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COMMUNICATION

Optimization of Selected Chromatographic Responses Using a Designed Experiment at the Fine-Tuning Stage in Reversed-Phase High-Performance Liquid Chromatographic Method Development

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ABSTRACT

This study evaluated the applicability of a designed experiment at the fine-tuning stage in reversed-phase high-performance liquid chromatographic (HPLC) method development. Using acetaminophen, theophylline, and caffeine as model drugs, a 3^2 factorial design was used to optimize selected chromatographic responses. The effects of the ratio of water to acetonitrile (%v/v) in the mobile phase and mobile phase flow rate on the theoretical plate number of acetaminophen peak, capacity factor of acetaminophen, resolution of acetaminophen and theophylline peaks, and the time for the elution of last peak (run time) were determined. Polynomial equations were derived to evaluate the quantitative relationships between the experimental factors and responses. A solution space was found by overlaying contour plots. Results indicated that, once the mobile phase that provides reasonably good retention and resolution has been identified, the strategy of using a designed experiment is advantageous over the conventional one-factor-at-a time approach since it would enable the analyst to optimize important responses, including run time with a minimum number of experiments.

Key Words: *Designed experiment; High-performance liquid chromatography; Optimization.*

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INTRODUCTION

Combined with multiple regression and suitable optimization techniques, designed experiments can be used to achieve desirable responses (1,2). Factorial and simplex types of designed experiments have been used in high-performance liquid chromatographic (HPLC) method development studies to optimize chromatographic responses (3–8). The use of such statistical tools, especially at the fine-tuning stage of HPLC method development studies, is advantageous. This is because traditional HPLC method development techniques are time and energy consuming and uneconomical, resulting in waste of organic solvents and other mobile phase components; further, they may not necessarily yield methods that would meet suitability requirements during validation.

Preliminary studies conducted in our quality control laboratory using a ternary solvent system containing water, methanol, acetonitrile, and glacial acetic acid, a C18 column, and a mixture of acetaminophen, caffeine, and theophylline resulted in the following elution order: acetaminophen, followed by theophylline, followed by caffeine. Based on the results, it was decided that the following chromatographic responses needed to be optimized if validation of the method was to be successful: theoretical plate number of acetaminophen (TPN_A); capacity factor of acetaminophen K'_A; resolution between the two closely eluting peaks, acetaminophen and theophylline, R_{AT}; and the retention time of the last eluting compound (run time). It was thought that it would be possible to achieve desirable levels for these four responses ($TPN_A > 1000$, K'_A 0.5–10, $R_{AT} > 3.5$, and run time 9–10 min) by changing the levels of water-to-acetonitrile ratio (W-to-A ratio) in the mobile phase and mobile phase flow rate.

Therefore, the present study was undertaken to demonstrate the use of a 3^2 factorial design to characterize the relationship between chromatographic factors and responses and to optimize responses using the response surface method (2).

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Waters 501 HPLC pump, a model VK 6 universal injector, a Lambda-Max model 480 LC spectrophotometer (Waters Associates, Milford, MA); and an HP3396 integrator (Hewlett Packard, Palo Alto, CA). Separations were performed on a 250 mm × 4.6 mm (id) reversed-phase column containing silica packing material 5 μm in diameter and bonded with octadecyl (C18) silane (CSC, Montreal, Quebec, Canada).

Chemicals and Reagents

HPLC-grade acetonitrile and methanol (EM Science, Gibbstown, NJ); acetaminophen, caffeine, and theophylline (Sigma, St. Louis, MO); and analytical reagent quality glacial acetic acid (BDH, Toronto, Ontario, Canada) were used.

Chromatographic Conditions

Experimental Design

The three levels of W-to-A ratio were 70%:5% v/v, 69%:6% v/v, and 68%:7% v/v, and the three levels of flow rate were 0.9, 1.0, and 1.1 ml/min, for a total of nine experimental runs. A 69.4%:5.6% v/v W-to-A ratio was used in combination with three levels of flow rate (0.9, 1.0, and 1.1 ml/min) to conduct three additional experiments.

Samples

Individual samples of acetaminophen (50 ppm), theophylline (5 ppm), and caffeine (5 ppm) solutions were obtained by dissolving each analyte separately in appropriate quantities in a solvent containing water, methanol, acetonitrile, and glacial acetic acid (70%:24%:5%:1% v/v). A mixture containing acetaminophen (10 ppm), theophylline (5 ppm), and caffeine (5 ppm) was also prepared using the same solvent.

Calculation of Chromatographic Responses

In all experiments, the mixture was used to generate chromatograms, and individual samples were used for peak identification. Only chromatograms generated with the mixture were used in the calculation of chromatographic responses.

RESULTS AND DISCUSSION

Analytes and Their Detection

Two of the three model drugs used in the present study, acetaminophen and caffeine, can occur together in oral dosage forms (9). The choice of the concentration of acetaminophen (50 ppm) and caffeine (5 ppm) was based on the proportion of these two drugs expected to

Table 1

Experimental and Predicted Values for Different Chromatographic Response Variables at Various Experimental Conditions

Experiment Number	Levels of Independent Variables		Responses			
	W/A Ratio[a]	Flow Rate (ml/min)	R_{AT}[b]	TPN_A[c]	K'_A[d]	Run Time (min)
1	68/7 (−1)[e]	0.9 (−1)	3.82	895.7	0.67	9.92
			(3.84)[f]	(910.8)	(0.67)	(9.87)
2	69/6 (−0.17)	0.9 (−1)	3.07	880.9	0.53	8.09
			(3.03)	(857.4)	(0.53)	(8.18)
3	70/5 (+1)	0.9 (−1)	4.26	1117.1	0.60	9.41
			(4.27)	(1125.5)	(0.60)	(9.37)
4	68/7 (−1)	1.0 (0)	3.81	863.1	0.76	9.02
			(3.83)	(874.7)	(0.76)	(9.02)
5	69/6 (−0.17)	1.0 (0)	3.08	860.7	0.61	7.34
			(3.04)	(836.3)	(0.61)	(7.33)
6	70/5 (+1)	1.0 (0)	4.29	1112.5	0.68	8.49
			(4.31)	(1125.3)	(0.68)	(0.50)
7	68/7 (−1)	1.1 (+1)	3.87	909.1	0.75	8.27
			(3.83)	(882.4)	(0.75)	(8.32)
8	69/6 (−0.17)	1.1 (+1)	2.98	810.9	0.60	6.69
			(3.06)	(858.8)	(0.60)	(6.61)
9	70/5 (+1)	1.1 (+1)	4.39	1189.9	0.68	7.74
			(4.36)	(1168.7)	(0.68)	(7.77)
10	69.4/5.6 (0.25)	0.9 (−1)	3.55	925.1	0.56	8.76
			(3.16)	(907.5)	(0.52)	(8.13)
11	69.4/5.6 (0.25)	1.0 (0)	3.54	895.1	0.66	8.60
			(3.18)	(893.8)	(0.60)	(7.27)
12	69.4/5.6 (0.25)	1.1 (+1)	3.60	922.9	0.71	7.61
			(3.20)	(923.8)	(0.59)	(6.55)

Experiments 10–12 are additional experiments.
[a] Water-to-acetonitrile (%v/v) ratio in mobile phase.
[b] Resolution of acetaminophen and theophylline peaks.
[c] Theoretical plate number of acetaminophen peak.
[d] Capacity factor of acetaminophen.
[e] Transformed value.
[f] Predicted value.

be present in the dosage form (9). Theophylline was co-resolved with the other two analytes since it could be present as an impurity in raw materials of caffeine (10). For simultaneous detection of the three analytes, 272 nm was chosen as the wavelength.

Calculation of Chromatographic Responses and Construction of Polynomial Equations

Chromatographic responses were calculated from the corresponding chromatograms. Table 1 summarizes the experimental design and shows the levels of the chromatographic factors studied along with the responses.

The relationship between the particular chromatographic response and the chromatographic parameters are shown by polynomial equations 1 to 4.

$$P = 853.4 + 3.7x + 21.8x^2 + 125.3y + 146.7y^2 + 17.9xy \quad (1)$$

$$C = 0.60 + 0.04x - 0.05x^2 - 0.04y + 0.12y^2 \quad (2)$$

$$R = 3.05 + 0.02x + 0.01x^2 + 0.24y + 1.02y^2 + 0.02xy \quad (3)$$

$$T = 7.24 - 0.79x + 0.07x^2 - 0.26y + 1.52y^2 + 0.01xy \quad (4)$$

where P, C, R, and T represent TPN_A, and K'_A, R_{AT}, and run time, respectively, and x and y represent the flow rate and W-to-A ratio, respectively. In the above equations, values of x and y are transformed values that can range between -1 and $+1$.

A typical method of construction of polynomial equations for the 3^2 design was followed to derive Equations 1 to 4 (2). The general form of equation using transformed values for the present two independent variables is

$$Y = B_0 + B_1 n_1 + B_{11} n_1^2 + B_2 n_2 + B_{22} n_2^2 B_{12} n_1 n_2$$

where Y is the chromatographic response of interest, the B's are the coefficients, and the n's are independent variables. It is assumed that interactions other than $n_1 n_2$ are absent. A multiple regression software program (Statistica for Windows Operating System, 3rd Ed., StatSoft, Inc., Tulsa, OK, 1997) was used for the computation of coefficients.

Examining of the Coefficients

Tests of significance were performed using the t test procedure for all coefficients. Flow rate was unimportant for R_{AT} and TPN_A, but it influenced K'_A and run time. The linear term of flow rate had a significant ($p < .05$) synergistic and antagonistic effect on K'_A and run time, respectively. The quadratic term of flow rate had a significant ($p < .05$) antagonistic effect on K'_A. Water:acetonitrile ratio was important for all four responses. Both the linear and the quadratic terms of the W-to-A ratio had a significant ($p < .05$) synergistic effect on R_{AT} and TPN_A. The linear term of the W-to-A ratio had a significant ($p < .05$) antagonistic effect on K'_A and run time, whereas the quadratic term of the same variable had a significant ($p < .05$) synergistic effect on these two responses. Interaction was not important for all four responses. Both the significant and nonsignificant coefficients were retained in Eqs. 1–4.

Model Verification and Optimization

R^2 values for TPN_A, K'_A, R_{AT}, and run time were 0.967, 0.999, 0.995, and 0.997, respectively, and the agreement between the predicted and observed responses of the experimental runs and the three additional runs (checkpoints) selected within the experimental space (Table 1) confirmed model fit. Therefore, Eqs. 1–4 were used to define response surfaces and to generate contour plots as a function of W-to-A ratio and flow rate of the mobile phase. Contour plots showed that a combination of reduced flow rate and high W-to-A ratio is important to obtain desired values for all four responses.

Figure 1 shows the contour plot for run time. The shaded area represents the solution space and encompasses the constraints (K'_A 0.5–10, $TPN_A > 1000$, resolution >3.5, and run time 9–10 min) on responses.

The solution space indicated that a combination of a low flow rate (0.9 ml/min) and high W-to-A ratio in the range 69.9%:5.1% to 70%:5% v/v should provide required levels for all four responses (Fig. 1). Conditions used in experiment 3 (flow rate 0.9 ml/min and W-to-A ratio 70%:5% v/v) satisfied the constraints imposed on responses (Table 1). These conditions also provided ac-

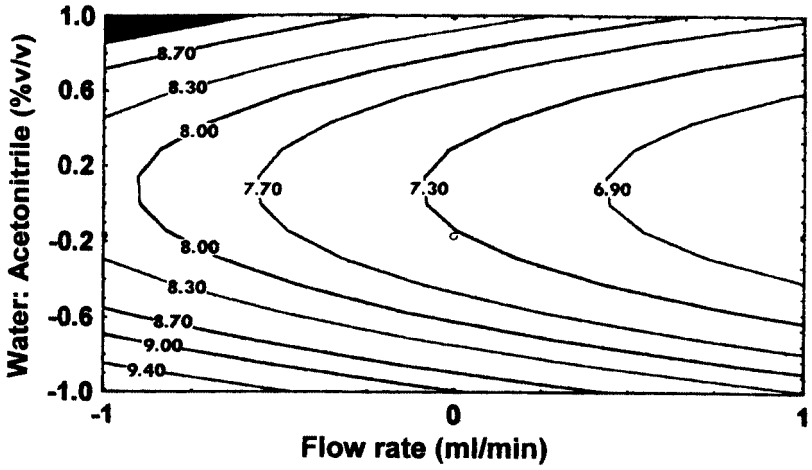

Figure 1. Contour plot for time for the elution of last peak (caffeine) as a function of flow rate and ratio of water to acetonitrile in the mobile phase.

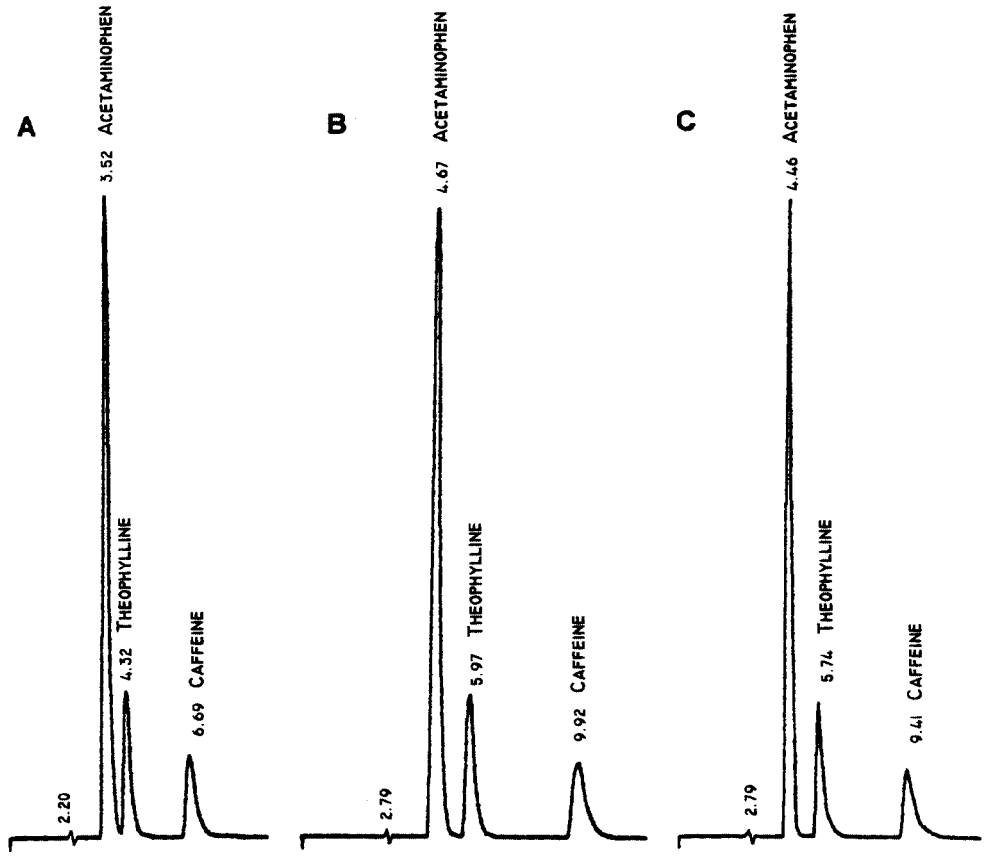

Figure 2. Chromatograms for different experimental conditions: (A) water:acetonitrile (69%:6% v/v) and flow rate 1.1 ml/min; (B) water:acetonitrile (68%:7% v/v) and flow rate 0.9 ml/min; (C) water:acetonitrile (70%:5% v/v) and flow rate 0.9 ml/min.

ceptable theoretical plate number (>1000) for theophylline and caffeine peaks (data not shown).

Representative chromatograms from three experimental runs, including trial 3, are shown in Fig. 2A–2C. Although a combination of low flow rate (0.9 ml/min) and low W-to-A ratio (68%:7% v/v) provided satisfactory values for K'_A, R_{AT}, and run time, optimization of these three chromatographic responses together with TPN_A could be accomplished only with a combination of high W-to-A ratio (70.5% v/v) and low flow rate (0.9 ml/min) (Table 1; Figs. 2A–2C).

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