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# Improved HPLC Method with the Aid of Chemometric Strategy: Determination of Loxoprofen in Pharmaceutical Formulation

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## Abstract

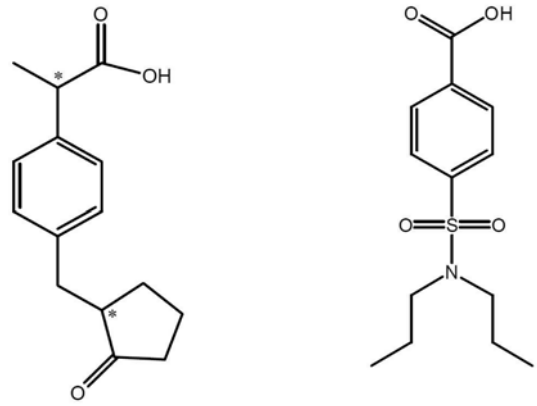
Loxoprofen belongs to a class of Nonsteroidal anti-inflammatory drug acts by inhibiting isoforms of cyclo-oxygenase 1 and 2. In this study an improved RP-HPLC method was developed for the quantification of loxoprofen in pharmaceutical dosage form. For that purpose an experimental design approach was employed. Factors-independent variables (organic modifier, pH of the mobile phase and flow rate) were extracted from the preliminary study and as dependent variables three responses (loxoprofen retention factor, resolution between loxoprofen probenecid and retention time of probenecid) were selected. For the improvement of method development and optimization step, Derringer's desirability function was applied to simultaneously optimize the chosen three responses. The procedure allowed deduction of optimal conditions and the predicted optimum was acetonitrile: water (53:47, v/v), pH of the mobile phase adjusted at to 2.9 with *ortho* phosphoric acid. The separation was achieved in less than 4minutes. The method was applied in the quality control of commercial tablets. The method showed good agreement between the experimental data and predictive value throughout the studied parameter space. The optimized assay condition was validated according to International conference on harmonisation guidelines to confirm specificity, linearity, accuracy and precision.

**Keywords:** Central composite design, derringer's desirability function, loxoprofen, multiple response optimization, probenecid, reversed-phase HPLC

## 1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain and inflammation. NSAIDs produce their therapeutic effect by inhibiting the cyclooxygenase (COX) enzymes, which are involved in the biosynthesis of prostaglandins (PGs).[1,2] Loxoprofen, 2-[4-(2-oxocyclopentylmethyl) phenyl]-propionate with two chiral centers, is marketed as an equal parts mixture of four stereoisomers. Loxoprofen sodium is an important non-steroidal anti-inflammatory drug (NSAID) of the 2-arylpropionic acid group used for the treatment of rheumatoid arthritis and osteoarthritis. Loxoprofen is a prodrug which produces effects after being absorbed from the gastrointestinal tract followed by conversion to an active metabolite. Loxoprofen has an activity to treat inflammatory rheumatoid diseases and relieve acute pain.[3,4,5] It is effective against period pains, pain after surgery and fever. Loxoprofen available in pharmaceutical formulations as tablets and transdermal patches.[6,7]

A literature search revealed that seven methods are available for the determination of loxoprofen in pharmaceutical formulations and biological fluids. Hideo Naganuma *et al*[8] developed a simple and sensitive high-performance liquid chromatographic procedure to determine loxoprofen and its diastereomeric alcohol metabolites in




Loxoprofen (LOX) Probenecid (SI)

**Fig.1.** The chemical structures of analyte (LOX) and internal standard (IS)

biological specimens by fluorescence labeling. Hisomu Nagashima *et al*[9] reported a column liquid chromatography method for the simultaneous determination of the enantiomers of loxoprofen sodium and its metabolites in human urine. Hideko Kanazawa *et al*[10] established the chiral separation of loxoprofen in plasma by chiral column liquid chromatography with a circular dichroism-based detector. Kyo-Seok Choo *et al*[11] developed a simultaneous determination of loxoprofen and its diastereomeric alcohol metabolites in human plasma and urine by a simple HPLC-UV detection method. Hea-Young Cho *et al*[12] reported a direct and simultaneous analysis of loxoprofen and its diastereomeric alcohol metabolites in human serum by on-line column switching liquid chromatography. Tomonori Murakami *et al*[13] developed a method for identification of degradation products in loxoprofen sodium adhesive tapes by liquid chromatography-mass spectrometry and dynamic pressurized liquid extraction-solid-phase extraction coupled to liquid chromatography-nuclear magnetic resonance spectroscopy. Although all the above methods have been applied for quantification of loxoprofen in biological matrices, due to their complexity they may not be suitable for routine analysis. The Japanese pharmacopeia[14] describes an HPLC method for determination of loxoprofen in bulk drug. This HPLC method has reportedly a run time of about 7.0 min, but did not provide the details on the chromatographic variables, viz. capacity factor, resolution, asymmetric factor.

None of the above methods reported above employed a systematic optimization procedure for the separation and quantification of loxoprofen, but a time-consuming trial-and error approach resulting in an apparent optimum only. Information on the sensitivity of the factors on the analytes separation and interaction between factors is not available. In this work a chemometric procedure is applied to realize the above objective[15,16,17,18,19.] However, since the HPLC method is intended to be applied for the pharmaceutical or industrial environment, there is a need to optimize multiple responses (analysis time and resolution) simultaneously.[20,21,22,23] To achieve global optimization of multiple responses the Derringer's desirability function (Multi-Criteria decision making) has been applied.[24,25,26,27,28] Hence there was a need to develop an improved HPLC method for determination of loxoprofen in pharmaceutical formulations applying a chemometric protocol.

The aim of this work is to (i) develop an improved HPLC method suitable for the routine quality control analysis of loxoprofen in a pharmaceutical laboratory and (ii) provide information on the sensitivity of chromatographic factors and their interaction effects on the separation characteristics. The chromatographic factors that had significant effects on analysis time were optimized using a central composite design and response surface methodology.

# 2. Experimental

## 2. 1. Apparatus

Chromatographic measurements were made on a Shimadzu (Tokyo, Japan) model which consisted of a LC10AD and LC10 ADvp solvent delivery module, SPD 20 PDA detector, a Rheodyne injector (model 7125, USA) valve fitted with a 20 $\mu$l loop, and PDA detector (SPD-20). 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 a Branson sonicator (Branson Ultrasonics Corporation, USA). Absorbance spectra were recorded using an UV–Visible spectrophotometer (Model UV-1601PC, Japan) employing a quartz cell with 1.00 cm of path length.

## 2. 2. Softwares

Experimental design, data analysis and desirability function calculations were performed using Design-Expert® trial version 7.0.0. (Stat-Ease Inc., Minneapolis).

## 2. 3. Chemicals and Reagents

Working standards of loxoprofen (99.79%) were donated by M/S Micro labs limited, Hosur. India. Probenecid (PRB) (≥99%) was purchased from Fluka, Buchs, Switzerland. Acetonitrile (ACN) of HPLC grade and dipotassium hydrogen phosphate and phosphoric acid were of analytical-reagent grade supplied by M/S SD Fine chemicals, Mumbai, India. The HPLC grade water was prepared using a Milli-Q Academic system, Millipore, Bangalore, India. The pharmaceutical Loxomac® tablets (containing loxoprofen 60 mg) were purchased from Macleods Pharmaceuticals, Mumbai, India.

## 2. 4. Standard Solutions

Stock standard solutions of loxoprofen and probenecid (1 mg/ml) were prepared in mobile phase. The prepared stock solution was stored at 4 °C protected from light. Working standard solutions were freshly obtained by diluting the stock standard solutions with mobile phase during the analysis day. Calibration curves reporting peak area ratios of loxoprofen to that of the IS versus drug concentrations were established in the range of 1.5–15 $\mu$g/ml for loxoprofen, in the presence of probenecid (7.5 $\mu$g/ml) as an internal standard. Standard solutions prepared for the optimization procedure constituted loxoprofen and IS at 15 and 7.5 $\mu$g/ml, respectively.

## 2. 5. Sample Preparation

Twenty tablets were weighed and finely powdered. In the case of capsule dosage, the contents of the capsule were mixed thoroughly. An amount of capsule/tablet powder equivalent to 15 mg loxoprofen was accurately weighed and transferred in a 10 ml volumetric flask; a suitable quantity of IS was added followed by 5 ml of mobile phase. This mixture was subjected to sonication for 10 min for complete extraction of the drug and the solution was made up to the mark with mobile phase to obtain a concentration of loxoprofen and IS of 15 and 7.5 $\mu$g/ml, respectively. The solution was centrifuged at 4000 rpm for 10 min; the clear supernatant was collected and filtered through a 0.2 $\mu$m membrane filter (Gelman Science, India) and 20 $\mu$l of this solution was injected for HPLC analysis.

## 2. 6. Chromatographic Procedure

Chromatographic separations were carried out on a Phenomenex® C18 analytical column (150 mm × 4.6 mm i.d., 5 $\mu$m) connected with a Phenomenex® C18 guard cartridge (4 mm × 3 mm i.d., 5 $\mu$m). The mobile phase consisted of acetonitrile: water; the pH of the mobile phase was adjusted to 2.9 with 10% ortho phosphoric acid. In order to increase the sensitivity for the less concentrated compound and to decrease the background of mobile phase, a wavelength of 220 nm was selected for detection. The injection volume of the sample was 20 $\mu$l. The HPLC system was used in an air-conditioned laboratory atmosphere (20 ± 2 °C).

## 2. 7. Validation

Validation studies were conducted using the optimized assay conditions based on the principles of validation described in the ICH guidelines "Text on Validation of Analytical Procedures"[29] and "Q2B, Validation of Analytical Procedures: Methodology".[30] Key analytical parameters, including, specificity, accuracy, precision, linearity, detection limit and quantitation limit were evaluated. To study specificity, a placebo containing starch, lactose monohydrate, aerosil, hydroxy propyl methylcellulose, titanium dioxide and magnesium stearate was used. Calibration curves were constructed in the range of 0.05 to 1.0% of the target analyte concentration for the limit of detection and quantification.[31] Also the robustness of the proposed method was assessed with respect to small alterations in the ACN concentration (53% ± 0.5) and the pH value (2.9 ± 0.2).

# 3. Results and Discussion

## 3. 1. Optimization Design and Analysis

The central composite design can be applied to optimize the separation and to assist in the development of a better understanding of the interaction of several chromatographic factors on separation quality.[32] In this work, the important chromatographic factors were selected and optimized by a central composite design experiment. 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 the key factors selected for optimization process were ACN concentration (A), pH of the mobile phase (B) and flow rate (C). Table 1 shows the set of conditions applied to these factors to optimize the determination of loxoprofen. As can be seen in this table, the conditions for each factor were limited: ACN concentration (45–55%), pH (2.5–3.0) and flow rate (1.0–1.5 ml/min). As response variables, the retention time of IS ($tR_2$), capacity factor ($k$) and the resolution between two pairs, loxoprofen-IS ($Rs_{1,2}$) were chosen. All experiments were performed 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

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

where Y is the response to be modeled, β is the regression coefficient and $X_1$, $X_2$ and $X_3$ represent factors A, B and C, respectively. To obtain a simple and yet realistic model, the insignificant terms ($P > 0.05$) are eliminated from the model through a 'backward elimination' process. 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.[33] In the present study, the adjusted $R^2$ values were

**Table 1.** Central composite rotatable design arrangement and responses[a]

| Design points | Factor levels A (%v/v) | B (pH) | C (ml/min) | Responses $k_1$ | $tR_2$ | $Rs_{1,2}$ |
|---|---|---|---|---|---|---|
| 1 | 52.97 | 2.60 | 1.42 | 2.00 | 3.15 | 4.60 |
| | | | | 2.01 | 3.15 | 4.61 |
| 2 | 50.00 | 2.75 | 1.30 | 2.43 | 4.21 | 5.87 |
| | | | | 2.43 | 4.21 | 5.88 |
| 3 | 50.00 | 2.50 | 1.30 | 3.05 | 5.49 | 6.99 |
| | | | | 3.05 | 5.49 | 6.99 |
| 4 | 52.97 | 2.90 | 1.42 | 2.06 | 3.85 | 4.65 |
| | | | | 2.08 | 3.82 | 4.66 |
| 5 | 45.00 | 2.75 | 1.30 | 3.40 | 5.28 | 7.87 |
| | | | | 3.41 | 5.28 | 7.88 |
| 6 | 47.03 | 2.60 | 1.18 | 2.88 | 5.61 | 7.10 |
| | | | | 2.86 | 5.62 | 7.11 |
| 7 | 50.00 | 2.75 | 1.30 | 2.43 | 4.21 | 5.87 |
| | | | | 2.43 | 4.21 | 5.88 |
| 8 | 47.03 | 2.90 | 1.42 | 2.97 | 4.41 | 6.65 |
| | | | | 2.95 | 4.41 | 6.60 |
| 9 | 50.00 | 2.75 | 1.30 | 2.43 | 4.21 | 5.87 |
| | | | | 2.43 | 4.21 | 5.88 |
| 10 | 50.00 | 2.75 | 1.30 | 2.43 | 4.21 | 5.87 |
| | | | | 2.43 | 4.21 | 5.88 |
| 11 | 55.00 | 2.75 | 1.30 | 1.81 | 3.23 | 4.29 |
| | | | | 1.79 | 3.24 | 4.23 |
| 12 | 52.97 | 2.60 | 1.18 | 1.99 | 4.02 | 5.00 |
| | | | | 2.00 | 4.01 | 5.04 |
| 13 | 50.00 | 3.00 | 1.30 | 2.11 | 3.79 | 5.07 |
| | | | | 2.11 | 3.64 | 4.86 |
| 14 | 47.03 | 2.90 | 1.18 | 2.99 | 5.60 | 7.10 |
| | | | | 2.97 | 5.61 | 7.11 |
| 15 | 50.00 | 2.75 | 1.10 | 2.44 | 5.24 | 6.28 |
| 16 | 50.00 | 2.75 | 1.30 | 2.43 | 4.21 | 5.87 |
| 17 | 50.00 | 2.75 | 1.30 | 2.43 | 4.21 | 5.87 |
| 18 | 50.00 | 2.75 | 1.50 | 2.45 | 3.50 | 5.49 |
| 19 | 47.03 | 2.60 | 1.42 | 2.86 | 4.42 | 6.64 |
| 20 | 52.97 | 2.90 | 1.18 | 2.06 | 3.15 | 4.56 |

[a] Randomized

well within the acceptable limits of $R^2 \geq 0.80$[34] which revealed that the experimental data show a good fit with the second-order polynomial equations. For all the reduced models, P values < 0.05 are obtained, implying that 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[35]. In this study, the ratio was found to be in the range of 15.61 to 29.31, which indicates an adequate signal and therefore the model is significant for the separation process. The coefficient of variation (CV) 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%.[35] The CV for all the models was found to be less than 10%.

As can be seen in table 2, the interaction term with the largest absolute coefficients among the fitted models is A (+0.28) of the $tR_2$ model. The positive interaction between A and C is statistically significant (P = 0.0001) for $tR_2$. The study reveals that changing the fraction of ACN from low to high results in a rapid decline in the retention time of loxoprofen, both at low and high flow rate. 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 optimization of the chromatographic separation.

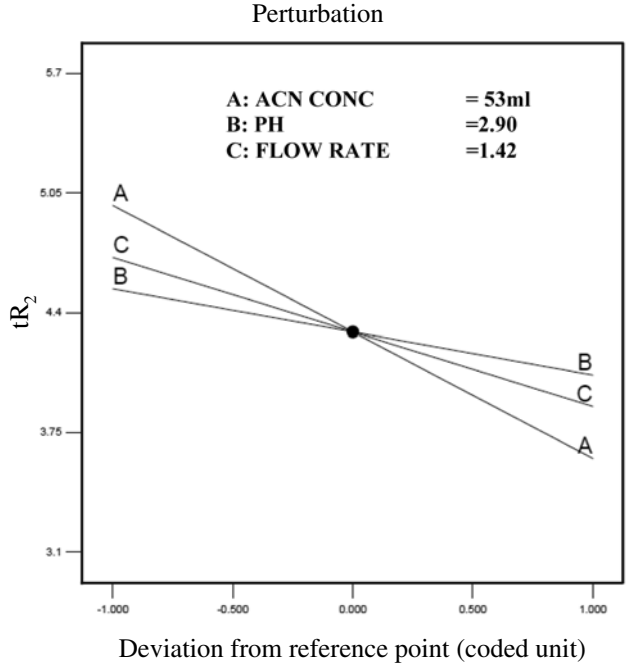



**Fig 2.** Perturbation plot showing the effect of each of the independent variables on $tR_2$ while keeping other variables at their respective midpoint levels

**Table 2.** Reduced response models[a] and statistical parameters obtained from ANOVA (after backward elimination)

| Response | Regression model | Adjusted $R^2$ | Model P-value | %C.V | Adequate precision |
|---|---|---|---|---|---|
| $k_1$ | 2.49 – 0.46A | 0.8439 | 0.000 | 6.67 | 29.31 |
| $tR_2$ | 4.30 – 0.68A – 0.23B – 0.40C + 0.28AC | 0.8009 | 0.000 | 8.19 | 15.61 |
| $Rs_{1,2}$ | 5.87 – 1.08A – 0.28B – 0.18C | 0.9185 | 0.000 | 4.83 | 28.65 |

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

In order to gain a better understanding of the results, the predicted models are presented in Fig. 2 in a perturbation plot.[36] This graph 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 steep slope or curvature in a factor indicates that the response is sensitive to that factor. Hence, the plot shows that factor A mostly affected the analysis time ($tR_2$), followed by factor C and then B.

## 3. 2. Multi-Criteria Decision Making

In the present study, to optimize three responses with different targets, Derringer's desirability function, was used.[23] The Derringer's desirability function, D, is defined as the geometric mean, weighted, or otherwise, of the individual desirability functions. The expression that defines the Derringer's desirability function is:

$$D = \left[ d_1^{p1} \times d_2^{p2} \times d_3^{p3} \times \ldots\ldots \times d_n^{pn} \right]^{1/n} \quad (2)$$

Where *p*i is the weight of the response, *n* the number of responses and *di* is the individual desirability function of each response obtained from the transformation of the individual response of each experiment. The scale of the individual desirability function ranges between $d_i = 0$, for a completely undesired response, to $d_i = 1$ for a fully desired response. Weights can range from 0.1 to 10. Weights lower than 1 give less emphasis to the goal, whereas weights greater than 1 give more emphasis to the goal (in both cases, *d*i varies in a non linear way while approaching to the desired value). With a weight of 1, $d_i$ varies in a linear way. In the present report we chose weights equal to 1 for all the six responses. A value of D different to zero implies that all responses are in a desirable range simultaneously and consequently, for a value of D close to 1, the combination of the different criteria is globally optimal, so as the response values are near target values.

**Table 3.** Criteria for the optimization of the individual responses.

| Response | Lower limit | Upper limit | Criteria Goal | Criteria Importance |
|---|---|---|---|---|
| $k_1$ | 1.8 | 3.413 | Target = 2 | 4 |
| $tR_2$ | 3.156 | 5.621 | Minimize | 5 |
| $Rs_{1,2}$ | 4.24 | 7.88 | Minimize | 4 |

The criteria for the optimization of each individual response are shown in Table 3, and it is proposed for selecting an optimum experimental condition for analyzing routine quality control samples. As can be seen in Table 3, two responses ($tR_2$ and $Rs_{1,2}$) were minimized in order to shorten the analysis time. On the other hand, $k_1$ was targeted at 2.00. The importance can range from 1 (the least important) to 5 (the most important), which gives emphasis to a target value. For instance, a high importance value of 5 was assigned to the $tR_2$ response as a short analysis time is usually preferred for routine analysis. 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. 3. The coordinates producing the maximum desirability value ($D$ = 0.971) were: ACN concentration of 53%, pH of the mobile phase 2.9 and flow rate of 1.42 ml/min. The predicted response values corresponding to the latter value of $D$ were: $k_1 = 2.0$, $tR_2 = 3.25$ min, $Rs_{1,2} = 4.32$. The prediction efficiency of the model was confirmed by performing the experiment under the optimal condition and the corresponding chromatogram is shown in Fig. 4.The agreement between experimental and predicted responses for the predicted optimum are shown in Table 4. The errors for retention factor, retention time and resolution were found to be in good agreement:[37] 2.04, 0.61 and 1.88%, respectively.

## 3. 3. Assay Method Validation

The optimised assay method is specific in relation to the placebo used in this study because there was no excipient peak co-eluted with the analytes and IS (Fig. 4). An excellent linearity was established at five levels in the range of 1.5–15 $\mu$g/ml for loxoprofen with an $R^2$ better than 0.9989. The slope and intercept for the calibration curve of loxoprofen were 0.322 and –0.005, respectively. The LOD and LOQ for loxoprofen were estimated as 1.74 and 5.89

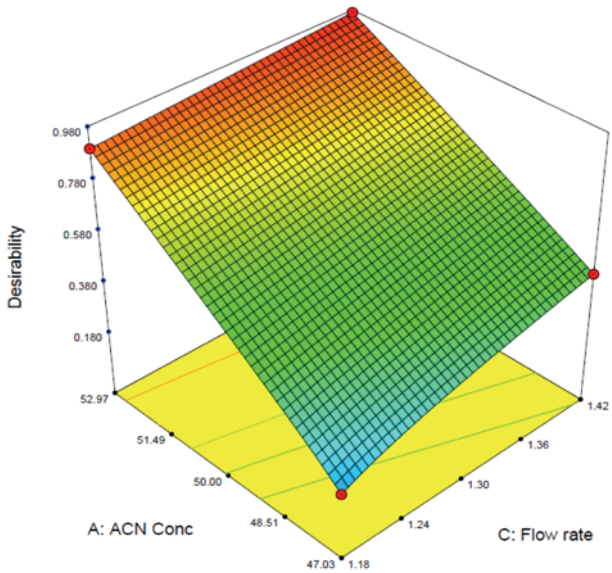



**Fig. 3.** Graphical representation of the overall desirability function D. ACN concentration (A) is plotted against flow rate (C) with factor B held constant at 2.75 pH

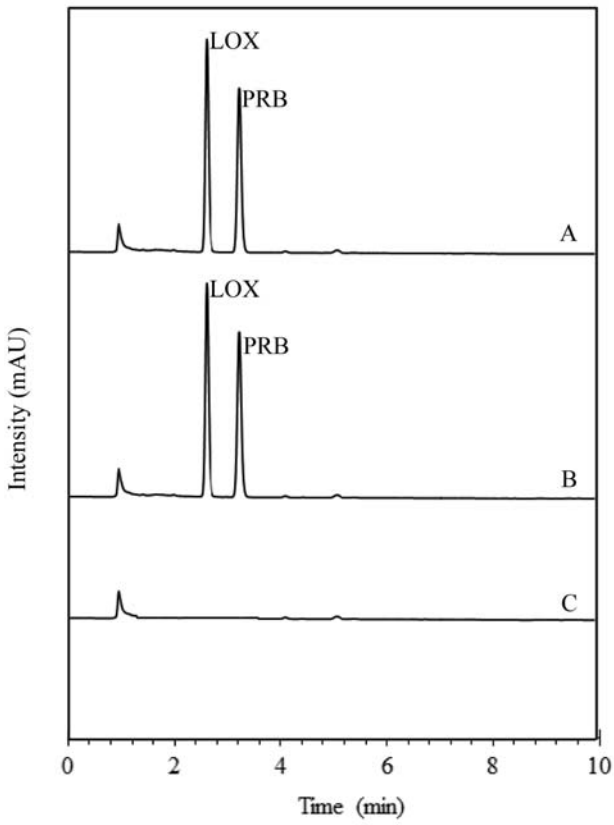



**Fig. 4.** Chromatograms corresponding to (A) a real sample of Loxomac tablets containing Lox( 10.08 μg/ml), IS (7.48 μg/ml): (B) a synthetic mixture of lox (10.06 μg/ml), IS (7.48 μg/ml): (C) mobile phase solution under optimum condition

ng/ml, respectively. Accuracy ($n = 9$), assessed by spike recovery, was found to be 99.86 and within acceptable ranges of 100 ± 2%.[38] The intra and inter-assay precision ($n = 6$) was confirmed since, the %C.V. were well within the target criterion of ≤2.[39] The robustness study reveals that small changes did not alter the retention times, retention factor and resolutions more than 3% and therefore it would be concluded that the method conditions are robust.

### 3. 4. Application of the Method

The proposed RP-HPLC method was applied to the quantitative analysis of real samples (Loxomac® tablets) containing loxoprofen. Representative chromatograms are presented in Fig. 4. The results achieved when analyzing Loxomac® tablets was 861.08 (0.09) mg of Loxoprofen, with the values in parenthesis representing the %C.V. of the six replicates. Good agreement was found between the assay results and the label claim of the product. The %C.V. tablets was <2, indicating the precision of the analytical methodology.

## 4. Conclusion

Statistically-based experimental designs proved to be a valuable approach in optimizing selectivity-controlling parameters for the determination of loxoprofen in commercial tablets. The significant factors were optimized by applying the central composite design and surface response methodology. The objective responses, resolution and the analysis time, were then simultaneously optimized by applying the Derringer's desirability function, a multi-criteria decision making tool. The improved method showed higher sensitivity and shorter analysis time than the existing methods making it viable to be implemented for routine quality control analysis in a pharmaceutical laboratory. The method was validated; the validation study supported the selection of the assay conditions by confirming that the assay was specific, accurate, linear, precise, and robust.

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**Table 4.** The comparison of experimental and predictive values of different objective functions under optimal conditions.

| Optimum conditions | ACN (%) | pH | Flow (ml/min) | $tR_2$ | $k_1$ | $Rs_{1,2}$ |
|---|---|---|---|---|---|---|
| I | Desirability value (D) = 0.971 | | | | | |
| | 52.97 | 2.90 | 1.42 | | | |
| | | Experimental | | 3.23 | 1.96 | 4.24 |
| | | Predictive | | 3.25 | 2.0 | 4.32 |
| | Error | | | 0.61 | 2.04 | 1.88 |

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## Povzetek


Loksoprofen spada v skupino nesteroidnih protivnetnih zdravil. Deluje preko inhibicije izoform ciklooksigenaze 1 in 2. V pričujoči študiji smo razvili izboljšano RP-HPLC metodo za kvantifikacijo loksoprofena v obliki farmacevtskega pripravka. Za ta namen smo uporabili eksperimentalni načrt. Iz preliminarne študije smo določili faktorje – neodvisne spremenljivke (organsko topilo, pH mobilne faze, pretok), kot odvisne spremenljivke pa smo izbrali tri tipe odziva (retencijski faktor loksoprofena, ločljivost med loksoprofenom in probenecidom ter retencijski čas probenecida). Za izboljšavo razvoja metode in njene optimizacije smo uporabili Derringerjevo funkcijo zaželenosti in tako hkrati optimizirali izbrane tri odzive. Postopek je dopuščal določitev optimalnih pogojev. Predvideni optimum je bil: razmerje acetonitril : voda (53:47, v/v), pH mobilne faze uravnan na 2, 9 z ortofosforno kislino. Ločba je potekla v manj kot 4 min. Metodo smo uporabili za kontrolo kakovosti komercialnih tablet. Pri metodi smo ugotovili skladnost med eksperimentalnimi podatki in predvidenimi vrednostmi v celotnem izbranem območju parametrov. Optimizirani postopek smo validirali glede na smernice Mednarodne konference za harmonizacijo (ICH) in s tem potrdili specifičnost, linearnost, točnost in natančnost.