur-Marne, France). A Waters column (Nova pak C_{18}, 5µm, 150 mm x 3.9 mm I.D) was used at controlled temperature in an Interchim oven TM N 701 (Montluçon, France). Overall temperature control was maintained within ± 1°C with a variation from 25°C to 50°C. The detection wavelength was 254 nm. The flow-rate used varied from 0.6 to 1.6 mL/min. The mobile phase was an ACN/water mixture with percentage of ACN varying from 40 to 80 %.

Chemometric Methodology

The chemometric approach is based on factorial designs. Two-level factorial designs give a fitting of a first order (linear) model to the data.[12] If the effects of each of the three factors do not vary linearly, a design which requires 13 experiments to detect curvature in the response can be used. Thus, the Box and Benhken design[13-15] was developed, specifically, to enable a second order response surface to be fitted to the data, as it provides sufficient information for the fitting of a quadratic model to a data set. Such models are amenable to regression analysis. For three factors this takes the form of:

$$y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 \quad (1)$$

where y is the response or dependent variable, and x_1, x_2, x_3 are the logarithms respectively of, percentage of ACN θ (%) in the ACN/water mixture, mobile phase flow rate F (mL/min) and column temperature T(°C). x_1, x_2, and x_3 were coded to have a variation range from -1 to +1. The a terms represent the parameters of the model.

Simplex Optimization

To optimize the mathematical model (y) given by the experimental design, a simplex method was used. The y value was calculated for m sets of starting conditions where m was given by the number of factors to be optimized plus 1. Therefore, in this case m was 4. The point, corresponding to the lowest value of y, was then reflected in relation to the surface defined by the three other points to give a fifth set of starting conditions. Once again, the point with the lowest y was reflected and the process repeated, sequentially, until the same mobile phase composition, flow rate and column temperature continued to be selected.

Table 1

Estimates of Regression Parameters for the Two Regression Models

Independent Variables	Parameter Terms	(a) N/L	$Ln(k'_{diazepam})$
Intercept	a_0	+ 130.23	+2.87
x_1	a_1	+ 12.31	- 1.73
x_2	a_2	+ 9.41	+ 0.03
x_3	a_3	+ 2.31	- 0.15
x_{12}	a_{11}	- 10.25	+ 0.04
x_2^2	a_{22}	- 16.52	+ 0.00
x_3^2	a_{33}	- 13.62	- 0.03
x_1x_2	a_{12}	- 7.40	+ 0.01
x_1x_3	a_{13}	- 15.40	+ 0.06
x_2x_3	a_{23}	+ 19.00	+ 0.02

(a) inverse of the height to a theoretical plate (mm^{-1})

RESULTS AND DISCUSSION

Column Plate Height

The experimental H were calculated from the chromatograms. All experiments were repeated three times. The coefficient of variation of the H values was less than 3 % in most cases, indicating a high reproductibility and good stability for the chromatographic system. Using the experimental design, the column efficiency represented by the height to a theoretical plate (H) was modelled by the two order polynomial (Eq.1) where y is equal to 1/H. From the full regression model (Table 1) a student T-test was used to provide the basis for the decision as to whether or not the model coefficients were significant. Results of the student T-test show that no variables can be excluded from the model. This generated model was assessed statistically using a Fischer Snedecor test (F-test) and a coefficient of multiple determination R^2. These criteria were respectively equal to 180 and 0.971. These values show a good validity for the model. Experimental and calculated values of the H are summarized in Table 2. These values were chosen in the parameter space, but not among the 13 experiments given by the experimental design, to show that the H-model was within the range. The variation of H versus column temperature and mobile phase flow rate was similar to those obtained in recent works.[11,16]

Table 2

Calculated and Experimental H Values (H_{cal} and H_{exp}) for Different Values of F (mL/min), T(°C), and θ(%)

F(mL/min)	T(°C)	θ(%)	H_{cal} (10^3 mm)	H_{exp} (10^3 mm)
0.8	25	45	11.01	11.40
1.1	35	45	8.36	8.42
1.4	35	55	7.86	7.98
0.8	45	55	8.88	8.93
0.8	35	55	8.17	8.39
1.4	45	55	7.51	7.75
1.1	25	55	9.17	8.99
0.8	45	75	9.09	9.14
1.4	45	75	8.12	8.22
1.1	45	45	7.98	8.02
0.8	25	65	7.92	8.00
0.8	35	65	7.79	7.85
1.1	25	75	7.96	8.02
1.4	45	65	7.71	7.83
1.4	25	55	10.33	10.21
1.1	35	55	7.66	7.99
0.8	35	45	9.19	9.43
1.4	25	65	9.44	9.34
1.4	45	75	8.12	8.44
1.4	25	55	10.33	10.12
1.1	45	75	8.15	8.65
1.1	45	65	7.81	7.92
0.8	25	55	8.85	8.75
1.1	45	55	7.71	7.84
1.4	25	45	12.58	12.81
1.4	25	75	9.09	9.14
1.4	45	65	7.71	7.95
0.8	45	45	9.48	9.65
1.1	25	45	11.60	12.00
1.4	35	45	8.44	8.66
0.8	45	65	8.83	9.00

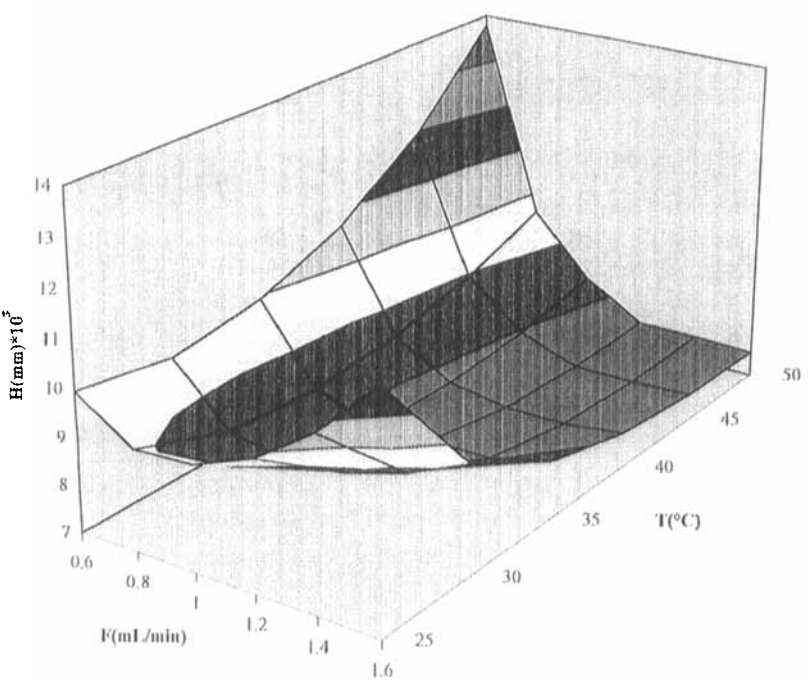

Figure 1. Response surface of the height to a theoretical plate (H) versus temperature/flow rate (percentage of ACN kept at 53 %).

For a constant percentage of ACN in the ACN/water mixture, the surface S presented a minimum (maximum column efficiency) for a couple, column temperature/flow rate (Figure 1). Equation 1, shows that for a constant flow rate (or temperature) the variation of H versus the ACN percentage presents a minimum (maximum column efficiency). The response surfaces generated for the H model are given in Figures 2 and 3. This variation, as in the case of the binary CH_3OH/H_2O mixture,[11,16] can be explained by the decrease in solvent polarity and viscosity with an increase in organic modifier (OM) percentage. In addition, in these experiments there was a specific organization of ACN molecules in the ACN/water mixture. In this mixture, ACN is organized in agregates or loosely defined clusters.[17-19]

For the low region of θ, when θ increased, the number and size of the clusters increased. The non polar solute such as a naphtalene molecule, was embedded in the clusters which increased its solubility.

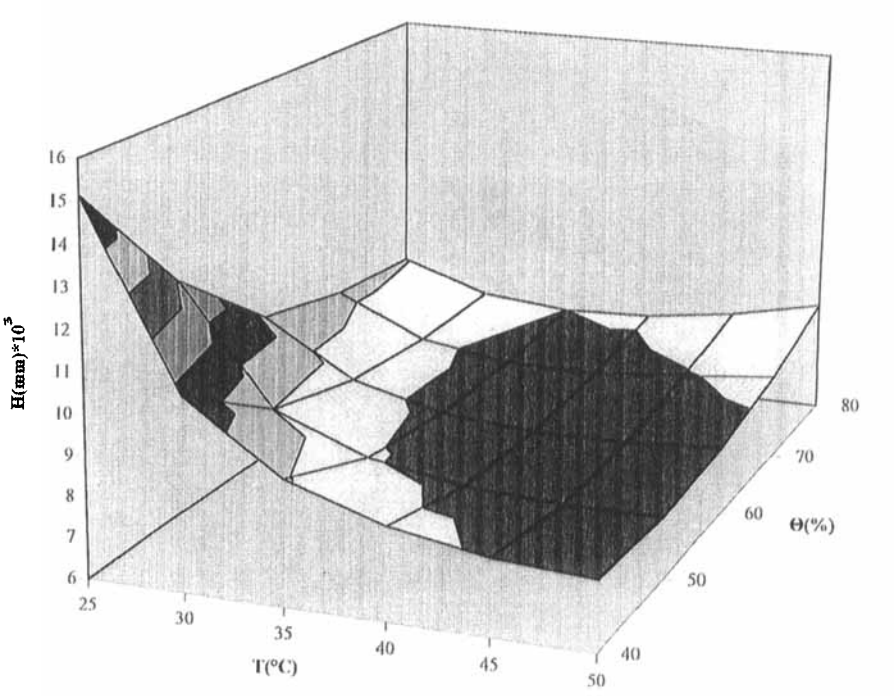

Figure 2. Response surface of the height to a theroetical plate (H) versus temperature/percentage of ACN (flow rate kept at 1.40 mL/min).

Therefore, the mass transfer of the solute in both the stationary and mobile phases increased, by decreasing solvent polarity and its viscosity (OM = CH_3OH or ACN) and increasing the molecule solubility (more specifically for OM = ACN). The factor peak band broadening due to mass transfer decreased. Thus, the plate height decreased. But, the rapid decrease in the capacity factor with an increase of θ necessitated a higher plate number. Thus, beyond an optimal mobile phase composition, this second effect supplanted the increase in the column efficiency referred to above and the plate number decreased. The optimum value of the column efficiency was determined using the simplex optimization method. The results obtained were θ = 53 % of ACN, F = 1.40 mL/min and T = 45°C. The corresponding calculated H value was 0.0075 mm. The experimental value of H obtained for this condition was 0.0080 mm. The theoretical result was also good. In a previous paper,[11] the column efficiency was studied in a CH_3OH/water mixture. The optimum column efficiency in this mixture was calculated as being equal to 0.0138 mm.

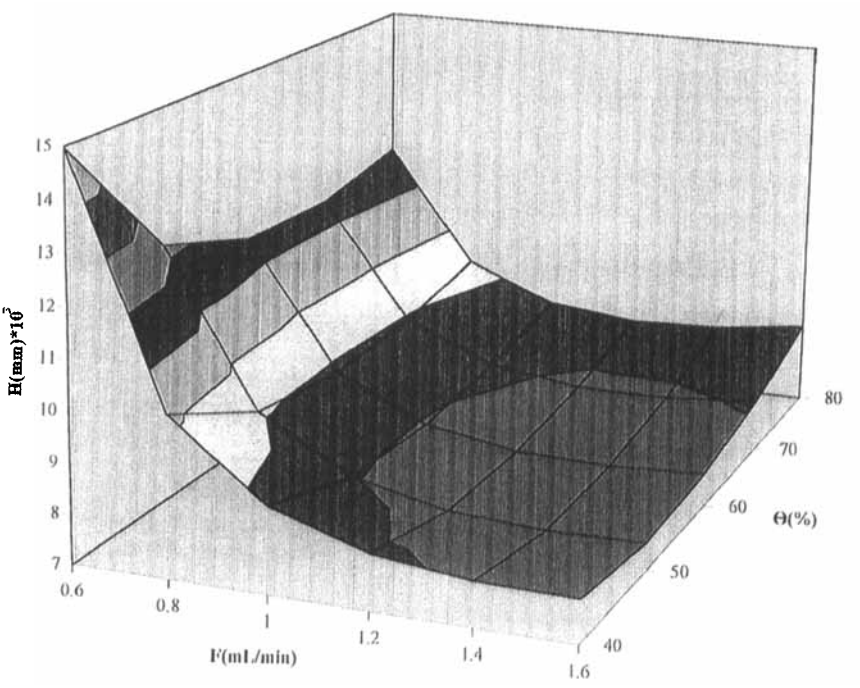

Figure 3. Response surface of the height to a theoretical plate (H) versus flow rate/percentage of ACN (column temperature kept at 45° C).

In the optimum conditions, column efficiency is approximately two times poorer than with acetonitrile. Table 3 shows the calculated and experimental H values for different percentages of organic modifier (OM) in the OM/water mixture (OM = ACN or OM = CH_3OH). The values were found to be markedly dependent on the organic component in the eluent. In CH_3OH/Water, the plate height was $\geq$ 0.013mm corresponding to a plate number N $\leq$ 11540 and in ACN/Water H $\geq$ 0.007mm corresponded to N $\leq$ 21430. From the data in Table 3, it was shown that for a given θ-value, in the ACN/water mixture, the column was two or five times more efficient than with methanol. For example, if F = 1.6 mL/min, T = 50°C and θ = 70% in the OM/Water mixture, the experimental H value was 0.00821 mm for OM = ACN and 0.01875 mm for OM = CH_3OH. Another work[20] has reported that the column efficiency was poorer than with ACN. However, the authors did not systematically note the experimental column efficiency in a large variation range of percentages of OM in the mixture OM/Water, flow rate, and column temperature.

Table 3

Calculated and Experimental H (10^3 mm) Values for Different Percentages of Organic Modifier OM in the Mixture OM/Water Mixture (OM = ACN or OM = CH_3OH)

		Acetonitrile		Methanol	
	θ(%)	H_{cal}	H_{exp}	H_{cal}	H_{exp}
A	45	11.01	11.40	51.00	49.89
	50	9.63	9.54	34.81	34.78
	55	8.85	8.75	29.90	29.82
	60	8.30	8.21	28.78	28.72
	65	7.92	8.00	29.80	29.75
	70	7.66	7.70	33.00	32.97
B	45	8.30	8.20	22.78	22.54
	50	7.92	7.82	20.30	20.00
	55	7.69	7.75	19.83	19.78
	60	7.58	7.66	20.53	20.72
	65	7.54	7.65	22.45	22.68
	70	7.57	7.68	25.84	26.01
C	45	7.52	7.53	14.42	14.27
	50	7.53	7.57	13.82	14.01
	55	7.62	7.55	13.99	14.10
	60	7.77	7.83	14.76	14.85
	65	8.00	8.10	16.17	16.25
	70	8.30	8.21	18.43	18.75

A. F = 0.8 mL/min; T = 25°C
B. F = 1.0 mL/min; T = 38°C
C. F = 1.6 mL/min; T = 50°C

A new equation was developed relating H to the nature of OM and its percentage in the mixture OM/water. For a given couple, temperature/flow rate, the 1/H model [Eq.1] can be rearranged to obtain the analytical equation relating 1/H to the percentage of OM:

$$1/H_{OM} = \alpha_1 + \alpha_2 Ln\theta + \alpha_3 (Ln\theta)^2 \qquad (2)$$

Table 4

Values of ξ_1, ξ_2,ξ_3, of Equation 4 ($V(\theta)$ in 10^{-1} mm^{-1}) for Three Values of Column Temperature/Flow Rate

F(mL/min)	T(°C)	ζ_1	ζ_2	ζ_3	r^2*
0.8	25	96.10	-50.81	7.19	0.98
1.0	38	115.89	-57.97	7.74	0.97
1.6	50	114.88	-55.22	6.99	0.98

* Correlation coefficients of the fits.

where α_1, α_2, α_3 were constants and H_{OM} is the height equivalent to a theoretical plate determined on naphtalene in the OM/Water mixture. Therefore the following can be written:

$$1/H_{ACN} - 1/H_{CH3OH} = V(\theta) \quad (3)$$

$$V(\theta) = \xi_1, + \xi_2 Ln\theta + \xi_3(Ln\theta)^2 \quad (4)$$

Values of the constants ξ_1, ξ_2, ξ_3 (determined using experimental data from Table 3) and correlation coefficients for the fits are given in Table 4. In each case, these constants were of the same signs and similar ranges. In the interval [45%,80%], the polynomial $V(\theta)$ was always greater than zero indicating that $H_{ACN} \leq H_{OM}$. This conclusion can be equally supported by the fact that the methanol solution is dominated by competitive hydrogen bonding and the availability of free "methanol" for solute solvatation decreases rapidly with an increasing fraction of water.

However, ACN solution chemistry is governed by clusters of ACN where the naphtalene molecule is preferentially solvated. ACN molecules increase solute naphtalene solubility in an aqueous solution and consequently its transfer between the two phases (mobile and stationary).

Separation Optimization

This separation was studied in a CH_3OH/water mixture.[11] The separation analysis time was determined as being equal to 18 min.

It is of interest to decrease this value and to obtain an efficient separation in a minimum analysis time. To reduce the time a new response function ζ is proposed. ζ is defined as:

$$\begin{aligned} \zeta &= \text{Min}(R_{ij}) && \text{if Min}(R_{ij}) \leq R_l \\ \zeta &= R_l + 1/t_a && \text{if not} \end{aligned} \qquad (5)$$

where $\text{Min}(R_{ij})$ = the resolution for the worst separated pair of peaks on the chromatogram.

R_l is the limit resolution accepted. If the separation is to be presented to industrial process control engineers, the objective might be to optimize the analysis time and not be to demanding for the value of the resolution of the worst separated pair of peaks. In our application, R_l was 0.8.

Therefore, if the resolution for the worst separated pair of peaks is inferior to the chosen limit resolution, then the ζ-function is equal to the resolution. If not, separation conditions were obtained and then the analysis time t_a intervened in the form 1/ta. Thus, the ζ-function was maximal when both efficient separation conditions and a minimal analysis time were obtained. The resolution between two peaks is given by the well known equation:

$$R_{ij} = \frac{\sqrt{N}}{4}\left(\frac{k'}{1+k'}\right)\left(\frac{\alpha-1}{\alpha}\right) \qquad (6)$$

where L is the column length, N is the column plate number = L/H, α is the separation factor given by the ratio of the capacity factor k' for the two solutes between which resolution is being calculated. The coefficient of variation of the k' values was less than 2%. Using the experimental design, ln(k') for each of the ten compounds and the column dead time t_o were modelled by a two order polynomial.

All the correlation coefficients were higher than 0.991. The student T-test confirmed that, for each compound, the k' value was not mobile phase flow rate dependent On the contrary t_o depended entirely on it. Obviously, the t_o model was found to be the same if ACN was used instead of CH_3OH (polynomial coefficients of the t_o model are given in Ref. 11). The analysis time t_a was given by the retention time of the last compound on the chromatogram (diazepam)

$$t_a = t_o\,(1 + \exp(\text{Lnk}'_{\text{diazepam}})) \qquad (7)$$

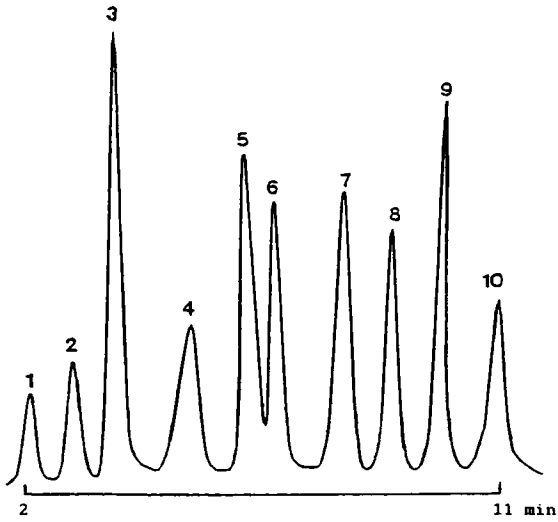

Figure 4. Benzodiazepine chromatogram in the conditions: ACN/water (60/40)(V/V), F = 1.00 mL/min, T = 47°C. Number above peaks refers to the 10 compounds: see experimental reagents.

Table 5

Comparison of Calculated $(t_a)_c$ and Experimental $(t_a)_{exp}$ Analysis Time (in min) for Different Experimental Conditions

θ(%)	F(mL/min)	T(°C)	$(t_a)_c$	$(t_a)_{exp}$
55	1.4	35	13.65	13.50
55	0.8	45	20.69	20.30
75	0.8	45	5.69	5.60
75	1.1	25	4.66	4.60
65	1.4	45	5.90	6.01
65	1.4	25	6.77	6.60
45	1.4	45	31.49	31.30
45	1.4	25	42.00	41.00

For example, the corresponding polynomial coefficients of $Ln(k'_{diazepam})$ are given in Table 1. Experimental and calculated values of t_a for different values of the three factors are given in Table 5. Computer simulations have begun to play an increasing role in the optimization separations.[21-25] The utility

Table 6

Result of the Simplex Process for the Optimization of the ξ-Function

θ(%)	F(mL/min)	T(°C)	ζ
80.00	1.60	25	0.0101
75.85	1.57	32	0.0129
72.48	1.65	45	0.0581
70.44	1.60	30	0.0542
78.12	1.75	40	0.0121
74.65	1.23	42	0.0226
69.75	1.00	46	0.2081
65.45	1.45	25	0.0201
69.63	1.32	35	0.1998
66.00	1.20	48	0.3888
77.45	1.19	38	0.0123
68.12	1.33	50	0.3423
64.13	1.00	44	0.3951
67.14	1.20	46	0.3884
65.47	0.80	50	0.5411
55.22	0.90	48	0.8532
54.96	0.85	48	0.8534
50.01	0.80	37	0.8273
45.05	0.70	34	0.8134
52.41	0.75	42	0.8346
54.23	0.84	48	0.8496
50.74	0.92	50	0.8411
56.12	1.02	47	0.8694
59.56	1.00	45	0.8864
59.95	1.01	47	0.8920

of the ζ-method, is that it takes into account the analysis time t_a and the simultaneous variation of column efficiency with the three factors, mobile phase composition, its flow rate, and the column temperature. The experimental design reduced the number of experiments to be carried out. Therefore, knowing the variation of H, k', α and t_a with the mobile phase composition, its flow-rate and column temperature, the ζ-values can be

calculated for different values of the three factors. ζ-reached its maximum for θ = 60%, F = 1.00mL/min, T = 47°C (values determined using the simplex method (Table 6). The chromatogram with these conditions is given in Figure 4. The analysis time was 11 min.

CONCLUSION

The use of an ACN/water instead of a CH_3OH/water mixture improved column efficiency. The results are corroborated by simple new equations relating the column efficiency to both the nature of the organic modifier and its percentage in the OM/water mixture, and by the organization of the ACN molecules in the H_2O molecules. In addition, the separation of these compounds and the analysis time were both optimized with a new response function developed in our laboratory. The results showed that the analysis time was reduced by 50 % when ACN was chosen instead of CH_3OH.

AKNOWLEDGMENT

The authors thank V. David Maupoil for her help.

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Received January 1996
Accepted April 24, 1996
Manuscript 4066