Multicriteria optimization of an liquid chromatographic method for the simultaneous separation and estimation of zaltoprofen and paracetamol in human plasma sample: Application of chemometric protocols

R. Sathiyasundar*, P. Venkatesan, M. Swamivelmanickam, R. Suresh, K. Valliappan and K. Kannan

Department of Pharmacy, Faculty of Engineering and Technology, Annamalai University, Annamalainagar, India

ABSTRACT

The new drug combination of zaltoprofen and paracetamol are widely used as analgesic and antiinflammatory medication. There is no method reported for simultaneous estimation of this combination for plasma samples. On considering these facts a new reliable, sensitive and accurate reverse phase liquid chromatographic method has been developed for simultaneous quantitative estimation of these drugs in human plasma sample using probinicotid as internal standard. In the preliminary screening steps, a factorial design was employed to identify the factors that had significant effects on the selected chromatographic responses. Based on this responses analyses time (tR3), Capacity factor (k1) and selectivity (α) was optimized using Rotatable Central Composite Design (RCCD) and Response Surface Methodology (RSM). Further, this optimized condition was used to analyze the spiked plasma samples, reconstituted by a plasma protein precipitation method. The sample was efficiently separated using Reverse Phase Liquid Chromatography method equipped with PDA detector. The chromatographic separation was carried out using supelcosil LC-8 (150mm × 4.6mm I.D and 5µm particle size) analytical column measured at 254 nm. The developed RP-HPLC method can be applied for the estimation of quantitative pharmacokinetics in preclinical and clinical studies.

Key words: Paracetamol, Zaltoprofen, human plasma samples, Rotatable Central Composite Design, Response Surface Methodology

INTRODUCTION

A potent anti-inflammatory drug zaltoprofen (ZLP), a is 2-Arylpropionic acid derivative is chemically (2RS)-2 (10-Oxo-10, 11-dihydrodibenzo [b, f] thiepin-2yl) propanoic acid. Therapeutically it is used as high potent inhibitory effects on acute and chronic inflammation with less adverse effects on the GIT than other NSAIDs. ZLP exerts anti-inflammatory and analgesic effect by inhibition of bradykinin through B-receptor-mediator peripheral responses in prostaglandin. The 2-Aryl Propanoic acid derivatives are an important group of chiral NSAIDs, most of which are marketed as racemates[1, 2]. The other potent analgesic and antipyretic drug paracetamol(PAR) is a paraaminophenol derivatives and is the active metabolite of phenacetin. This drug is widely used as over the counter medication for more than thirty years and it is accepted as a very effective drug for the relief of pain and fever in adults and children. In oral dosage form acetaminophen has excellent bioavailability, achieves faster peak plasma concentrations and is uniformly distributed throughout most of the body fluids [3, 4]. The RP-HPLC methods are mostly applied for the pharmaceutical analysis and bio-analyticals. The Chemometric technique is the most suitable option for the optimization of more than one response at a time utilization of multicriteria decision making approach. The chemometrics may accomplish a range of goal in chromatographic research: including rapid method development, better use of chromatographic data and explain the chromatographic interactions [5].
The preparation of extract of an analyte is based on its partition between an aqueous phase and an immiscible organic phase. The extraction of analyte is influenced by a wide variety of factors, for example, pH and ionic strength of the aqueous solution, type and volume of the extraction solvent, sample weight, shaking time, sonication time, temperature, wash volume. An ideal extraction procedure required minimal extraction steps which ought to impart clean extracts as well as maximum recoveries for all of the extracted analytes [6, 7]. Due to the large number of possible extraction factors, it would be impossible to perform optimization by trial and error or single-factor approach. The limitation of the single-factor approach is that too many experiments are needed to be carried out, especially when the number of the examined factors becomes larger, without necessarily finding the true optimum. Secondly, the information concerning the sensitivity of factors on analytes separation and interaction between factors are being ignored [8]. The review of literature revealed that various analytical methods for the determination of PAR which employed techniques such as RP-HPLC and HPTLC methods [9, 10]. There are very few HPLC methods have been cited in the literature for ZLP enantioseparation and separation from biological fluids [11, 12]. ZLP officials in Japanese pharmacopeia [13] and Paracetamol official in Indian pharmacopeia, British Pharmacopoeia and United State pharmacopeia [14, 16] in which an HPLC-UV method is available for estimation. In best of our knowledge, there seems to be no report concerning method for the simultaneous determination of PAR and ZLP in biological fluids. On considering these factor objective of the study was fix to develop and optimize an RP-HPLC method for the simultaneous estimation of PAR and ZLP in spiked human plasma sample, using experimental design. The significance of the studied factors was evaluated with the aid of a full factorial design whilst the optimum chromatographic conditions were estimated by a CCD using both a graphical and Derringer’s desirability (mathematical) global optimization approach.

MATERIALS AND METHODS

2.1 Chemical and reagents
Working standards of paracetamol (99.69%) were gifts from Ranbaxy Laboratories Ltd., New Delhi, India. Zaltoprofen (99.78%) were donated by Sunglow pharmaceutical Ltd., Pondicherry. Probinicid (Internal Standard) was purchased from Sigma Chemical Co, MO, USA. HPLC grade Methanol (MeOH) and ortho phosphoric acid and other reagents of analytical grade reagents were from SD Fine Chemicals (Mumbai, India). HPLC-grade water was prepared by use of Milli-Q Academic water purified.

2.2 Instrumentation
This study was performed with a Shimadzu (Japan) chromatograph equipped with an LC-20 AD and LC-20 AD VP solvent-delivery module, an SPD-20A PDA detector, and a Rheodyne model 7125 injector valve fitted with a 20µL sample loop. The system was controlled through a system controller (SCL-10A) and a personal computer using a Shimadzu chromatographic software (LC Solution, Release 1.11SP1) installed on it. The mobile phase was degassed using Branson sonicator (Branson Ultrasonics Corporation, USA). Absorbance spectra were recorded using a UV-Visible spectrophotometer (Model UV-1601PC, Japan) employing quartz cell of 1.00cm of path length. The chromatographic analyses were done on a Supelcosil LC-8 (150mm × 4.6mm I.D and 5µm particle size) analytical column. The human plasma was gifted from Raja Muthiyar Medical College and Hospital, Annamalai University, Annamalainagar, Tamilnadu, India-608002.

2.3 Standard solutions
Standard stock solutions of PAR, ZLP (1.0 mg/ml) were prepared in the mobile phase. Working standard solutions were freshly obtained by diluting the standard stock solutions with mobile phase during the analysis time. Calibration curves reporting peak area ratios of PAR, ZLP to that of the IS versus drug concentrations were established in the range of 1.0 -5.0µg/mL for PAR, ZLP, in presence of probinicid 5.0 µg/ml as internal standard. Standard solution prepared for the optimization procedure constituted 4.0µg/mL of PAR and ZLP.

2.4 Software
Experimental design, data analysis and desirability function calculations were performed by using Design-Expert 8.0.0 (Stat-Ease Inc., Minneapolis) and Individual desirability function were performed by JMP-Software (SAS). The rest of the calculations for the analysis were performed by the use of Microsoft Excel 2010 software (Microsoft, USA).

2.5 Sample extraction technique
1.0 ml blank plasma and equal quantity of ethyl acetate added in a glass-stoppered 15 ml centrifuge tube were spiked with the working solutions of PAR and ZLP to that of the IS versus drug concentrations were established in the range of 1.0 -5.0µg/mL for PAR, ZLP, in presence of probinicid 5.0 µg/ml as internal standard. Standard solution prepared for the optimization procedure constituted 4.0µg/mL of PAR and ZLP.

1.0 ml blank plasma and equal quantity of ethyl acetate added in a glass-stoppered 15 ml centrifuge tube were spiked with the working solutions of PAR and ZLP to achieve a concentration of 500 ng ml^{-1} each. The samples was gently shaken for 5.0 min and centrifuging on a laboratory centrifuge (Remi®, R&C, Remi Equipment, Mumbai, India) at 4500 rpm for 5 min. The supernatant organic layer was transferred to Petridish and the contents were evaporated to dry air method. The residue was reconstituted in 100 µL of mobile phase and vortex for 60 seconds.
Aliquots of 20 µL were injected into the chromatographic system. The same procedure was carried out for blank plasma samples to check the cleanliness of the extracts. To assess the efficiency of the extraction procedure, the spiked plasma sample was extracted according to the above procedure, but the addition of IS after extraction. The recoveries of each drug and IS from spiked plasma were determined by comparing the peak area of each analyte after extraction with the respective non-extracted standard solution at the same concentration both low and high concentration for each compound was checked. The concentration of the IS where established in 5.0 µg/ml.

RESULTS AND DISCUSSION

3.1 Preliminary experiments:
The most frequently used stationary phases in RP-HPLC such as C18, C8, C6 and CN bonded phases. It is known that solutes behave differently in each column yet to be paid different interactions between them and the organic chain bonded to silica, giving rise to differences in separation [17]. Moreover the optimized mobile phase mixtures for one column will not inevitable are optimum for the other. Thus, the choice of the column that will give desired results is not often straightforward and, because of that, these stationary phases were selected for this study. Column chemistry, organic modifier, solvent strength, temperature and flow rate were then varied to determine the best chromatographic separation. In this study, the critical chromatographic factors for optimization were based on preliminary experiments and prior knowledge of literature, as well as certain instrumental limitations.

The analytes ZLP and PAR are predominantly polar and have low molecular mass, therefore, a Supelcosil LC-8 and binary mobile phases consisted of MeOH and Water (pH 3.5). Commonly alcohols can have hydrogen bonding with water and also dipole-dipole interactions will aid miscibility. The mobile phase conditions that the first-eluting component does not interfere with peaks of solvent and plasma components. Other criteria viz., Analysis time, appropriate k range (1<k<10) for eluted peaks, resolution, tailing factor, sample sensitivity and solvent noise were also considered. The retention time of the analyses found to be decreased with an increase in the MeOH concentration. It was noticed that the capacity factor for PAR was too low (k<1) at higher MeOH concentrations, whereas, lowerMeOH concentrations gave too high k value but resulting in excessively long runtime. Instant of MeOH tried in MeCN broadening the ZLP, wide range of pH tried and observed the excess of peak tailing, fronting, and peak broadening.

3.2 Central composite design and analysis
A rotatable CCD for three factors was applied to optimize the separation of PAR, IS and ZLP The selection of factors for optimization was based on preliminary experiments as well as certain instrumental limitations. From preliminary experiments, a C18 column as a stationary phase and a binary mobile phase consisted of MeOH and Water were employed in which concentration of MeOH content and pH of water was varied. The flow rate of mobile phase could also moderately influence selectivity of analytes. Therefore, the key factors selected for optimization process are concentration of methanol, pH of water, and flow rate of mobile phase. The levels of each factor studied for finding out optimum values and responses, and the ranges of each factors used were: MeOH concentration (50-60% v/v), pH of water (3-4.0) and flow rate (0.8-1.2 mL/min). As response variables, the capacity factor of PAR (k1), the retention time and concentration (50-60% v/v), pH of water (3-4.0) and  flow rate (0.8-1.2 mL/min). As response variables, the capacity factor of PAR was too low (k<1) at higher MeOH concentrations. The most frequently used stationary phases in RP-HP LC such as C18, C8, C6 and CN bonded phases. It is known that solutes behave differently in each column yet to be paid different interactions between them and the organic chain bonded to silica, giving rise to differences in separation [17]. Moreover the optimized mobile phase mixtures for one column will not inevitable are optimum for the other. Thus, the choice of the column that will give desired results is not often straightforward and, because of that, these stationary phases were selected for this study. Column chemistry, organic modifier, solvent strength, temperature and flow rate were then varied to determine the best chromatographic separation. In this study, the critical chromatographic factors for optimization were based on preliminary experiments and prior knowledge of literature, as well as certain instrumental limitations.

The analytes ZLP and PAR are predominantly polar and have low molecular mass, therefore, a Supelcosil LC-8 and binary mobile phases consisted of MeOH and Water (pH 3.5). Commonly alcohols can have hydrogen bonding with water and also dipole-dipole interactions will aid miscibility. The mobile phase conditions that the first-eluting component does not interfere with peaks of solvent and plasma components. Other criteria viz., Analysis time, appropriate k range (1<k<10) for eluted peaks, resolution, tailing factor, sample sensitivity and solvent noise were also considered. The retention time of the analyses found to be decreased with an increase in the MeOH concentration. It was noticed that the capacity factor for PAR was too low (k<1) at higher MeOH concentrations, whereas, lowerMeOH concentrations gave too high k value but resulting in excessively long runtime. Instant of MeOH tried in MeCN broadening the ZLP, wide range of pH tried and observed the excess of peak tailing, fronting, and peak broadening.

The statistical parameters obtained from the ANOVA for the reduced models are given in Table 2. Since R^2 always decreases when a regressor variable is eliminated from a regression model, in statistical modeling the adjusted R^2, which takes the number of regressor variables into account, is usually selected [18]. In the present study, the adjusted
R² were well within the acceptable limits of $R^2 > 0.80$ [19] which revealed that the experimental data shows a good fit with the second-order polynomial equations. For all the reduced models, P value of <0.05 are obtained, implying these models are significant. The adequate precision value was found to be more than 4, i.e., in the range of 15 to 28, which indicates an adequate signal and therefore the model is significant for the separation process. The CV for all the models was found to be less than 10 %, which indicates that the model can be considered reasonably reproducible. As shown in Table 2.0, the interaction term with the largest absolute coefficients between the fitted models is AB (+16.16) of tR3 model.

### Table 1.0 Experimental responses and rotatable central composite design arrangements

<table>
<thead>
<tr>
<th>Std</th>
<th>Run</th>
<th>Point Type</th>
<th>MeOH</th>
<th>pH</th>
<th>Flow</th>
<th>$k_1$</th>
<th>tR3</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>1</td>
<td>Axial</td>
<td>46.59</td>
<td>3.25</td>
<td>1.00</td>
<td>1.381</td>
<td>39.453</td>
<td>0.812</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>Center</td>
<td>55.00</td>
<td>3.25</td>
<td>1.00</td>
<td>1.244</td>
<td>16.155</td>
<td>2.343</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>Axial</td>
<td>55.00</td>
<td>3.67</td>
<td>1.00</td>
<td>1.313</td>
<td>19.756</td>
<td>3.331</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>Center</td>
<td>55.00</td>
<td>3.25</td>
<td>1.00</td>
<td>1.244</td>
<td>16.157</td>
<td>2.343</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>Fact</td>
<td>60.00</td>
<td>3.50</td>
<td>0.80</td>
<td>1.738</td>
<td>11.562</td>
<td>2.508</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>Center</td>
<td>55.00</td>
<td>3.25</td>
<td>1.00</td>
<td>1.277</td>
<td>16.159</td>
<td>2.523</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>Fact</td>
<td>50.00</td>
<td>3.00</td>
<td>1.20</td>
<td>0.924</td>
<td>25.838</td>
<td>0.983</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>Fact</td>
<td>60.00</td>
<td>3.00</td>
<td>1.20</td>
<td>0.844</td>
<td>11.967</td>
<td>2.281</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>Fact</td>
<td>50.00</td>
<td>3.00</td>
<td>0.80</td>
<td>1.618</td>
<td>32.135</td>
<td>1.985</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>Center</td>
<td>55.00</td>
<td>3.25</td>
<td>1.00</td>
<td>1.244</td>
<td>16.156</td>
<td>2.343</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>Fact</td>
<td>60.00</td>
<td>3.00</td>
<td>0.80</td>
<td>1.781</td>
<td>17.674</td>
<td>2.244</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>Fact</td>
<td>60.00</td>
<td>3.50</td>
<td>1.20</td>
<td>0.816</td>
<td>7.798</td>
<td>2.576</td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>Axial</td>
<td>63.41</td>
<td>3.25</td>
<td>1.00</td>
<td>1.174</td>
<td>7.104</td>
<td>2.182</td>
</tr>
<tr>
<td>17</td>
<td>14</td>
<td>Center</td>
<td>55.00</td>
<td>3.25</td>
<td>1.00</td>
<td>1.254</td>
<td>16.156</td>
<td>2.345</td>
</tr>
<tr>
<td>13</td>
<td>15</td>
<td>Axial</td>
<td>55.00</td>
<td>3.25</td>
<td>0.66</td>
<td>2.492</td>
<td>24.739</td>
<td>2.522</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>Axial</td>
<td>55.00</td>
<td>2.83</td>
<td>1.00</td>
<td>1.204</td>
<td>15.943</td>
<td>2.606</td>
</tr>
<tr>
<td>14</td>
<td>17</td>
<td>Axial</td>
<td>55.00</td>
<td>3.25</td>
<td>1.34</td>
<td>0.701</td>
<td>12.174</td>
<td>2.452</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>Fact</td>
<td>50.00</td>
<td>3.00</td>
<td>1.20</td>
<td>0.903</td>
<td>23.002</td>
<td>2.825</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>Center</td>
<td>55.00</td>
<td>3.25</td>
<td>1.00</td>
<td>1.244</td>
<td>16.156</td>
<td>2.343</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>Fact</td>
<td>50.00</td>
<td>3.50</td>
<td>0.80</td>
<td>1.848</td>
<td>28.95</td>
<td>1.616</td>
</tr>
</tbody>
</table>

*Randomized*

### Table 2.0 Reduced response models and statistical parameters obtained from ANOVA for CCD

<table>
<thead>
<tr>
<th>Responses</th>
<th>Model</th>
<th>Model p-value</th>
<th>Adequate precision</th>
<th>Adjusted R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1 = +1.25-0.034A+0.024B-0.48C$</td>
<td>0.0001</td>
<td>27.813</td>
<td>0.9804</td>
<td></td>
</tr>
<tr>
<td>$tR3 = +16.16-8.44A-0.72B-3.14C+0.35AC$</td>
<td>0.0001</td>
<td>18.626</td>
<td>0.9600</td>
<td></td>
</tr>
<tr>
<td>$+0.29BC+2.47A2+0.55B2+0.77C2$</td>
<td>0.0001</td>
<td>15.499</td>
<td>0.9565</td>
<td></td>
</tr>
</tbody>
</table>

This positive interaction between A and B is statistically significant (P=0.0001) for tR3. The positive interaction indicates the synergistic and the negative interaction indicate the aggressive effect. The non-parallel lines obtained from the AB interaction plot were support this observation. This study reveals that changing the fraction of MeOH from low (-1) to high (+1) results in a rapid decline in tR3 both at the low and high level of aqueous pH. Further, at low level of factor A, an increase in the aqueous pH results in marginal increase in the retention time and capacity factor. Therefore, when the MeOH concentration is set at its highest level, the aqueous pH has to be at its lowest level shorten the runtime and capacity factor. Especially this interaction is synergistic, as it led to a decrease in analysis time. The existence of such interactions emphasizes the necessity to carry out active multifactor experiments for optimization of the chromatographic separation. In order to gain a better understanding of the results, the predicted models and the perturbation plot, this plot show that the factor A, mostly affected the analysis time (tR3) and capacity factor ($k_1$) followed by factor C and then B.

### 3.3 Multi Criteria Decision Making:

Derringer’s desirability function was employed for global optimization of three responses and to select different optimal conditions for the analysis of plasma samples, to substantiate the flexibility of the optimal strategy and to search for an optimum experimental condition for analyzing plasma samples. The criteria for the optimization of each individual response are shown in Table 3.0 and it was proposed for selecting an optimum experimental condition for the plasma sample. For instance, the high value of $k_1$ has to be selected for the separation of first eluted peak to avoid the initial plasma interference. Therefore, $k_1$ was target at 1.25 and importance value of 3 was assigned, the importance can range from 1 to 5, which gives emphasis to a target value.
Table 3.0 Criteria for the optimization of the individual responses for the analysis of human plasma samples

<table>
<thead>
<tr>
<th>Responses</th>
<th>Lower limit</th>
<th>Upper limit</th>
<th>Criteria</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$</td>
<td>1.0</td>
<td>2.50</td>
<td>Target = 1.25</td>
<td>3</td>
</tr>
<tr>
<td>$t_R$</td>
<td>7.10</td>
<td>30.0</td>
<td>Minimize</td>
<td>5</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>0.81</td>
<td>1.61</td>
<td>Inrange</td>
<td>3</td>
</tr>
</tbody>
</table>

As shown in under criteria, the responses of $t_R$ were minimized, in order to reduce the total run time, on the other hand, selectivity ($\alpha$) value were in the range of 1.0 to 16. The following conditions and restrictions above, the optimization procedure was carried out. The response surface obtained from the global desirability function is shown in Fig 1 and the individual desirability of each response was shown in Fig 2. From the figure it can be concluded that there was a set of coordinates, producing high desirability value ($D = 0.925$) were MeOH concentration 60% v/v, Aqueous pH 3.5, Flow rate 0.98 ml min\(^{-1}\). The optimized condition for plasma assay was, there for C\(_{}\)8 column with MeOH: Water (pH 3.5) 60:40 % v/v as mobile phase at a flow rate of 0.98 mL min\(^{-1}\) and PDA detection at 254 nm. The predicted response values corresponding to the D values were confirmed experimentally. It is observed that the experimental $k_1$ value (1.2) is well within the acceptable limit and the analysis time ($t_R$) 10.5 min. The observed difference between the predicted and experimental response is obtained to be in good agreement, the percentage of prediction error (P.E) was calculated by the equation (3.0), [20], and the difference should be 0.0-6.0%.

\[
\text{Predicted Error} = \frac{\text{Experimental} - \text{Predicted}}{\text{Predicted}} \times 100
\]  

(3.0)

The chromatogram obtained under optimum condition is presented in Fig 3. This approach offers flexibility to the chromatographer to slide $k_1$ values depending upon the environment of the analyte under consideration.
Fig 1.: (a) Graphical representation of maximum global desirability function for optimal plasma condition. (b) Response surfaces plots for response $t_{R_3}$ of MeOH concentration (B) and pH (C) held constant at flow rate $k_1$

**Desirability**

Fig 2.: The individual desirability of each responses ($k_1$; Retention time ($t_{R_3}$); Separation factor ($\alpha$))

Fig 3.: (a) Typical chromatograms of spiked plasma extract containing PAR, IS and ZLP; Mobile phase: MeOH/ Water (pH 3.5) 60:40% v/v at a flow rate of 0.98 mL min$^{-1}$
3.0 Method Validation:

Linearity was established at five levels over the concentration ranges of 1.0-5.0 µg/mL for PAR and ZAL respectively, in presence of probenecid, IS (5000 ng/mL). The homocedasticity for the calibration curves was verified by applying Cocharan’s test, and in that, no statistical difference (P > 0.05) was found between variants. In addition, the mean (n = 6) of regression coefficient values were more than 0.999 for both the analytes One way ANOVA for linearity of analysts which results Fcalc values less than the Ftab at 5.0 % significance level, indicating that there was no significant difference between replicate determinations for each concentration level. The LOD and LOQ were estimated as for PAR, ZLP 13.59, 41.19 ng ml⁻¹ and 16.46, 49.90 ng ml⁻¹ respectively. There was no plasma peaks co-eluted with the analytes and IS, indicating that the optimized bio-analytical method is selective and specific in relation to the blank plasma used in this study. Accuracy, and precision were determined by replicating analysis at each level (n = 6) and mean % recovery (n =18) were determined. The recoveries of PAR and ZLP at each level were found well within the acceptable criteria of bias ±2.0%. The mean % recovery (n = 18) for each analyte was also tested for significance by using the Student t-test. Since the tcalc is less than the theoretical t value (tcalc = 2.620), at 5% significance level, the null hypothesis (99.20 and 98.3%) were accepted. These results indicate that the method is accurate and therefore the absence of interference from blank plasma used in this study. The intra and inter-day precision was confirmed since, the % RSD was well within the target criterion of ≤ 2.0. The stability of analytes and the IS stock solution in MeOH (100 ng ml⁻¹ each) was also checked over a 12.0 h period, at 4.0 h sampling intervals. The percentage responses for the aged solutions were calculated using freshly prepared solutions. The results show that the sample and standard solutions of analytes and IS were stable for 12.0 h, as during this time the result does not decrease below the minimum percentage.

CONCLUSION

A reliable, sensitive and accurate new RP-HPLC method for the simultaneous estimation of PAR and ZLP in human plasma has been developed and optimized. It may conclude that experimental designs coupled with MCDM approach is a convenient analytical tool to develop new RP-HPLC method from the perspective of reducing development time and thus the cost of analysis by saving time and laboratory resources. This approach can provide essential information regarding the sensitivity of various chromatographic factors and their interaction effects on the attributes of separation. The validation study supported the selection of the assay conditions by confirming that the assay was accurate, linear, precise, and matrix effect could not observed. It can be applied for the quantitative pharmacokinetics and pharmacodynamics, in preclinical and clinical studies.

Acknowledgements

All the authors’ is grateful to University Grants Commission (UGC), New Delhi, India, for the financial assistance through UGC-BSR fellowship and to UGC SAP-DRS Phase II sponsored Department of Pharmacy, Annamalai University for providing the facilities to carry this research work. The authors are deeply indebted to Dr. R. Manavalan, UGC-Basic Science Research Faculty Fellow (UGC-BFF), Govt. of India, for kind support. All the authors declared no conflict of interest.

REFERENCES