Prediction of chiral separation of ketoprofen using experimental design

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The paper demonstrates how experimental design could be applied to predict chiral resolution and run time for indirect chiral high performance liquid chromatography (HPLC) analysis of ketoprofen. An attempt is made to establish quantitative relationship between chromatographic variables and the response factors (resolution and retention time). The effects of important chromatographic variables on chiral resolution and retention time have been highlighted by way of interaction studies. This technique enables optimal utilization of resources by avoiding trial and error approach. The study advocates that experimental design is a prospective tool to predict and optimize chromatographic conditions for chiral HPLC analysis.

Ketoprofen, (+)-2-(3-benzoylphenyl)propanoic acid, is a member of the 2-aryl-propanoic acid series of non-steroidal anti-inflammatory drugs (Fig. 1). Ketoprofen (KT) contains one stereogenic center and is currently available in the racemic form. Today, it is well documented that chiral Pharmaceuticals demonstrate different pharmacodynamic and pharmaco-kinetic properties. The prostaglandin synthetase inhibiting effect of ketoprofen is mostly attributable to the S-(-)-enantiomer (eutomer) and the R-(+) enantiomer (distomer) is regarded as being much less active. A change in the relative rate of absorption of the enantiomers may influence the pharmacodynamic properties of the drug. Therefore, the stereoselective determination of KT enantiomers in plasma is of potential clinical importance and hence KT was chosen as the model drug for the study.

Enantioselective studies require analytical methods capable of resolving and quantifying individual enantiomer of a chiral drug. One of the most popular and powerful analytical gadgets is chiral HPLC. Under this, two approaches are available, namely, the direct and indirect chiral HPLC. Direct chiral HPLC requires chiral stationary phases that are exorbitantly expensive. Hence in this work, indirect chiral analysis is employed to achieve chiral separation. This technique relies on chiral derivatization of functional groups (such as amino, hydroxyl, carbonyl and carboxylic acid) in the drug molecule to give diastereomers that are suitable for analysis on achiral stationary phases in a reversed-phase HPLC mode.

Experimental designs have been employed to optimize HPLC analysis systematically. The success of this approach depends on correctly selecting the chromatographic separation variables that can affect the baseline enantioseparation and judging the extent to which these can be experimentally varied to achieve acceptable chiral resolution.

In the present study, experimental design is applied to chiral HPLC analysis of KT with an objective to: (i) develop mathematical models to predict chiral separation; (ii) optimize indirect chiral HPLC analysis; and (iii) study the effects of significant chromatographic variables on chiral resolution and retention time through interaction studies.

Materials and Methods
(R/S)-ketoprofen, S-ketoprofen, probenecid (internal standard) and L-leucinamide hydrochloride were obtained from Sigma (India Office, Bangalore), ethylchloroforamte was procured from Fluka (Buchs, Switzerland). Indomethacin was a gift from M/S

![Chemical structure of ketoprofen; *indicates stereogenic center.](image)
Micro Labs Ltd. (Hosur, India) and S-naproxen kindly provided by M/S Shasun Chemicals (Chennai, India). Acetonitrile used was HPLC grade, while all other reagents employed were of analytical grade supplied by M/S SD Fine Chemicals (Mumbai, India). Water (HPLC-grade) was generated using Milli Q Academic, Millipore (Bangalore, India).

**Chromatographic apparatus and conditions**

The chromatograph consisted of a Shimadzu (Japan) model LC10AD and LC10ADvp solvent delivery module, SPD-10A UV-visible detector, a Rheodyne model 7125 injector valve fitted to a 20 µl volume sample loop and a Shimadzu chromatographic workstation CLASS LC10 ver.1.63. Diastereomers of KT were resolved on a Supelcosil ODS analytical column (25x0.46 cm I.D., 5µm particle size) in the reversed-phase partition chromatographic condition. The system was used in an air-conditioned HPLC laboratory atmosphere (20±2°C). Before analysis, the mobile phase was degassed using Branson sonicator (Branson Ultrasound Corporations USA) and filtered through a 0.2 µ filter (Gelman Science, India). Sample solutions were also filtered through a 0.2 µ filter. The system was equilibrated before making an injection. The column was monitored for UV absorbance at a detection wavelength of 256 nm. The wavelength of 256 nm was selected from the overlain spectra of the t-leucinamide derivatives KT and probenecid (PB). The standard solutions of KT & PB (10 µg/ml) were derivatized as per the procedure of Bjorkman and the UV spectra were recorded using UV-visible Spectrophotometer (UV-1601PC, Shimadzu) using reagent blank.

**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. S-(−)-1-(naphthen-1-yl) ethylamine was applied for the separation of NSAIDs from the group of 2-arylpionic acids. The method of Bjorkman was adopted for the resolution of racemic KT.

To a mixture of 0.5 ml solution of racemic KT (10 µg/ml) and 0.5 ml of solution of PB (4 µg/ml), were added 100 µl of 50 mM triethylamine in dried acetonitrile and the tube was vortex-mixed briefly. To this mixture were added at 30s intervals, 50 µl of 60 mM ethyl chloroformate in acetonitrile and 50 µl of 1M L-leucinamide hydrochloride in methanol containing 1M triethylamine. After 2 min, 50 µl of HPLC-grade water were added. Aliquots of 10-20 µl injected into the HPLC system.

The order of elution of the enantiomer was determined by testing the retention time of a peak eluted from a pure authentic sample of S-KT solution. It was confirmed that the L-leucinamide derivative of S-KT enantiomer eluted later than the one of the R-enantiomer from the reversed-phase column.

**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 chromatographic variables such as solvent strength, solvent type, buffer concentration, pH, column dimension, column packing, particle size, column temperature, special additives such as ion-pair reagents and flow rate may affect the retention time and chiral resolution. To identify the key factors that could influence quality separation (baseline resolution) a cause-and-effect analysis was performed (Fig. 2). Based on the analysis it was decided to choose the strength 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.

**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. The coded value of any intermediate level was calculated using the expression:

\[
X_i = \frac{(X - X_{xp})}{(X_{hi} - X_{lo})/2} \quad \ldots (1)
\]
Table 1—Controlling chromatographic parameters

<table>
<thead>
<tr>
<th>Factors (variables)</th>
<th>Notation</th>
<th>Unit</th>
<th>Level</th>
<th>Coding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic modifier</td>
<td>A</td>
<td>% v/v</td>
<td>Low: 45</td>
<td>High: 55</td>
</tr>
<tr>
<td>Buffer concentration</td>
<td>B</td>
<td>mM</td>
<td>Low: 10</td>
<td>High: 25</td>
</tr>
<tr>
<td>Flow rate</td>
<td>C</td>
<td>ml/min</td>
<td>Low: 1</td>
<td>High: 1.5</td>
</tr>
</tbody>
</table>

$X_i$ 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, $2^3$ experiments have to be performed for a complete factorial design. Eight sets of coded conditions were used to form the design matrix of $2^3$ factorial design. The detailed methods of designing such a matrix are dealt in literature [20-22]. The run order of the experiments made at random to avoid systematic errors creeping in the results. Chiral resolution and retention time on changing factors according to the design matrix are shown in Table 2. The Shimadzu Class LC10 chromatographic workstation computes and gives retention time (minutes) and resolution values directly. The resolution values also may be calculated from equation [15].

**Development of mathematical model**

Most approaches to optimize resolution involve various means of computer-assisted retention time mapping. In some instances, analyte resolution is determined as a function of mobile phase composition [23,24]. In the present study, mathematical model is developed for predicting both optimum chiral resolution and run time. To represent the results in the form of a mathematical model it is required to express the response factor, here chiral resolution and retention time, as a function of the variables whose effects on it have been investigated. Thus

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

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 [25].

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

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 for
chiral resolution (Table 3). In the same way, ANOVA was carried out for the retention time of peaks 1 and 3. To determine the significance of the factors, F-test at 99% confidence level was carried out from which it was found that three main effects and one two-factor interaction have significant effects on chiral resolution. Therefore, terms with only these factors constituted the response function. Thus, the selected regression model for resolution was reduced to the following workable form.

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

where $X_1$, $X_2$ and $X_3$ represent factors A, B and C; $X_1X_2$ and $X_1X_3$ represent AB and AC interaction respectively. The regression coefficients $b_1, b_2, b_3$ and $b_4$ 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. The values of the coefficients were determined using the SPSS/PC+ version 5.0 software package. Consequently the following regression model was developed to represent chiral resolution.

$$R_s = 2.33 - 0.57X_1 + 0.03X_2 - 0.13X_3 - 0.04X_1X_2 \quad \ldots (5)$$

The values for the factors $(X_1, X_2, X_3)$ 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, a mathematical model was developed for the retention time of the 1-leucinamide derivative of S-KT (peak 1) to know at what time the first peak leaves the column. This helps to make sure that peak 1 does not overlap with the solvent front while predicting mobile phase composition. F-test at 99% confidence level was performed to identify the factors that have significant effects on the retention time (peak 1). It was noticed that three main factors and two of the two-factor interactions have significant effects and only these factors are incorporated in the model. Hence the partial polynomial (3) was reduced to the following practical form.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_2X_3 + b_5X_4 \quad \ldots (6)$$

Substituting the coefficient values for significant factors in equation (6) resulted in the model for retention time for peak 1:

$$t_{RI} = 5.60 - 1.32X_1 - 0.02X_2 - 1.11X_3 + 0.04X_2X_3 + 0.26X_4 \quad \ldots (7)$$

### Table 2—Chiral resolution and retention time

<table>
<thead>
<tr>
<th>Factor levels</th>
<th>Resolution ($R_s$)</th>
<th>Retention time ($t_{RI}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak 1 ($t_{R1}$)</td>
<td>Peak 2 ($t_{R2}$)</td>
</tr>
<tr>
<td>A B C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+1 -1 +1</td>
<td>1.95</td>
<td>1.95</td>
</tr>
<tr>
<td>-1 +1 -1</td>
<td>2.77</td>
<td>2.80</td>
</tr>
<tr>
<td>-1 +1 +1</td>
<td>2.80</td>
<td>2.70</td>
</tr>
<tr>
<td>+1 +1 +1</td>
<td>1.72</td>
<td>1.72</td>
</tr>
</tbody>
</table>

$d_{t_{R1}}$, $d_{t_{R2}}$ and $d_{t_{R3}}$ represent the retention time of peak 1, peak 2 and peak 3 respectively; $R_s$ denotes the resolution of L-leucinamide derivatives of (±)-ketoprofen. $T_1$, $T_2$ and $T_3$ indicate trials 1, 2 and 3 respectively.

### Table 3—Anova for chiral resolution (peaks 1, 2)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of square</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>Calculated F ratio (F$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.762704</td>
<td>1</td>
<td>7.762704</td>
<td>14304.55$^*$</td>
</tr>
<tr>
<td>B</td>
<td>0.018740</td>
<td>1</td>
<td>0.018740</td>
<td>34.87$^*$</td>
</tr>
<tr>
<td>C</td>
<td>0.387604</td>
<td>1</td>
<td>0.387604</td>
<td>722.63$^*$</td>
</tr>
<tr>
<td>AB</td>
<td>0.033004</td>
<td>1</td>
<td>0.033004</td>
<td>61.53$^*$</td>
</tr>
<tr>
<td>AC</td>
<td>0.001838</td>
<td>1</td>
<td>0.001838</td>
<td>3.43</td>
</tr>
<tr>
<td>BC</td>
<td>0.001504</td>
<td>1</td>
<td>0.001504</td>
<td>2.80</td>
</tr>
<tr>
<td>ABC</td>
<td>0.003037</td>
<td>1</td>
<td>0.003037</td>
<td>5.66</td>
</tr>
<tr>
<td>Error</td>
<td>0.008582</td>
<td>16</td>
<td>0.000536</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.126977</td>
<td>23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^*$indicates significant factor, $F_{critical} = F_{0.01,1,16} = 8.53$
On the same pattern another model was developed for retention time of peak 3, the late eluting peak from the column, with an objective to predict the run time of the analysis.

\[ t_{R3} \text{(peak 3)} = 7.96 - 2.50X_1 - 0.03X_2 - 1.54X_3 + 0.04X_1X_2 + 0.47X_1X_3 \]  

\[ \ldots (8) \]

**Adequacy of the model**

To check the adequacy of the model the coefficient of determination \( R^2 \) was employed. The significance of individual effects was evaluated using ANOVA technique. The regression model (5) developed for predicting \( R_s \) showed \( R^2 = 0.9999 \). The models (7) and (8) developed for predicting the retention time of peak 1 and peak 3 showed \( R^2 = 0.9965 \) and \( R^2 = 0.9871 \) respectively.

**Results and Discussion**

The diastereomers of chiral KT was formed at ambient temperature in less than 3 minutes by utilizing L-leucinamide and ethylchloroformate. The amide derivatives, formed by the reaction of the carboxylic moiety of the NSAID with the chiral coupling component (L-leucinamide), are separated on an achiral column in the reversed-phase system.

In the experimental domain reasonably good separation of peaks was observed except few cases with excess resolution. The run time (retention time of the last-eluting solute; PB) was around 13 min. So it was decided to optimize separation of peaks (resolution ca. 2) and reduce the run time (ca. 10 min). To achieve this end, the predictive power of the regression models developed was used.

Different theoretical chromatographic conditions were selected to check the validity of the models in predicting quality separation of the peaks (peaks 1, 2 and 3) and short chromatographic time. It is observed that in almost all the mobile phase compositions tested the run time was well within 10 min and hence didn’t warrant further tuning of separation. If required run time can be further reduced by diminishing the separation between peaks 2 and 3. The predicted and measured chiral resolution for peak 1, 2 and retention time for peaks 1 and 3 are presented in Table 4. The predicted and measured chiral resolution and retention time are found to be in good agreement, with a difference of 1-6%.

Figure 3 depicts a typical chromatogram under the chromatographic conditions -0.4, -1, -0.2 representing the mobile phase composition (48% v/v acetonitrile, 10 mM phosphate buffer) and a flow rate of 1.2 ml/min. L-leucinamide derivatives of the KT enantiomers and the internal standard, (PB), were eluted at 6.36, 6.95 and 9.06 min respectively.

![Typical Chromatogram](image)

**Table 4—Predicted and measured response factors**

<table>
<thead>
<tr>
<th>Factor levels</th>
<th>Resolution (( R_s )) Peaks (1,2)</th>
<th>Retention time (( t_{R1} ))</th>
<th>Peak 1</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \Delta R_s )</td>
<td>( \Delta R_s )</td>
<td>( \Delta t_{R1} )</td>
<td>( \Delta t_{R1} )</td>
</tr>
<tr>
<td>0; -1; -1</td>
<td>2.43</td>
<td>2.46</td>
<td>0.03</td>
<td>1.21</td>
</tr>
<tr>
<td>0; +1; -1</td>
<td>2.49</td>
<td>2.44</td>
<td>0.05</td>
<td>2.04</td>
</tr>
<tr>
<td>-0.4; -1; -1</td>
<td>2.97</td>
<td>2.66</td>
<td>0.01</td>
<td>0.37</td>
</tr>
<tr>
<td>-0.4; +1; -1</td>
<td>2.70</td>
<td>2.67</td>
<td>0.03</td>
<td>1.12</td>
</tr>
<tr>
<td>-0.4; -1; 0.2</td>
<td>2.57</td>
<td>2.53</td>
<td>0.04</td>
<td>1.58</td>
</tr>
</tbody>
</table>

\( P \) indicates predicted values; \( M \) denotes measured values; \( \Delta R_s \) represents deviation in the \( P \& M \) values of resolution of peak (1,2); \( \Delta t_{R1} \) & \( \Delta t_{R1} \) denote difference in the \( P \& M \) values of retention time of peaks 1 & 3 respectively.

**Fig. 3—Representative HPLC chromatogram of derivatized (+)-KT**. Sample: refers to KT along with the internal standard PB, blank; produced by mixing the reagents without addition of KT & PB. Peaks: 1 = R & 2 = S-ketoprofen diastereomers, 3 = amide derivative of PB.
To select the suitable internal standard for the indirect chiral analysis three drugs viz. S-naproxen (S-NP), probenecid (PB) and indomethacin (IN) were examined. All the chosen internal standard drug candidates were derivatized using L-leucinamide and ethylchloroformate as described earlier. The L-leucinamide derivative of S-NP gave a single peak, which overlapped with the amide derivative of S-KT. Hence S-NP could not be used as internal standard. The amide derivative of both PB and IN gave single peak. These peaks did not interfere with derivatized peaks of both the enantiomers of KT in the chromatographic conditions employed for the analysis. Hence, both PB and IN qualified to be suitable internal standards. But PB was preferred as the internal standard since it eluted from the column earlier compared to IN thereby reducing the run time.

Interaction analyses were carried out to understand and appreciate the effect of chromatographic variables (A, B and C) on chiral resolution and retention time in the established experimental domain. The possibility of three-factor interaction in chiral resolution (peaks 1, 2) was rejected on the basis that the ABC interaction factor is not significant as indicated by the ANOVA for chiral resolution (Table 3). Likewise, ANOVA for retention time (peaks 1 and 3) revealed no three-factor interaction. Therefore only effect of two factor interactions with respect to resolution (L-leucinamide derivatives of the enantiomers of KT; peaks 1 and 2) and retention time (L-leucinamide derivative of PB; peak 3) were considered and analyzed.

Interaction studies of factors A, B and C in relation to resolution of peaks 1, 2

In the present study one of the goals is to separate peaks of L-leucinamide derivatives of the KT enantiomers and PB, peaks 1, 2 and 3 respectively. Mathematical model for retention time was developed only for peaks 1 and 3. Peak 2, the derivative of the enantiomeric pair of peak 1, eluted from the column in less than one minute, after peak 1, as indicated in Table 2. Hence, it was considered a futile exercise to develop a model for predicting retention time of peak 1.

The interaction plot AB shows non-parallel lines suggesting an interaction between factors (A) and (B). It is evident from AB interaction plot that with change in the strength of acetonitrile (A) from low (45% v/v) to high (55% v/v), there is a steady drop in the resolution both at the low and high level of buffer (B) concentration. It is observed that, in the experimental domain, a shift in the buffer concentration to a higher level results in a marginal increase in resolution ($R_s$). Besides the positive effect value of B (+ 0.06) confirms that effect of increasing the level of B is to increase $R_s$. This may be due to the fact that a decrease in the fraction of (A) results in an increase in the retention factor ($k$) leading to higher $R_s$.

BC interaction plot exhibits parallel lines indicating the absence of interaction between the factors (B) and (C) in relation to resolution of peaks (1 and 2) in the experimental domain. It is observed that changing buffer concentration from low level (10 mM) to high (25 mM) level, irrespective of the level of flow (C), there is a gradual increase in the scale of resolution. But the magnitude of the increase is higher when flow is set at low level.

Similarly, the interaction behaviour of factors A and C with respect to resolution revealed that there exists no interaction between the factors A and C. Thus changing the fraction of acetonitrile both at low and high levels of flow (C) results in a decrease in the magnitude of resolution. Further it is noticed that the resolution is higher when the mobile phase flow is set at low level (1 ml/min) compared to that at a high flow (1.5 ml/min) as confirmed by the negative effect value of C (-0.25). This pattern could be attributed to the better efficiency of the column when set at low level of (C) against a high level.

Interaction studies of factors A, B and C in relation to retention time; peaks 1 and 3

As indicated earlier, a mathematical model was developed, for the L-leucinamide derivative of PB (peak 3), to predict the run time of the analysis. The AB interaction plot exhibits intersecting lines demonstrating the possible interaction between the factors (A) and (B). The study reveals that changing the fraction of acetonitrile from low to high results in a rapid decline in the retention time both at low and high buffer concentration. An increase in the fraction of acetonitrile results in a decrease in polarity and hence the L-leucinamide derivatives leave the column faster in the reversed-phase mode. Further at low level of (A) an increase in the buffer concentration results in a marginal decrease in the retention time. This may be due to reduced silanol effects as a result of higher buffer concentration used. This observation is supported by the negative value of B (-0.05).

The effect of fraction of acetonitrile (A) on mobile phase flow rate (C) is subsequently investigated. AC interaction plot shows typical non-parallel lines confirming interaction between the factors (A) and (C). Analysis of the interaction plot divulges that
varying the concentration of acetonitrile from low to high level results in a decrease in retention time (run time here) both at high and low mobile phase flow rates (C). Further, it is noticed that a decrease in flow rate is to increase the retention time and vice-versa. This is in accordance with negative effect value of C (-0.308). But it is observed that the retention time is reduced drastically at a high flow setting. This could be attributed to the synergistic effect of increasing fraction (A) with high level of (C).

Further studies on the effect of buffer concentration (B) on the mobile phase flow rate (C) with respect to retention time exposed the absence of interaction between factors (B) and (C). It is noticed that shifting the level of factor (B) from low to high, in the experimental domain, there is no major impact on the retention time both at low and high levels of (C). As expected at low flow level the retention time is higher as compared to high level of (C).

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