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Mathematical Model for Customizing Chiral High Performance Liquid Chromatographic Analysis Employing Factorial Experimental Design

K. VALLIAPPAN*, K. KANNAN, R. MANAVALAN AND C. MURALIDHARAN[1]
Institute of Pharmaceutical Technology, Faculty of Engg. Tech.,
Annamalai University, Annamalainagar-608 002
[1]Dept. of Production Engineering, Faculty of Engg. Tech.,
Annamalai University, Annamalainagar-608 002.

This paper deals with the development of a mathematical model to predict the baseline enantioseparation and to customize chiral HPLC analysis using factorial experimental design. An attempt is made to establish quantitative relationship between chromatographic separation variables and the response factor (resolution). The significance of the chromatographic factors and adequacy of the model developed was checked using F-test and coefficient of determination (R^2) respectively. The trends of important effects of chromatographic factors on chiral resolution have been presented in graphical form. The study suggests that factorial design is a potential tool to customize chiral HPLC analysis.

Pharmaceutical analysts who deal with the development of HPLC method generally use experimental designs based on the variation of one factor at a time. This approach, however, is time consuming and might fail due to the many variables and interactions involved[1]. Accordingly, formal optimization methods are generally preferred. Factorial designs have been employed to optimize HPLC separation systematically[1-3]. Factorial experimental design is a convenient, time saving means of establishing quantitative relationship between chromatographic parameters and baseline separation. The success of this approach depends on correctly selecting the chromatographic separation variables, which can affect the baseline enantioseparation and judging the extent to which these can be experimentally varied to achieve acceptable chiral resolution.

Pharmaceutical enantiomers exhibit different pharmacodynamic and pharmacokinetic properties[4-7]. To understand and assess such differences chiral analytical techniques are required. Therapeutic agents often contain chemical functional groups such as amino, hydroxyl, carbonyl and carboxylic acid. These can be reacted with enantiomerically pure chiral derivatizing agent to give diastereomers suitable for analysis on achiral HPLC columns in a reversed-phase mode. Flurbiprofen, (fig.1), [FL, (±)-2-(2-fluoro-4-phenyl) propionic acid], is chosen as the model drug for the study. FL is a chiral orally effective nonsteroidal antiinlfammatory drug (NSAID) used in the management of arthritic disorders. It contains one stereogenic center[8] and is marketed as a racemate. In the present study, a mathematical model is developed, using a 2^3 factorial design, with an objective to customize chiral HPLC analysis and to establish quantitative relationship between chromatographic separation variables and enantioseparation.

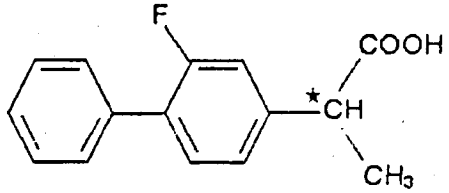

Fig.1: Chemical structure of (±)-flurbiprofen; ★ indicates stereogenic center

For correspondence
E-mail: valliappank@vsnl.net

EXPERIMENTAL

(R/S)- Flurbiprofen was a gift from FDC Limited, Mumbai, India. Ethyl chloroformate, triethylamine and L-leucinamide hydrochloride were purchased from Fluka, Buchs, Switzerland. Acetonitrile and water used were of HPLC grade while all other reagents employed were of analytical grade supplied by SD Fine Chemicals, Mumbai.

Chromatographic apparatus and conditions:

The chromatograph consisted of a Shimadzu (Japan) model LC10AD and LC10AD*vp* solvent delivery module, SPD-10A UV-Visible detector set at 260 nm, a Rheodyne model 7125 injector valve fitted to a 20 µl loop and a Shimadzu chromatographic workstation CLASS LC10 ver. 1.63. Stereoselective separation of the diastereomers was accomplished on a Supelcosil ODS analytical column (25 x 0.46 cm I.D., 5 µm particle size). The system was used in an air-conditioned HPLC laboratory atmosphere (20±2°). Before analysis, the mobile phase was degassed using Branson sonicator and filtered through a 0.2 µm membrane filter. Sample solutions were also filtered through a 0.2 µm membrane filter. The system was equilibrated before making an injection.

Derivatization procedure:

To investigate stereoselective drug disposition of chiral NSAID, α-methylbenzylamine is most commonly used as an optically active coupling component following activation of the carboxylic moiety with thionyl chloride or 1,1'-carbonyldiimidazole[9]. Other reagents described of which the most important seems to be S-(-)-1 (naphthen-1-yl) ethylamine, which was applied for the separation of NSAIDs from the group of 2-arylpropionic acids[10]. In 1985, Bjorkman[11] described the formation of diastereomeric derivatives of indoprofen following formation of the mixed anhydride with a chloroformate and further reaction with aminoacid derivative L-leucinamide, a reaction that is known for protein synthesis. This method was adopted for the resolution of racemic FL for the following reasons. The procedure was easy to perform, led to diastereomeric derivatives with suitable chromatographic properties for resolution in reversed-phase HPLC. Besides, the time required to complete one analysis was less than 20 min.

To 5 µg of racemic FL, 100 µl of 50 mM triethylamine in dried acetonitrile were added, and the tube was vortexed briefly. To this mixture were added at 30 s intervals, 50 µl of 60 mM ethyl chloroformate in acetonitrile and 50 µl of 1 M L-leucinamide hydrochloride in methanol containing 1 M triethylamine. After 2 min, 50 µl of HPLC-grade water were added. Aliqouts of 10-25 µl injected into the HPLC system. The amide derivative of the R-FL enantiomer was eluted earlier than the S-FL enantiomer from the reversed-phase column[12].

Factorial design:

The factorial experimental design was carried out in the following sequence. Initially, the chromatographic variables were identified and their useful limits established. Experiments were performed as per the design matrix developed. Subsequently the significant variables were identified. Based on this a mathematical model was developed and its adequacy was checked statistically.

Identification of chromatographic variables:

Various HPLC parameters such as flow rate, fraction of organic modifier, buffer concentration and pH may affect the retention time and chiral resolution[13-15], and it was decided to chose the volume of acetonitrile (A), buffer concentration (B) and mobile phase flow rate (C) as the variables for the factorial experiment. In the study, mobile phase pH was fixed at 6.5 as this could influence the stability of the diastereomeric derivative[11].

Selection of the useful limits of chromatographic variables:

The two levels selected for each of the three variables are shown in Table 1. For the convenience of recording and processing the experimental data, the high and low levels of the variables were coded as +1 and -1, respectively. Based on chromatographic experience and prior knowledge from literature the range of each variable was established[13]. The coded value of any intermediate level was calculated using the expression[16],

$$X_i = \frac{(X - X_{av})}{(X_{hi} - X_{lo})/2} \qquad (1)$$

X_i is the required coded value of a variable; X is any value of the level between X_{hi} and X_{lo}; X_{hi} is the high level; X_{lo} is the low level and X_{av} is the average of X_{hi} and X_{lo}

Design matrix development:

With three factors, 2^3 experiment (8 total) has to be performed for a complete factorial design. Table 2, shows 8 sets of coded conditions used to form the design matrix of 2^3 factorial design. The detailed methods of designing such matrix have been reported in literature[17-19].

TABLE 1: CONTROLLING CHROMATOGRAPHIC PARAMETERS

Factors	Notation	Unit	Level		Coding	
			Low	High	Low	High
Organic modifier	A	%v/v	45	60	-1	+1
Buffer concentration*	B	mM	10	60	-1	+1
Flow rate	C	ml/min	1	2	-1	+1

* Potassium dihydrogen phosphate

TABLE 2: DESIGN MATRIX

Experimental runs	Run order	Design Factor		
		A	B	C
1	1	-1	-1	-1
2	5	+1	-1	-1
3	3	-1	+1	-1
4	6	+1	+1	-1
5	2	-1	-1	+1
6	7	+1	-1	+1
7	4	-1	+1	+1
8	8	+1	+1	+1

(A) indicates organic modifier concentration (%v/v); (B) indicates phosphate buffer concentration (mM); (C) indicates flow rate (ml/min)

The experiments were performed in two replicates and run order of the experiments was made at random to avoid systematic errors creeping in the results.

The experiments were conducted as per the conditions dictated by the design matrix (Table 2) and the experimental retention time and chiral resolution on changing factors according to the design matrix are shown in Table 3. The Shimadzu Class LC10 chromatographic workstation computes and gives resolution value directly or it may be calculated from equation[13].

Development of mathematical model:

Most approaches to optimize resolution involve various means of computer-assisted retention time mapping. In some instances, sample resolution is determined as a function of mobile phase composition[20]. In the present study, the second approach is adopted as in the set experimental domain baseline chiral separation was

TABLE 3: RETENTION TIME AND CHIRAL RESOLUTION

Run order	Retention time (min) Replicate				Resolution (R_s) Replicate		Total R_s
	I		II		I	II	
	t_{R1}	t_{R1}	t_{R2}	t_{R2}	R_s	R_s	
1	17.24	18.96	17.21	18.94	3.02	3.03	6.05
5	5.95	6.35	5.96	6.34	1.95	1.94	3.89
3	16.75	18.67	16.79	18.62	3.47	3.46	6.93
6	6.05	6.52	6.00	6.50	2.25	2.27	4.52
2	8.88	9.69	8.85	9.67	2.36	2.37	4.73
7	3.00	3.20	3.00	3.21	1.42	1.40	2.82
4	8.63	9.55	8.60	9.50	2.84	2.82	5.66
8	3.06	3.29	3.08	3.29	1.71	1.70	3.41

t_{R1} and t_{R2} represents the retention time of peak 1 and peak 2 respectively; R_s denotes the resolution of L-leucinamide derivatives of (±)-flurbiprofen

achieved within a reasonably good run time of less than 15 min. Hence a mathematical model was developed for predicting optimum chiral resolution.

To represent the results in the form of a mathematical model it is required to express the response factor, chiral resolution, as a function of the variables whose effects on it have been investigated. Thus

$$Y=f\ (X_1,X_2,X_3,X_4,X_5,...,X_n) \quad (2)$$

where Y is the response factor and $(X_1...X_n)$ are variables. The aim being to develop a model that would serve the purpose adequately, it was decided to use the following partial polynomial[21].

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_1X_2+b_5X_1X_3+b_6X_2X_3+b_7X_1X_2X_3 \quad (3)$$

The above model includes the main effects of the variables and their first order interaction. To determine the significant factors of the model, analysis of variance (ANOVA) was performed (Table 4). To determine the significance of the factors F-test at 99% confidence level was carried out form which it was found that three main effects and two of the two-factor interaction have significant effects. Therefore, terms with only these factors constituted the response function. Thus, the selected regression model was reduced to the following workable form.

$$Y= b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_1X_2+b_5X_1X_3 \quad (4)$$

Where X_1, X_2 and X_3 represents factors A, B and C respectively. X_1X_2 and X_1X_3 represent AB and AC interaction respectively. The regression coefficients b_1,b_2,b_3,b_4 and b_5 are one half the corresponding effects estimates and b_0 is the grand average. By substituting the values of the coefficients in the above equation, the desired mathematical model could be formed[17]. The values of the coefficients were determined using the SPSS/PC + version 5.0 software package[22]. Consequently the following regression model was developed to represent chiral resolution.

$$R_s=2.375-0.545X_1+0.189X_2-0.298X_3-0.037X_1X_2+0.025X_1X_3 \quad (5)$$

The values for the factors $(X_1..X_3)$ in these models are to be used in the coded form that can be obtained using the expression (1) as described earlier. It is important to realize that the predictions using this model will remain valid only in the defined experimental domain. Similarly, one may develop a mathematical model for the run time also[1].

Adequacy of the model:

To check the adequacy of the model the coefficient of determination R^2 was employed[22]. R^2 is a measure of the goodness of fit of particular model. The significance of individual effects was evaluated using ANOVA technique. The regression model (5) developed for predicting R_s showed $R^2=0.9975$, indicating that 99.75% of variation in R_s is explained by the model.

RESULTS AND DISCUSSION

The effect of different chromatographic variables on

TABLE 4: ANOVA FOR CHIRAL ANALYSIS

Source of Variation	Sum of Square	Degrees of Freedom	Mean square	Calculated F ratio (F^0)
A	4.763306	1	4.763300	47633*
B	0.573800	1	0.573800	5738*
C	1.422050	1	1.422050	4221*
AB	0.021750	1	0.021750	218*
AC	0.015000	1	0.015000	105*
BC	0.000006	1	0.000006	-
ABC	0.000506	1	0.001000	10
Error	0.000860	8	0.000108	-
Total	6.792790	15	-	-

*Significant factor, (A) indicates organic modifier concentration (%v/v); (B) indicates phosphate buffer concentration (mM); (C) indicates flow rate (ml/min)

chiral resolution, based on the experimental observation, is shown in figs. 2, 3 and 4. These show the trends between cause and effect. It is evident, from fig. 2, that as the fraction of (A) in the mobile phase is set at a low level (45% v/v), there is a relative increase in the measure of resolution in all the four sets of conditions, compared to that at a high level (60% v/v). This may be due to the fact that a decrease in the (A) results in an increase in the retention factor (k) leading to a higher R_s.

Further, it is noticed that R_s attains a high value at low level of (A) with low flow rate (C) and high buffer

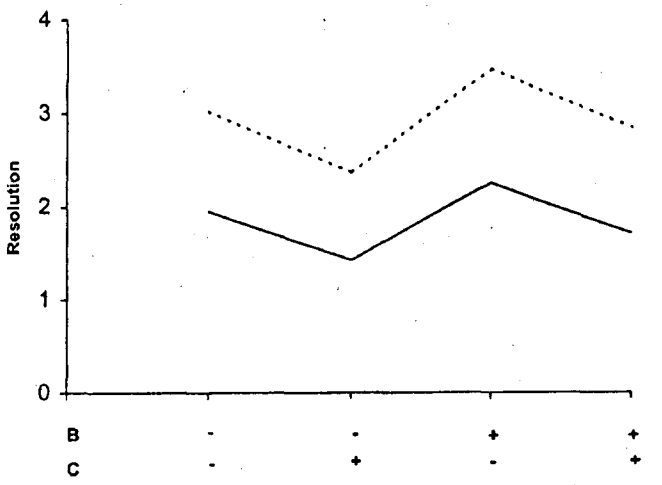

Fig. 2: Paired comparison of A with B and C; (—) indicates high level of A, whereas (----) indicates low level of A; A denotes organic modifier

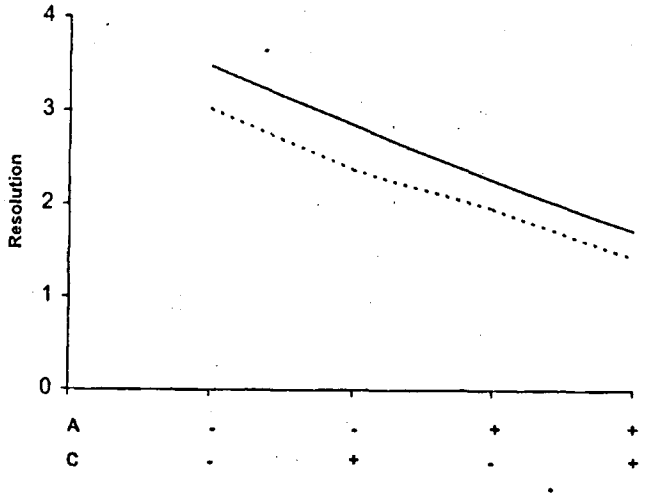

Fig. 3: Paired comparison of B with A and C; (—) indicates high level of B, whereas (----) indicates low level of B; B denotes buffer concentration

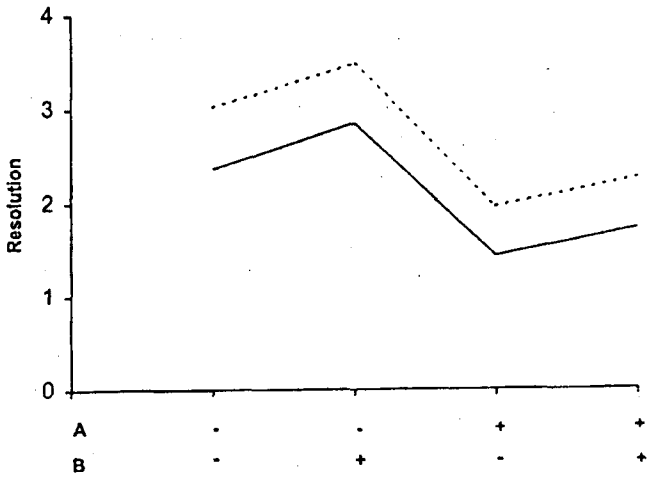

Fig. 4: Paired comparison of C with A and B; (—) indicates high level of C, whereas (---) indicates low level of C: C denotes flow rate (ml/min)

concentration (B). On the contrary, R_s value drops to a minimum value at fraction of (A) with high level of (C) and low level of (B). An explanation may be offered in the following lines. At low level of (A); an increase in buffer (KH_2PO_4) increases the polarity of the mobile phase and the more polar R-FL derivative elutes faster than the S-FL derivative offering a better selectivity. An increase in the salt concentration could reduce the silanol interaction minimizing the tailing effect. Synergic effect of both factors might have resulted in high R_s. But at high level of (A) with low level of (B) and high (C) level a reversal of the phenomenon is observed. At high (A) level the k value has decreased resulting in a lower selectivity. It also appears a high (B) level is not appropriate with high level (A) and a peak tailing was reflected by the asymmetry factor. The observed low R_s could be attributed to a combination of these effects.

Fig. 3 depicts the influence of buffer concentration (B) on R_s. It is noticed that R_s exhibits a maximum at high level (60 mM) of (B) with low level of (A) and (C) and a minimum at a low level (10 mM) of (B) with high level of (A) and (C). The first observation may be assigned to an increased column efficiency and improved selectivity as described earlier. The minimum R_s may be imputed to interplay of factors namely decreased k, inappropriate buffering and peak tailing.

Next, the effect of mobile phase flow rate (C) on R_s is studied. It is observed from fig. 4, that R_s is relatively higher at low level (1 ml/min) of (C) in all the four sets of

experimental conditions compared to that at a higher level (2 ml/min). This pattern could be attributed to the better efficiency of the column at lower level of (C) against a high level. Further, it is noted that R_s value reaches a maximum at low level of (C) with low fraction of (A) high level of (B). Similarly, R_s drops to a low minimum at a high (C) with high (A) and low (B) level. The explanations offered for the observations made in fig. 3, holds good for this situation also.

A typical theoretical HPLC condition was selected to check the validity of the model. The factors A,B and C were-1,0, and -1 respectively representing the fraction of organic modifier in the mobile phase, buffer concentration and mobile phase flow rate (45% v/v, 10mM, 1.5 ml/min). When this theoretical condition was tested with the HPLC system a satisfactory chiral separation was obtained. The actual R_s value for peak 1,2 is 2.67, which corresponded closely to the predicted value 2.69.

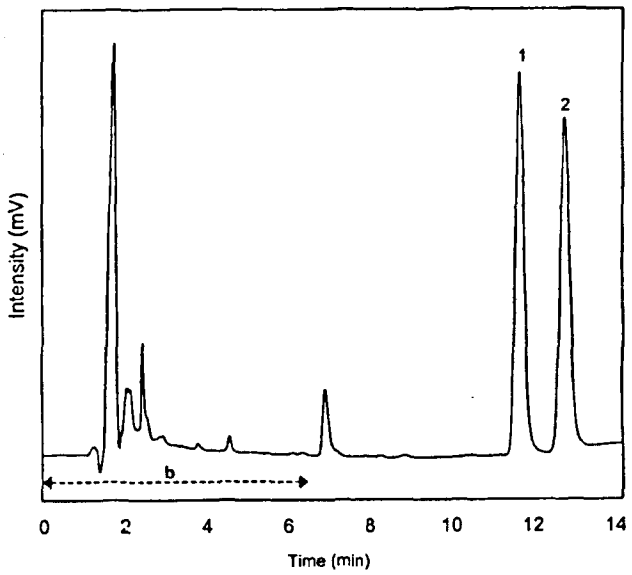

Fig. 5: Representative HPLC chromatogram of derivatized (±)-FL. Segment 'b' (time span:0.0 to 6.5 min.) of the chromatogram is the contribution due to blank; produced by mixing the reagents without addition of FL. Peaks : 1=R- and 2=S-flurbiprofen diastereomers

Fig. 5 depicts a representative chromatogram of derivatized (±)- FL. The retention time of the L-leucinamide derivatives of R-FL and S-FL (peaks 1 and 2) are 11.6 and 12.7 min respectively. The impurity peak of flurbiprofen, reported earlier[12], was observed at 6.9 min in the chromatographic condition employed in the study and this should not overlap with the internal standard peak (if used). The result of the study suggests that factorial experimental design is an effective and efficient tool to understand the effect of various chromatographic parameters on the response factor (resolution) and to develop mathematical model to customize chiral HPLC analysis.

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