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# EXPERIMENTAL DESIGN APPROACH TO OPTIMIZATION OF THE NEW COMMERCIAL RP-HPLC DISCRIMINATION CONDITIONS FOR THE ESTIMATION OF PARACETAMOL AND ZALTAPROFEN IN PHARMACEUTICAL FORMULATION

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**ABSTRACT:** A simple, rapid, RP-HPLC method developed for the simultaneous estimation of paracetamol and zaltaprofen in commercial pharmaceutical formulation by applying Derringer's desirability function. The dependent variables such as Capacity factor of first peak, α value and retention time of $tR_3$ as the responses with three important independent factors of organic phase composition, pH of mobile phase and flow rate, were used to design mathematical models. The experimental responses were fitted into a second order polynomial and the three responses were simultaneously optimized. The optimum assay conditions were: Methanol-Water (pH 3.5 adjusted with 10 % O-phosphoric acid) (60:40 % v/v)as the mobile phase and at a flow rate of 0.98 ml/min. while using this optimum condition, baseline separation with a minimum resolution of 2.0 and a run time of less than 10 min were achieved. This method showed good agreement between the experimental data and predictive value throughout the studied parameter space. The optimized assay condition was validated according to ICH guidelines to confirm specificity, linearity, accuracy and precision.

**INTRODUCTION:** Paracetamol (PAR) chemically 4-hydroxyacetanilide is a derivative of para-aminophenol and non-selective cox inhibitors; it has weak activity on prostaglandin synthetase in the inflamed peripheral tissues which have high concentration of peroxides. Therefore, it is a potent antipyretic and is rapidly absorbed on oral administration. Peak plasma levels are reached within one hour, it's metabolized in the liver and the metabolites excreted in urine as conjugation products of glucuronic and sulfuric acids. The ability of infant liver for glucuronidation of paracetamol is poor and this may result in enhanced toxicity of the drug in neonates[1, 2].

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Zaltaprofen (ZLP) is selective Cox-2 inhibitor and also inhibit the prostaglandin $E_2$ synthesis, it's chemically (2-RS) – 2 - (10-Oxo-10, 11-dihydrodibenzo [b, *f*] thiepin-2yl) propionic acid. Zaltoprofen is a unique compound that also has anti-bradykinin activity. Its analgesic effects may be a result of inhibition of bradykinin $B_2$ receptor-mediated bradykinin responses not only of cyclooxygenases but also of bradykinin-induced 12-lipoxygenase inhibitors. It has been used clinically for treatment of post-operative pain and low back pain for more than ten years[3, 4].

In this study, Chemometric approaches were employed to systematically optimize the relevant chromatographic parameters. The optimization of the method was performed in three steps (i) Initial experiments to decide basic requirements of the method (ii) screening to select important parameters and (iii) Response surfacing to find the optimum point[5]. In RP-HPLC method utilizes a

wide selection of chromatographic factor, viz., the type and concentration of organic modifier, pH, buffer molarity, temperature, flow rate, etc., optimization of the experimental conditions is a complicated process. Therefore, a systematic approach such as experimental design to optimize chromatographic separations is more essential. The best experimental design approach for the purpose of modeling and optimization are the response surface design[6].

The review of literature revealed that various analytical methods for the determination of PAR which employ techniques such as RP-HPLC[7] and HPTLC [8].Few HPLC methods have been cited in the literature for the enantioseparation[9] and biological fluids of ZLP [10].

In recently Pradnya *et'al* reported gradient elution method for simultaneous determination of related substances of ZLP and PAR[11].ZLP is official in Japanese pharmacopeia [12] and Paracetamol official in BP, EU and USP [13-15] in which a HPLC-UV method is available. Nevertheless, to the best of our knowledge, there seems to be no commercial methods reported for the simultaneous determination of PAR and ZLP in pharmaceutical preparations.

In the present work, a new commercial RP-HPLC method was developed, optimized and validated for the determination of PAR and ZLP present in commercial preparation. In order to understand the sensitivity of the chromatographic factors on the separation of analysis time, Chemometric protocols of response surface design and Derringer's desirability function were successfully employed.

## EXPERIMENTAL:

### Apparatus

In 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 an 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.

### Materials and reagents:

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

2.3 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 was performed by JMP-Software (SAS).

### Standard Solutions

Standard stock solutions of ZLP, PAR (1mg/ml) were prepared in 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 ZLP, PAR to that of the IS versus drug concentrations were established in the range of 1-5 µg/mL for PAR, ZLP, in presence of Probinicid5µg/mL as internal standard. Standard solution prepared for the optimization procedure constituted 4 µg/mL of ZLP, PAR.

### Validation Study:

The optimized method was validated according to ICHQ2 (R1) guidelines[16]. The validation parameters like specificity, linearity, Accuracy, precision; Limit of Detection (LOD) and Limit of Quantification (LOQ) and robustness were addressed. The ability of this method to separate the peaks of the compounds of interest of the compound indicates the specificity of the method. There is no interference from the extracted blank, extraction solvent, and excipients used for the drug

preparations on the retention times of the 2 compounds of interest. Linearity was established at five levels over the concentration ranges of 1-5 µg/mL for PAR and ZAL respectively, with regression coefficient values more than 0.999, which showed reproducibility. LOD and LOQ is13.59ng/ml, 41.19 ng/ml and 16.46ng/ml, 49.90 ng/ml of PAR, ZAL was founded respectively.

## RESULTS AND DISCUSSION:

### Preliminary experiments

Column chemistry (C18, C8, C6 and CN), solvent type (MeCN or MeOH), solvent strength and flow rate were varied to determine the best chromatographic conditions that give quality separation. The mobile phase conditions were optimized such that the first-eluting component does not interference with the peaks of solvent, excipient components. Other criteria viz., analysis time, appropriate *k* range ($1<k<20$) for eluted peaks, tailing factor, assay sensitivity and solvent noise were also considered.

The analytes PAR and ZLP are predominantly polar and have low molecular mass, therefore, a Supelcosil LC-8 column (150mm × 4.6 mm i.e., 5 µm) and binary mobile phases consisted of Methanol- Water (pH adjusted with 10 % O-phosphoric acid), were tried to examine initial separation condition that will provide minimal separation of analytes.

The retention time of the analytes found to be decreased with increase in the MeOH concentration. It was noticed that the *k* value for PAR was too low ($k_1<1$) at lower MeOH concentrations, whereas, higher Buffer concentrations gave too high *k* value for PAR ($k_1>3$) resulting in excessively long runtime.

It is well known that a multiple component mobile phase gives better separation efficiency than a binary component mobile phase, as it is convenient to vary 'solvent strength' and selectivity simultaneously to obtain desired retention times. Therefore, a different proposition of mobile phase Methanol- Water (pH 3.5) (50:50, 40:60, 60:40, 55:45 % *v/v*) were tried. Among these, the mobile phase composition ratio of 60:40 % v/v resulted in a quality separation in terms of peak symmetry, optimum resolution, reasonable run time and acceptable *k* values particularly for PAR.

To explore the possibility of a better separation C18 column was tested with the mobile phase composition, 60:40% *v/v*. It produced similar order of elution as that of C8 columns (PAR<IS<ZLP), but with varying retention times. CN,C6 and C18 columns have much weak retention of the analytes as compared with C8 columns, resulting unacceptable *k* value ($k_1<1$) especially for PAR and asymmetry factor. The results suggested that C8 column was the best choice since it provided acceptable *k* value ($k_1>1$), less peak tailing and greater plate number than the tested C18 columns.

### Optimization design and analysis

Before starting an optimization procedure, it is important to investigate the curvature term using Factorial design with center points. ANOVA generated for $2^k$ Factorial design shows that curvature is significant for all the responses ($k_1$, $\alpha$, *tR3*) since *p*-value is less than 0.05. This implies that a quadratic model should be considered to model the separation process [17].

In order to obtain second order predictive model, central composite design (CCD) is employed, which is a design type under RSM. CCD is chosen due to its flexibility and can be applied to optimize an HPLC separation by gaining better understanding of factor's main and interaction effects [18, 19].

The selection of factors for optimization was based on preliminary experiments and prior knowledge from literature, as well as certain instrumental limitations. From preliminary experiments, C8 as a stationery phase and a binarymobile phase consisted of Methanol- Water were employed in which concentration of MeOH content was varied. The mobile phase flow rate could also moderately influence selectivity in HPLC analysis. Therefore, the key factors selected for optimization process were MeOH concentration (A), pH of water (B) and flow rate (C).

**Table 1** shows the levels of each factors studied for finding out the optimum values and responses. As can be seen in this table, the ranges of each factors used were: MeOH concentration (50-60 % *v/v*), pH

of mobile phase (3.0-3.5) and flow rate (0.8-1.2 mL $min^{-1}$).

**TABLE 1. CENTRAL COMPOSITE ROTATABLE DESIGN ARRANGEMENT AND RESPONSES[a]**

| Factor levels | | | Responses | | |
|---|---|---|---|---|---|
| **MeOH** | **pH** | **Flow** | **$k_1$** | **$tR_3$** | **$\alpha_3$** |
| 46.59 | 3.25 | 1.00 | 1.381 | 39.453 | 0.812 |
| 55.00 | 3.25 | 1.00 | 1.244 | 16.155 | 2.343 |
| 55.00 | 3.67 | 1.00 | 1.313 | 19.756 | 3.331 |
| 55.00 | 3.25 | 1.00 | 1.244 | 16.157 | 2.343 |
| 60.00 | 3.50 | 0.80 | 1.738 | 11.562 | 2.508 |
| 55.00 | 3.25 | 1.00 | 1.277 | 16.159 | 2.323 |
| 50.00 | 3.00 | 1.20 | 0.924 | 25.838 | 0.983 |
| 60.00 | 3.00 | 1.20 | 0.844 | 11.967 | 2.281 |
| 50.00 | 3.00 | 0.80 | 1.618 | 32.135 | 1.985 |
| 55.00 | 3.25 | 1.00 | 1.244 | 16.156 | 2.343 |
| 60.00 | 3.00 | 0.80 | 1.781 | 17.674 | 2.244 |
| 60.00 | 3.50 | 1.20 | 0.816 | 7.798 | 2.376 |
| 63.41 | 3.25 | 1.00 | 1.174 | 7.104 | 2.182 |
| 55.00 | 3.25 | 1.00 | 1.254 | 16.156 | 2.345 |
| 55.00 | 3.25 | 0.66 | 2.492 | 24.739 | 2.522 |
| 55.00 | 2.83 | 1.00 | 1.204 | 15.943 | 2.606 |
| 55.00 | 3.25 | 1.34 | 0.701 | 12.174 | 2.452 |
| 50.00 | 3.50 | 1.20 | 0.903 | 23.002 | 2.825 |
| 55.00 | 3.25 | 1.00 | 1.244 | 16.156 | 2.343 |
| 50.00 | 3.50 | 0.80 | 1.848 | 28.950 | 16.161 |

[a] Randomized

As response variables, the capacity factors for the first eluted peak *PAR (k1),* α value of first eluted peak PAR, and the retention time of the last peak *ZLP (tR3),* were selected as responses. In the preliminary study, *α* value were found to be close to 1.5, hence these were considered as critical peaks and included as one of the response for the global optimization. Probenicid ($IS_1$) was used as an internal standard since it presented acceptable resolution and retention time with all the analytes.

All experiments were conducted in randomized order to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. Replicates (*n*=6) of the central points were performed to estimate the experimental error. For an experimental design with three factors, the model including linear, quadratic, and cross terms can be expressed as:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 z + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

Where, Y is the response to be modeled, β is the regression coefficient and $X_1$, $X_2$ and $X_3$ represents factors A, B and C respectively. Statistical parameters obtained from ANOVA for the reduced models are given in **Table 2**.

**TABLE 2. REDUCED RESPONSE MODELS[a] AND STATISTICAL PARAMETERS OBTAINED FROM ANOVA (AFTER BACKWARD ELIMINATION)**

| Response | Regression model | Model p-value | %CV | R-Square | Adj. R-Square |
|---|---|---|---|---|---|
| *K* | +1.24-0.48C+0.11$C^2$<0.0001 | 6.28 | 0.9659 | 0.9659 | |
| *tR3* | +16.16-8.44A+0.35AC+2.47$A^2$<0.0001 | | 9.420.9419 | | 0.9310 |
| $\alpha_3$ | +2.61+0.41A+0.22B-0.021C+1.78AC<0.0001 +1.52ABC+1.83$A^2$B | 9.90 | 0.9449 | 0.8530 | |

[a]Only significant coefficients with P < 0.05 are included. Factors are in coded levels.

**TABLE 3.CRITERIA FOR THE OPTIMIZATION OF INDIVIDUAL RESPONSES.**

| Response | Lower limit | Upper limit | Criteria | |
|---|---|---|---|---|
| | | | Goal | Importance |
| $k_1$ | 0.70 | 2.49 | Targets | 3 |
| $tR_3$ | 7.10 | 30.0 | Minimize | 5 |
| $\alpha_3$ | 0.81 | 16.16 | In Range | 3 |

The insignificant terms ($P > 0.05$) were eliminated from the model through backward elimination process to obtain a simple and realistic model. 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 [20].

In the present study, the adjusted $R^2$ were well within the acceptable limits of $R^2 \geq 0.80$, which revealed that the experimental data shows a good fit with the second-order polynomial equations[21]. For all the reduced models, $P$ value of < 0.05 is obtained, implying these models are significant. The adequate precision value is a measure of the signal (response) to noise (deviation) ratio". A ratio greater than 4 is desirable [22].

In this study, the ratio was found to be in the range of 16.95 –50.22, which indicates an adequate signal and therefore the model is significant for the separation process. The coefficient of variation (C.V.) is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if it is less than 10% [23] interaction term with the largest absolute coefficients among the fitted models is *AB* (+ 16.16) of $tR_3$ model.

The positive interaction between *A* and *C*is statistically significant (< 0.0001) for $tR_3$. The non-parallel lines obtained for the AC interaction plot **(Fig. 1)** support this observation. The study reveals that changing the fraction of MeOH from low to high results in a rapid decline in the retention time of ZLP both at the low and high level of flow rate. Further at low level of factor *A*, an increase in the Flow rate results in a marginal decrease in the retention time.

Therefore, when the MeOH concentration is set at its lowest level, the flow rate has to be at its highest level to shorten the run time. Especially this interaction is synergistic, as it led to a decrease in run time. The existence of such interactions emphasizes the necessity to carry out active multifactor experiments for the optimization of chromatographic separation.

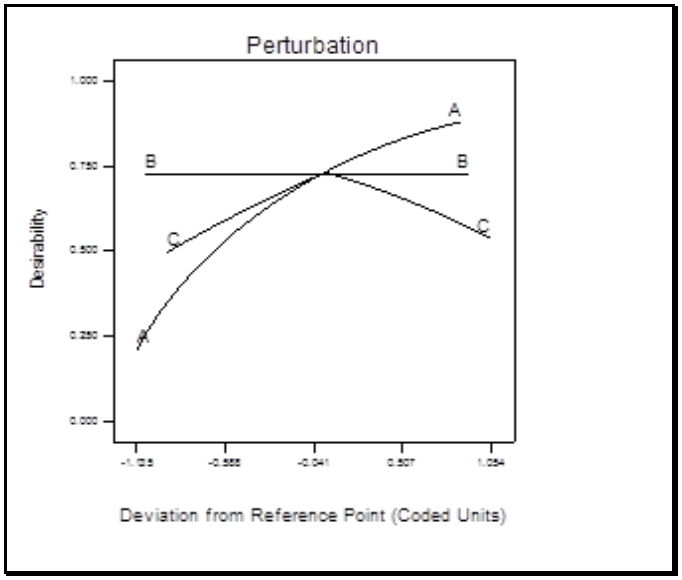


**FIG. 1. PERTURBATION PLOT SHOWING THE EFFECT OF EACH OF THE INDEPENDENT VARIABLES ON $TR_3$ WHILE KEEPING OTHER VARIABLES AT THEIR RESPECTIVE MID-POINT LEVELS.**

In order to gain a better understanding of the results, the predicted models are presented in the form of perturbation plot and 3D response surface plots. Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models, were chosen for the axes of the response surface plots. Consequently, factors A and C were selected for the response surface plots of $k_1$, $\alpha$, $tR3$, with factor B held constant usually at a central value of pH 3.5.

All these three-dimensional plots were beneficial to gain an overall understanding of the influence of MeOH content and flow rate on analysis time ($tR_3$). Perturbation plot provide silhouette views of the response surface plots, where it shows how the response changes as each factor moves from a chosen reference point, with all other factors held constant at the reference value. A steepest slope or curvature indicates sensitiveness of the response to a specific factor, MeOH (factor *A*) had the most important effect on retention time $tR_3$followed by

factor *C* and then *B*. The rest of the factors (pH of mobile phase and flow rate) had significant effect on $k_1$and$\alpha$.

**Multi criteria decision making**

Derringer's desirability function was employed for global optimization of four responses and to select different optimal conditions for the analysis of real samples

**Optimal condition for formulation assay**

The criteria for the optimization of each individual response are shown in **Table 3.** Criteria I have been proposed for selecting an optimum experimental condition for analyzing routine quality control samples. As can be seen under criteria I, the responses $tR_3$ was minimized, in order to shorten the analysis time.

In order to separate the first eluting peak (PAR) from the solvent front, *k*1 was targeted at 1.2 and α was fixed in the range of 0.8-16.16. Importance can range from 1 to 5, which gives emphasis to a target value. Following the conditions and restrictions above, the optimization procedure was carried out. The response surface obtained for the global desirability function is presented in **(Fig. 2),** from the figure it can be concluded that there was a set of coordinates producing high desirability value (*D* = 0.932).

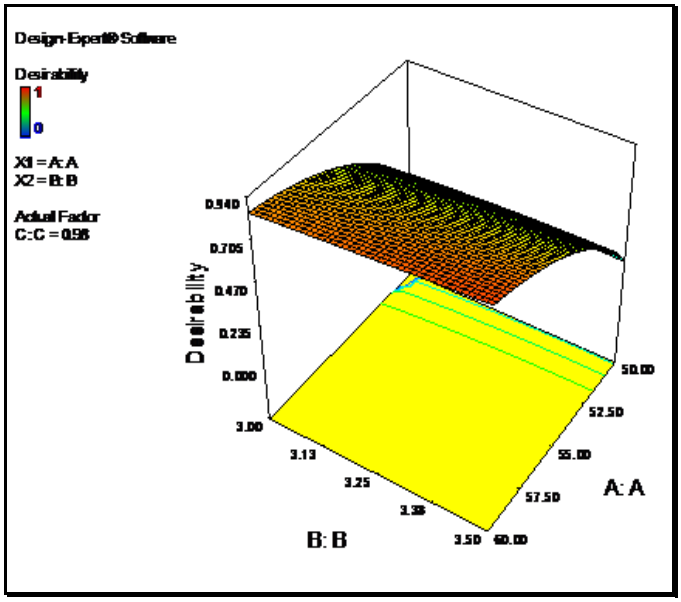


**FIG 2. RESPONSE SURFACES RELATED TO MeOH CONCENTRATION (%) AND FLOW RATE (ml/min): (A) CAPACITY FACTOR OF THE FIRST PEAK ($k_1$), (B) RETENTION TIME OF $tR_3$AND (C) α VALUE OF $tR_3$.**

The optimized formulation assay condition were, therefore, using C8 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 UV detection at 235 nm. The predicted response values corresponding to the latter value of *D* were: $k_1$ = 1.25, $\alpha$= 1.57, $tR_3$ = 8.56 min.

**Predicted Error =Experimental–Predicted/Predicted x 100 (2)**

**CONCLUSIONS:** In this study, an isocratic RP-HPLC-PDA method for the simultaneous determination of PAR and ZLP in pharmaceutical formulation was developed and optimized. Time of analysis and Capacity factor were simultaneously optimized by applying chemometrics tools: CCD and Derringer's desirability function. The employed optimization methodology reduces overall assay development time and provides essential information regarding the sensitivity of various chromatographic variables on separation attributes. It may concluded that experimental designs coupled with MCDM approach is a convenient analytical tool to develop new HPLC method from the perspective of reducing analysis time and thus the cost of analysis by saving time and laboratory resources.

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