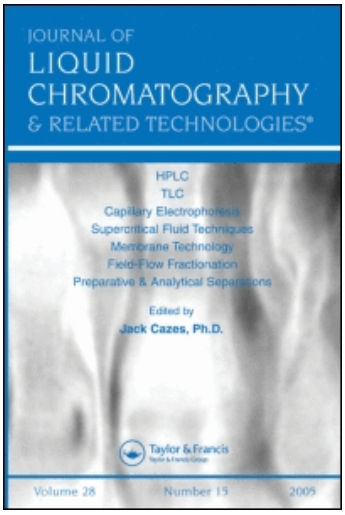

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Optimizing Mobile Phase Composition, its Flow Rate and Column Temperature in HPLC Using an Experimental Design Assisted with A Simplex Method

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OPTIMIZING MOBILE PHASE COMPOSITION, ITS FLOW RATE AND COLUMN TEMPERATURE IN HPLC USING AN EXPERIMENTAL DESIGN ASSISTED WITH A SIMPLEX METHOD

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ABSTRACT

In this work, a rapid procedure to separate ten compounds in high performance liquid chromatography is presented. The use of an experimental design assisted with a simplex method is proposed to separate these compounds with only thirteen chromatographic analyses to select the mobile phase composition, its flow rate and the column temperature. A flow rate of 0.77 ml/min with a percentage of methanol of 49,95% in the mixture methanol-water and a column temperature of 51.62°C gave the most efficient separation conditions.

INTRODUCTION

To optimize the mobile phase composition, its flow rate and column temperature, a sequential simplex method has been most frequently used (1,2,3). Little attention has been given to the optimization of these three factors taken simultaneously. The major emphasis of optimization schemes has been on optimizing

two, three or even four solvents (4,5,6). The methods often employed are based on window diagrams (7) and computer simulated techniques (8,9,10). Our aim, by doing this research, was to show the advantages of using an experimental design assisted with a simplex method to separate several compounds as ten benzodiazepines.

MATERIALS AND METHODS

Methanol and water were used in all experiments. Compounds: (1) Bromazepam (2) Nitrazepam (3) Flunitrazepam (4) Clobazam (5) Lorazepam (6) Oxazepam (7) Tofisopam (8) Chlordiazepoxide (9) Chlorazepate dipotassic and (10) Diazepam were obtained from HOFFMAN-LA ROCHE (Basel, Switzerland). They were diluted in methanol in a concentration range of 10-80 mg/ml. All solutions and solvents were degased by sonification.

CHROMATOGRAPHIC CONDITIONS

The HPLC system consisted of a HPLC Waters pump 501 (Saint-Quentin en Yvelines, France) a Merck L 4000 variable wavelength UV spectrophotometer detector and a Merck D 2500 Chromato Integrator (Nogent-sur-Marne, France). The reversed phase column used for the evaluation of the optimization scheme was a Waters steel Nova pak C18-60 Å column (15 cm x 3.9 mm ID, 4 µm particle size). The detection wavelength was 254 nm. The flow rate used varied from 0,6 to 1,6 ml/min. The volume percentage of methanol varied from 50 to 80% in the methanol-water mixture employed for the mobile phase. Weaker percentages were not used because of the excessively high column pressure obtained with 50% methanol with a flow rate of 1.6 ml/min. Column temperature was controlled with a Interchim crococil oven TM n°701 (Montluçon, France). Overall

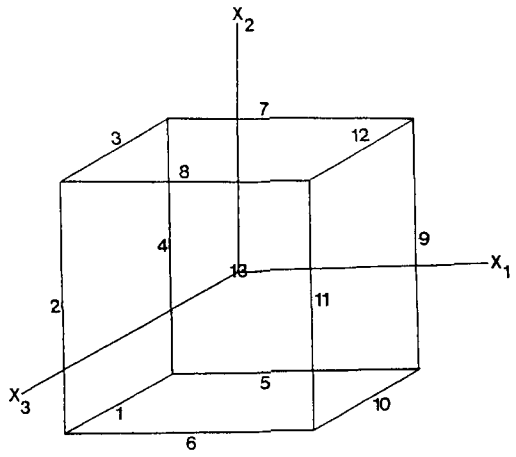

FIGURE 1. Modified Box and Behnken experimental design

temperature control was maintained within ± 1°C with a variation from 26°C to 50°C.

CHEMOMETRIC METHODOLOGY :

To optimize the separation of a number of compounds, the traditional approach would be to study separately, each factor which influences the separation. A chemometric approach is based on the use of a matrix of experiments which allows studying the simultaneous variation of all the factors. This way, the number of experiments can be reduced, compared with the traditional methods. A mathematical model was used which linked the observed response (Y) and the influencing factor (X_i). Variables were coded to have a range of variation from -1 to +1. The experimental quantitative factors which controlled the separation of benzodiazepines included the mobile phase composition, its flow rate and the column temperature.

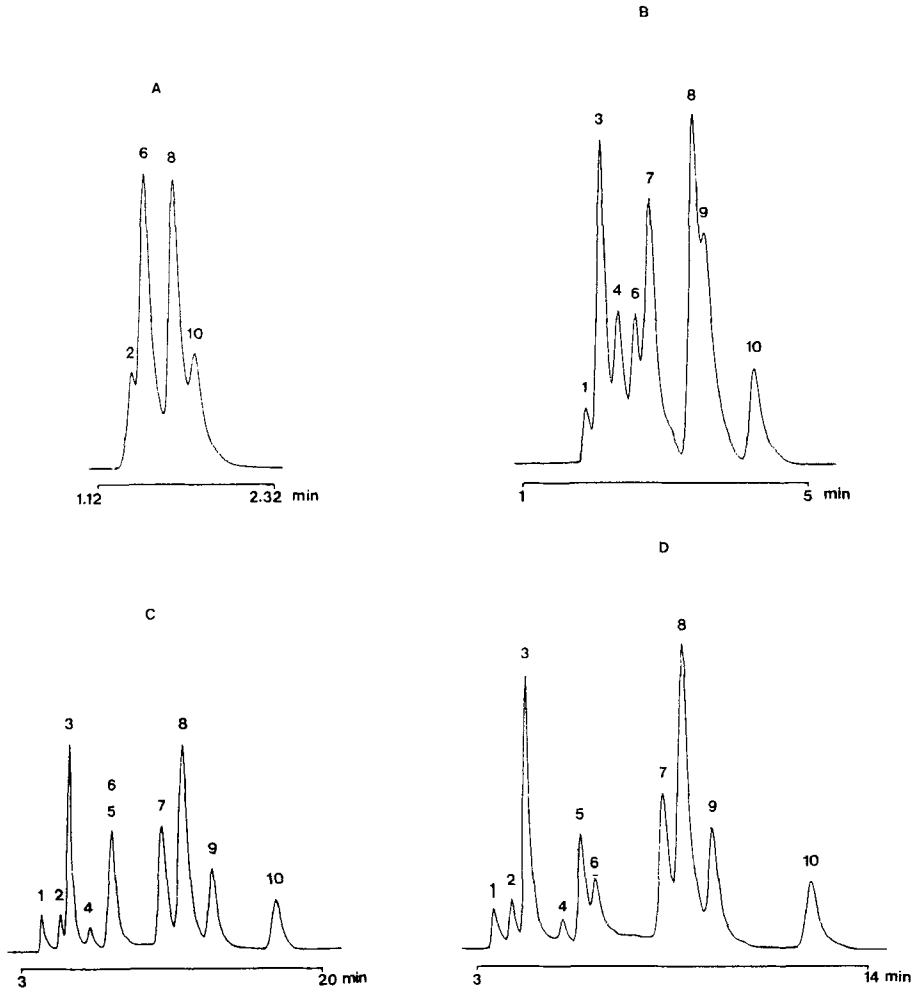

FIGURE 2. Representative chromatograms of 10 Benzodiazepines in the following conditions :

A - Methanol-eau, 80 : 20 v/v. Flow-rate 1 ml/min
Temperature = 26°C.
B - Methanol-eau, 63.24 : 36.76 v/v. Flow-rate 1 ml/min
Temperature = 26°C.
C - Methanol-eau, 50 : 50 v/v. Flow-rate 1 ml/min
Temperature = 26°C.
D - Methanol-eau, 50 : 50 v/v. Flow-rate 1 ml/min
Temperature = 50°C.
E - Methanol-eau, 49.95 : 50.05 v/v. Flow-rate 0.77 ml/min
Temperature = 51.62°C.
Numbers above peaks refer to the benzodiazepines in table 1.

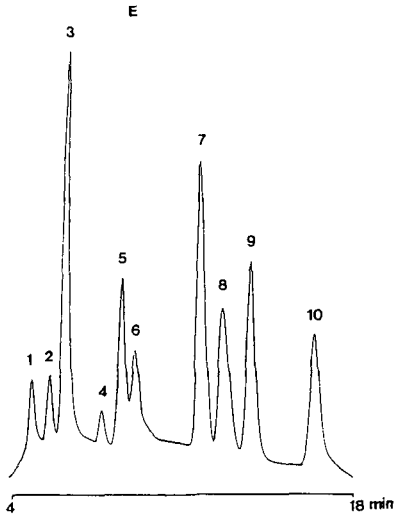

FIGURE 2 (continued)

RESULTS AND DISCUSSION

The principle of this procedure is based on a second order polynomial between the resolution of a pair of two adjacent peaks and the three main factors (mobile phase composition, mobile phase flow rate and column temperature). In order to investigate the effect of these three variables and their possible interactions in high performance liquid chromatography, a modified BOX and BENHKEN (11,12,13) experimental design was adopted (Fig. 1). Thirteen experiments were carried out and the resolutions were determined. Five chromatograms are shown in Figure 2.

The resolution factor reflects the degree of separation of a pair of peaks but doesn't take into account the quality of a separation.

The resolution of a pair of peaks could be measured from a chromatogram using retention time (t) and the peak widths (W_b) by the following equation :

$$R_s = 2 \frac{(t_2 - t_1)}{W_{b_1} + W_{b_2}} \qquad (1)$$

where the subscripts 1 and 2 refer to the first and second eluting peaks. Whatever the factor variation, all compounds were arranged on a chromatogram in the same order. The R_S value were then fitted into a second order polynomial.

$$\ln(1+R_S) = a_0 + a_1\ln(X_1) + a_2\ln(X_2) + a_3\ln(X_3) + a_{12}\ln(X_1)\ \ln(X_2) + a_{13}\ln(X_1)\ \ln(X_3) + a_{23}\ln(X_2)\ \ln(X_3) + a_{11}\ln^2(X_1) + a_{22}\ln^2(X_2) + a_{33}\ln^2(X_3) \qquad (2)$$

where X_1 is the mobile phase composition X_2 the flow rate, X_3 the column temperature and a_0, a_1, a_2, a_{11}, a_{22}, a_{33}, a_{12}, a_{13}, a_{23} the coefficients. The values of a_i and a_{ij} for each pair of peaks were determined using a basic program (table 1). The calculated R_S value for each pair of peaks for the thirteen experiments are given in Table 2. Good agreement was obtained between predicted data and experimental results. Using equation (2), R_S was calculated for different values of the three factors. In our case, the percentage (P) of methanol in the mixture varied from 50 to 80%. The column temperature (T) range varied from 26 to 50°C. The mobile phase flow rate (D) was varied between 0.6 to 1.6 ml/min. The highest resolution was used as a criterion of separation. A simplex method (14) was employed to carry out this optimization procedure.

In this process, the R_S value is calculated for m sets of starting conditions where m is given by the number of factors to be optimized plus 1. In this case, therefore m is 4. The

Table 1. Values of the coefficients a_0, a_1, a_2, a_3, a_{11}, a_{22}, a_{33}, a_{12}, a_{13}, a_{23} of the nine pairs of adjacent peaks and correlation coefficient r.

Peak pairs	a_0	a_1	a_2	a_3	a_{11}	a_{22}	a_{33}	a_{12}	a_{13}	a_{23}	r
(1,2)	0.3531	-0.5163	0.0592	-0.0869	0.1901	0.0577	-0.0099	-0.0556	0.0562	-0.0207	0.9989
(2,3)	0.0723	-0.3679	0.0062	0.0437	0.2649	0.0365	0.0249	-0.0305	-0.0256	0.0052	0.9879
(3,4)	0.6242	-0.5128	0.0078	0.0478	0.1120	-0.0593	0.0115	0.0303	-0.0636	0.0071	0.9991
(4,5)	0.2429	-0.4697	0.0116	-0.7640	0.2439	0.0011	-0.0003	0.0092	0.0296	0.0278	0.9894
(5,6)	0.3000	0.0249	-0.0003	0.0581	0.1576	-0.0394	0.0928	0.0118	-0.1517	-0.0033	0.9862
(6,7)	0.2903	-0.7328	0.0174	0.0292	0.4639	0.0103	0.0349	-0.0109	0.0200	0.0161	0.9923
(7,8)	0.7260	-0.1219	0.0297	-0.0776	-0.1270	0.0213	0.0631	0.0838	-0.0089	0.0366	0.9981
(8,9)	0.2624	-0.4840	0.0056	-0.0549	0.2512	0.0084	0.0348	0.0101	0.0105	0.0401	0.9991
(9,10)	1.1174	-0.0719	-0.6220	0.0051	-0.0307	-0.0166	-0.0719	-0.0224	-0.0123	0.0125	0.9874

(1,2) = (bromazepam, nitrazepam), (2,3) = (nitrazepam, flunitrazepam)
(3,4) = (flunitrazepam, clobazam), (4,5) = (clobazam, lorazepam), (5,6) = (lorazepam, oxazepam)
(6,7) = (oxazepam, tofisopam), (7,8) = (tofisopam, chlordiazepoxide), (8,9) = (chlordiazepoxide, chlorazepate dipotassic),
(9,10) = (chlorazepate dipotassic, diazepam).

Table 2. Resolution (R_s) between adjacent peaks, calculated for ten chromatographed benzodiazepines

Chromatographic conditions				Peak pairs[a]								
Experiment number	Methanol (% v/v)	Flow rate (ml/min)	Temperature (°C)	(1,2)	(2,3)	(3,4)	(4,5)	(5,6)	(6,7)	(7,8)	(8,9)	(9,10)
1	50	0.6	36	1.73	1.02	2.36	1.60	0.09	3.34	1.22	1.75	4.28
2	50	1.0	50	1.48	1.23	2.94	1.34	0.52	3.63	1.04	1.63	4.32
3	50	1.6	36	2.43	1.18	2.21	1.61	0.06	3.60	0.99	1.72	4.58
4	50	1.0	26	2.31	0.94	2.15	1.89	0.00	3.54	1.34	1.99	3.97
5	63	0.6	26	0.50	0.09	0.70	0.40	0.39	0.36	1.45	0.48	1.76
6	63	0.6	50	0.32	0.18	0.84	0.14	0.51	0.39	0.95	0.23	1.81
7	63	1.6	50	0.42	0.21	0.89	0.23	0.50	0.49	1.22	0.35	1.91
8	63	1.6	26	0.76	0.09	0.70	0.36	0.35	0.36	1.41	0.39	1.72
9	80	1.0	50	0.02	0.01	0.25	0.00	0.18	0.11	0.58	0.02	0.49
10	80	0.6	36	0.08	0.03	0.13	0.00	0.12	0.03	0.47	0.02	0.59
11	80	1.0	26	0.05	0.00	0.29	0.07	0.43	0.00	0.88	0.11	0.46
12	80	1.6	36	0.09	0.00	0.22	0.04	0.15	0.04	0.84	0.06	0.54
13	63	1.0	36	0.43	0.08	0.87	0.28	0.35	0.34	1.07	0.30	2.06

[a] see table 1

Table 3. Results of the simplex process

Experiment number	Percentage of Methanol (%)	Flow rate (ml/min)	Temperature (°C)	R_S
1	55.00	0.80	30.00	0.1865
2	59.71	0.85	31.18	0.1744
3	56.18	0.99	31.18	0.2286
4	56.18	0.85	34.62	0.2591
5	51.86	0.91	32.75	0.1278
6	54.47	1.03	35.76	0.2405
7	59.36	1.00	35.00	0.2060
8	57.17	0.93	39.15	0.3400
9	52.52	0.87	38.06	0.2380
10	56.10	0.74	38.86	0.3136
11	60.44	0.80	37.08	0.1716
12	54.63	0.80	42.01	0.3660
13	54.82	0.84	42.93	0.3941
14	58.31	0.98	43.87	0.3119
15	58.00	0.81	46.72	0.3479
16	56.66	0.66	43.90	0.4128
17	53.36	0.74	47.02	0.4784
18	57.30	0.68	42.52	0.3717
19	56.66	0.55	46.03	0.4273
20	53.92	0.62	48.80	0.5144
21	52.63	0.61	50.65	0.5595
22	49.95	0.77	51.62	0.5981

corresponding experimental conditions were :

[1] D = 0.80 ml/min T = 30,00°C p = 55,00%

[2] D = 0.85 ml/min T = 31.18°C p = 59.71%

[3] D = 0.99 ml/min T = 31.18°C p = 56.18%

[4] D = 0.85 ml/min T = 34,62°C p = 56.18%

The point corresponding to the lowest value of R_S is then reflected about the surface defined by the three other points to give a fifth set of starting conditions. Once again, the point with the lowest R_S is reflected and the process repeated sequentially until an apparent optimum has been obtained.

Twenty two iterative processes were performed by the computer and the results are given in Table 3. The maximum R_S (0.60) was the highest resolution for the worst separated pair of peaks. The

optimum conditions were a mobile phase flow rate of 0.77 ml/min with a percentage of methanol of 49.95% and a column temperature of 51.62°C. The corresponding chromatogram is presented in Figure 2. For the same values of percentage of methanol and flow rate, 50% and 1 ml/min peaks of Lorazepam and Oxazepam intermingled at 26°C. Nevertheless, at 50°C the two peaks were divided (Fig.2). The analysis time decreased (17.48 min and 12.44 min) respectively.

The separation of these ten compounds using this statistical method of optimization is readily possible from both an analytical point of view (resolution) and an economic point of view (analysis time).

The retention time t for each compound is given by the equation :

$$t = t_0 (1+k')$$

where k' is the capacity factor of the studied compound.

t_0 (min) is the retention time of an unretained peak as sodium nitrate.

Using the experimental design, ln(k) and t_0 were modelled by a two order polynomial.

The corresponding polynomial coefficients are given in Table 4.

The retention time for the ten benzodiazepines was calculated in accordance with the following experimental conditions

[1] p = 50% D = 1 ml/min T = 26°C

[2] p = 50% D = 1 ml/min T = 50°C

The predicted and measured chromatograms were similar (see Table 4). The effect of temperature was significant. Thus, when

Table 4. Values of the coefficients a_0, a_1, a_2, a_3, a_{11}, a_{22}, a_{33}, a_{12}, a_{13}, a_{23} and correlation coefficients r :
- for lnk of the ten compounds
- for t_0

Compound number[a]	a_0	a_1	a_2	a_3	a_{11}	a_{22}	a_{33}	a_{12}	a_{13}	a_{23}	r
1	0.1054	-1.0389	0.0287	-0.0977	0.0848	-0.0066	-0.0189	0.0264	0.0178	0.0101	0.9993
2	0.2709	-1.1345	0.0323	-0.1297	0.0561	-0.0100	-0.0269	0.0265	0.0361	0.0173	0.9991
3	0.2959	-1.1898	0.0277	-0.1180	0.0800	-0.0046	-0.0196	0.0260	0.0307	0.0154	0.9989
4	0.4906	-1.2460	0.0257	-0.1086	0.0704	-0.0095	-0.0208	0.0142	0.0203	0.0119	0.9985
5	0.5628	-1.3166	0.0292	-0.1369	0.0785	-0.0112	-0.0188	0.0114	0.0282	0.0222	0.9981
6	0.6477	-1.2610	0.0190	-0.1267	0.0648	-0.0084	-0.0163	-0.0001	0.0106	0.0153	0.9989
7	0.7366	-1.4250	0.0369	-0.1158	0.1344	-0.0136	-0.0078	0.0081	0.0293	0.0153	0.9990
8	1.0296	-1.3191	0.0356	-0.1342	0.0425	-0.0131	-0.0166	0.0196	0.0318	0.0211	0.9989
9	1.0874	-1.3712	0.0371	-0.1475	0.0540	-0.0095	-0.0154	0.0186	0.0384	0.0278	0.9995
10	1.3262	-1.4097	0.0320	-0.1257	0.0320	0.0027	-0.0253	0.0099	0.0567	0.0566	0.9996
t_0	0.9	0.0150	-0.4800	-0.0050	0.0125	0.1425	-0.0025	-0.0050	0.0101	0.0100	0.9999

[a]: see table 1

Table 5. Predicted (t^p) and measured (t^m) retention times for the ten benzodiazepines in the following conditions :
[1] Methanol = 50% v/v Flow-rate = 1 ml/min Température = 26°C
[2] Methanol = 50% v/v Flow-rate = 1 ml/min Temperature = 50°C

	[1]				[2]				
Compound Number[a]	t_I^m(min)	t_I^p(min)	Δt_I(min)	Δt_I(%)	t_{II}^m(min)	t_{II}^p(min)	Δt_{II} (min)	Δt_{II} (%)	$\Delta t^m_{(II-I)}$ min
1	4.32	4.24	0.08	1.89	3.50	3.43	0.07	2.04	0.82
2	5.35	5.30	0.05	0.94	4.00	3.91	0.09	2.30	1.35
3	5.82	5.73	0.09	1.60	4.41	4.34	0.07	0.37	1.41
4	7.02	6.93	0.09	1.30	5.46	5.38	0.08	1.50	1.56
5	8.22	8.12	0.10	1.23	5.98	5.93	0.05	0.84	2.24
6	8.22	8.19	0.03	0.37	6.37	6.17	0.20	3.20	1.85
7	11.03	11.00	0.03	0.27	8.30	8.18	0.12	1.50	2.73
8	12.18	12.17	0.01	0.08	8.84	8.69	0.15	1.73	3.34
9	13.90	13.90	0.00	0.00	9.70	9.55	0.15	1.57	4.20
10	17.48	17.04	0.44	2.58	12.44	12.28	0.16	1.30	5.04

[a]: see table 1

the retention time increased, the difference in the compound retention times between experiment [1] and [2] was greater.

CONCLUSION

This approach to the separation of ten compounds has allowed us to find optimum separation conditions with a limited number of experiments taking into account three elution factors. This procedure allowed us to carry out thirteen instead of twenty two experiments. Results demonstrate the importance of temperature and the need to control the temperature of the column.

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