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GLOBAL OPTIMIZATION USING DERRINGER'S DESIRABILITY FUNCTION: ENANTIOSELECTIVE DETERMINATION OF KETOPROFEN IN FORMULATIONS AND IN BIOLOGICAL MATRICES

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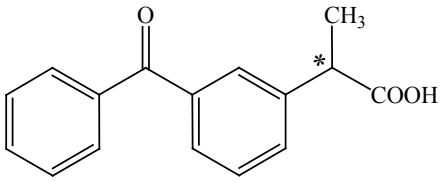
SUMMARY

A reversed-phase high-performance liquid chromatographic method for separation of the enantiomers of ketoprofen in formulations and in plasma matrices has been developed and optimized. A central composite design was used to develop response models and Derringer's desirability function was then used for simultaneous optimization of chiral resolution and analysis time. This procedure enabled discovery of two separate sets of optimum conditions for analysis of quality-control samples and plasma samples within the experimental domain. The optimum conditions predicted for quality-control samples were acetonitrile–dipotassium hydrogen phosphate buffer (10 mM, pH 6.5)–triethylamine 56:44:0.05 (*v*/*v*) as mobile phase and 1.13 mL min^{-1} as flow rate. Use of these conditions enabled baseline separation of the enantiomers of ketoprofen in approximately 4.2 min, which is quicker than the previously optimized method. The method was suitable for routine analysis of the enantiomers of ketoprofen in a commercial pharmaceutical preparation and in plasma samples.

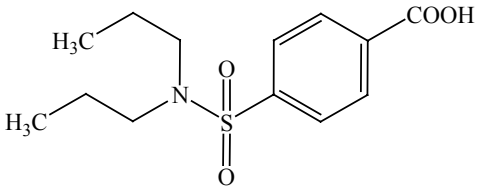
INTRODUCTION

Ketoprofen (Fig. 1), (±)-(*R*,*S*)-2-(3-benzoylphenyl)propionic acid, one of the most useful non-steroidal anti-inflammatory drugs, has received much attention in the past two decades. Its anti-inflammatory effect is approximately 160 times that of aspirin [1]. Although ketoprofen is used therapeutically as a racemic mixture, the anti-inflammatory effect resides almost exclusively in the *S* enantiomer; the *R* enantiomer is as much less active [2]. In-vivo experiments have proved that chiral inversion from *R* to *S*-ketoprofen can occur, the extent of inversion varying from one animal

species to another [3–5]. In sheep, the opposite *S* to *R* inversion has been reported [6] and in humans no inversion has been observed [7]. Enantioselective analysis of ketoprofen thus attracts attention in pharmacokinetic studies.



Ketoprofen



Probenecid (IS)

Fig. 1

Chemical structures of ketoprofen and the internal standard (IS); * indicates the stereogenic centre

The enantiomers of ketoprofen have substantially different pharmacological activity and benefits. The *S* enantiomer is used to reduce inflammation and relieve pains whereas the *R* enantiomer can be used as a toothpaste additive to prevent periodontal disease [11]. Thus the emergence of a new market strategy, the so called racemic switch or the production of single enantiomers of ketoprofen is expected to grow. Consequently, new ketoprofen drugs contain one enantiomer only, so the pharmaceutical industry requires rapid, accurate, and sensitive enantioselective methods for assessment of the enantiomeric purity of each enantiomer.

In addition to pharmacological and pharmacokinetic differences between the enantiomers of ketoprofen, there are also reports of pharmaceutical differences [8,9]. These reports are based on the hypothesis that chiral excipients may interact preferentially with one enantiomer leading to stereoselective release from a formulation containing a racemate [10]. It has been proved there is significant chiral discrimination in the release of

ketoprofen from formulations providing a chiral environment, for example when a chiral excipient, usually hydroxypropylmethylcellulose, is used.

All these studies further emphasize the need to perform stereoselective quality-control analysis of pharmaceutical formulations containing ketoprofen. In this context, the development of methods for enantioselective analysis of ketoprofen in quality-control and dissolution samples attracts attention.

Many high-performance liquid chromatographic (HPLC) methods for determination of the enantiomers of ketoprofen in formulations and biological matrices have been reported in the literature. These methods utilize a variety of chiral stationary phases [12–14], chiral mobile phases [1,15], or chiral derivatization reagents [7,16]. Diastereomeric methods employing a chiral derivatization reagent are more sensitive, versatile, and cost-effective. Hence, in this work, a diastereomeric method utilizing L-leucinamide as chiral derivatization reagent was used. Most of the studies mentioned above used univariate optimization, changing one factor at a time; this has the disadvantages of being time-consuming and of examining only a limited part of the experimental domain. These methods have also envisaged chiral optimization as the only objective, not considering analysis time as a second optimization criterion, so the long analysis times of these methods may limit their routine use. To overcome these problems, chemometric tools, for example experimental design coupled with global optimization, can be used.

Only two chemometrics-assisted enantioselective methods for ketoprofen have yet been reported – one employing a 2^3 factorial design without a global optimization technique [16] and another employing a 5^3 central composite design (CCD) combined with Derringer's desirability function, a global optimization technique for simultaneous optimization of resolution and analysis time [14]. Unfortunately, the long analysis time (13 min) of the latter method limits its routine use, necessitating re-optimization taking into consideration a different set of experimental factors and domains. Further, for any chromatographic method intended for routine application in a pharmaceutical or industrial environment, analysis time is usually optimized without losing resolution [17]. This prompted us to develop a rapid and accurate enantioselective method suitable for routine analysis of ketoprofen in formulation and plasma samples, by using a CCD and Derringer's desirability function. Derringer's desirability function was used because of its additional benefits – user flexibility in selecting optimum conditions for analysis of a variety of sample matrices, for example formulation and plasma samples, substantial timesaving, and more effective use of resources.

The objectives of this study were:

1. to investigate the effect of chromatographic factors and their interaction effects on separation behaviour with the aid of a CCD; and
2. to simultaneously optimize chiral resolution and analysis time and to deduce two separate sets of optimum conditions, for analysis of formulation and plasma samples, using Derringer's desirability function.

EXPERIMENTAL

Chemicals and Reagents

(*R*,*S*)-Ketoprofen, *S*-ketoprofen, probenecid (IS), and L-leucinamide hydrochloride were purchased from Sigma Chemical (Bangalore, India). Ethyl chloroformate was obtained from Fluka (Buchs, Switzerland). Acetonitrile (MeCN) and methanol (MeOH) of HPLC-grade and buffer salts and other reagents of analytical-grade were from SD Fine chemicals (Mumbai, India). Commercially available Rhofenid tablets containing 100 mg (*R*,*S*)-ketoprofen were purchased from Rhone–Poulenc (Mumbai, India). HPLC-grade water obtained by distillation in glass then passage through a Milli-Q Academic system (Millipore, Bangalore, India) was used to prepare all solutions.

Software

The chromatographic software, LC Solution-Release 1.11SP1 from Shimadzu was used for acquisition and treatment of chromatographic data. The homoscedasticity of the calibration plots was tested by Cochran's test using Matlab version 5.1.0.421 (The Math Works). Experimental design, data analysis, and desirability function calculations were performed by use of Design-Expert trial version 7.0.0. (Stat-Ease, Minneapolis, USA). This software was used because it enabled straightforward design of a multivariable experimental procedure.

Chromatographic Apparatus and Conditions

Chromatography was performed with Shimadzu (Tokyo, Japan) equipment comprising LC10AD and LC10 AD *vp* solvent-delivery modules, a Rheodyne (USA) model 7125 injection valve fitted with a 20-µL loop, and an SPD 10A UV–visible detector. The system was controlled by an SCL-10A system controller and a personal computer. Diastereoisomers of ketoprofen were resolved on a Phenomenex C_{18} analytical column (150

mm × 4.6 mm i.d., 5-µm particle) protected by a Phenomenex C_{18} guard cartridge (4 mm × 3 mm i.d., 5-µm particle); detection was at 256 nm. Mobile phases were mixtures of MeCN and 10 mM dipotassium hydrogen phosphate containing 0.05% (*v*/*v*) triethylamine. The pH of the aqueous phase was adjusted to 6.5 by addition of 10% phosphoric acid. Before analysis, mobile phases were degassed by use of a Branson sonicator (Branson Ultrasonics, USA). HPLC was performed in an air-conditioned laboratory atmosphere (20 ± 2°C).

Derivatization Procedure

Solutions of (*R*,*S*)-ketoprofen (20 µg mL^{-1}, 0.5 mL) and the IS (8 µg mL^{-1}, 0.5 mL) were mixed and evaporated to dryness in a clean Eppendorf tube using an Eppendorf (Hamburg, Germany) 5301 concentrator. The evaporated residue was then subjected to chiral derivatization by a method adapted from Foster and Jamali [7]. This procedure converts the enantiomers of ketoprofen into diastereoisomers, which can be resolved on a classic reversed-phase C_{18} column. To accomplish the conversion the residue was reconstituted in 100 µL 50 mM triethylamine in acetonitrile and vortex-mixed for 30 s. Solutions of ethyl chloroformate in acetonitrile (60 mM, 50 µL), L-leucinamide hydrochloride in methanol (1 M, 50 µL), and triethylamine in methanol (1 M, 50 µL) were added at 30-s intervals. The reaction was terminated after 2 min by addition of 50 µL HPLC-grade water. The derivatized samples containing ketoprofen diastereoisomers (20 µL) were then chromatographed.

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

Standard and Sample Preparation

Standard Solutions

Stock standard solutions of (*R*,*S*)-ketoprofen and IS (1 mg mL^{-1}) were prepared in methanol, protected from light, and stored at 4°C until used. Working standard solutions were freshly prepared on the day of analysis by diluting the stock standard solutions with methanol.

Separate calibration plots were constructed for *R* and *S*-ketoprofen by subjecting racemic (*R*,*S*)-ketoprofen to chiral derivatization and chro-

matography. Calibration plots reporting *R* and *S*-ketoprofen-to-IS peak-area ratio as a function of the concentration of each enantiomer were established by chromatography of solutions containing 1, 3, 5, 7, and 10 μg mL^{-1} of the enantiomers and 4 μg mL^{-1} probenecid as IS. Standard solutions used for optimization contained a mixture of *R*-ketoprofen (5 μg mL^{-1}), *S*-ketoprofen (5 μg mL^{-1}), and the IS (4 μg mL^{-1}).

Tablet Sample Preparation

Twenty tablets were weighed and finely powdered. An amount of tablet powder equivalent to 10 mg (*R*,*S*)-ketoprofen was accurately weighed and transferred to a 50-mL volumetric flask. A suitable quantity of IS was added, then 25 mL mobile phase. This mixture was subjected to sonication for 10 min for complete extraction of the drugs and the solution was diluted to volume with mobile phase to furnish final concentrations of (*R*,*S*)-ketoprofen and IS of 20 and 8 μg mL^{-1}, respectively. A portion (0.5 mL) of the resulting solution was then evaporated to dryness under vacuum at 60°C, using an Eppendorf 5301 concentrator, and the residue was subjected to chiral derivatization and chromatography.

Plasma Sample Preparation

Blank plasma (1 mL) was spiked with working solutions of (*R*,*S*)-ketoprofen and IS to achieve plasma concentrations of 10 μg mL^{-1} and 4 μg mL^{-1}, respectively. The samples were then acidified by addition of 100 μL 0.6 M sulphuric acid, vortex mixed for 30 s, centrifuged for 5 min at 3500 rpm (1878*g*) on a laboratory centrifuge (Remi, R&C, Remi Equipment, Mumbai, India), then extracted with 3 mL diethyl ether. The organic layer was then evaporated to dryness under vacuum at 60°C using an Eppendorf concentrator. The residue was then derivatized and chromatographed using the optimized separation conditions predicted for analysis of plasma samples.

To assess the efficiency of the extraction procedure, the spiked plasma sample was extracted by the above procedure, but with addition of IS after extraction. Percentage recovery was estimated by comparing the peak area of each enantiomer from spiked samples with that from blank plasma samples to which the drug had been added before the evaporation step.

$$\text{Recovery (\%)} = \frac{E_{(\text{spike})}/IS}{E_{(\text{non-spike})}/IS} \times 100 \qquad (1)$$

where, $E_{(spike)}$ is the area of each enantiomer in spiked plasma samples and $E_{(non\text{-}spike)}$ is the area of each enantiomer obtained by addition of the drug before the evaporation step. The reproducibility of the extraction procedure was determined by replicate ($n = 6$) extraction of spiked plasma samples.

Method Validation

The optimized assay was validated in accordance with current pharmaceutical regulatory guidelines "Q2A, Text on Validation of Analytical Procedures: Definitions and Terminology" [18] and "Q2B, Validation of Analytical Procedures: Methodology" [19]. The method was validated for linearity, limits of detection and quantitation, specificity, accuracy, precision, and robustness.

RESULTS AND DISCUSSION

Response-Surface Methodology

Response-surface methodology enables definition of quadratic models that accurately describe the response for all values of the chromatographic conditions in the experimental region [20–24]. To calculate quadratic regression model coefficients, each design variable must be studied at three distinct levels, at least, and, consequently, a CCD was used in this optimization study. Selection of factors for optimization was based on preliminary experiments, prior knowledge of the literature, and known instrumental limitations. For example, the pH of the aqueous phase was fixed at 6.5, because this could effect the stability of the diastereomeric derivative [25]. Literature data also suggested buffer concentration should be maintained at 10 mM, because this has least effect on chiral resolution and run time [16]. The mobile phase flow rate can also affect selectivity in HPLC analysis. The key conditions selected for optimization were, therefore, MeCN concentration (A) and mobile phase flow rate (B).

On the basis of preliminary experiments the factor space of this design was expanded within the ranges 45 to 58% (*v*/*v*) for MeCN concentration and 0.8 to 1.2 mL min^{-1} for flow rate. The retention factor of *R*-ketoprofen (k_1), the retention times of *R*-ketoprofen (t_{R1}), *S*-ketoprofen (t_{R2}), and the IS (t_{R3}), chiral resolution of *R*-ketoprofen from *S*-ketoprofen ($R_{S1,2}$), and resolution of *S*-ketoprofen from the IS ($R_{S2,3}$) were chosen as response variables. To provide a CCD for two factors, a full 2^2 factorial design was combined with four replicates of the centre points and 2×2

axial points, where one factor is set at an extreme level (±α) and the other factor is at its central level. The axial distance, α, was chosen as 1.414 to make this design rotatable. Replicates of the central points were performed to estimate the experimental error. Experiments were performed in random order to minimize the effects of uncontrolled variables that may introduce bias into the measurements.

The design matrix and experimental results are presented in Table I. Analysis of these data enables construction of a 'sequential model sum of squares' summary table for every response, indicating how terms of increasing complexity contribute to the total model. Investigation of the associated probability revealed that for all the responses quadratic models resulted in the best fit. The cubic models were aliased, as expected, because the central composite matrix provided very few unique design points to

Table 1

Central composite rotatable design arrangement[a] and responses

Design points	Coded factor levels		Responses					
	MeCN conc.	Flow rate	k_1	t_{R1} (min)	t_{R2} (min)	t_{R3} (min)	$R_{S1,2}$	$R_{S2,3}$
1	−1	−1	2.674	6.407	7.429	10.025	3.198	6.811
			2.663	6.389	7.407	9.971	3.145	6.646
2	+1	−1	1.324	4.053	4.529	5.423	2.132	3.645
			1.353	4.104	4.584	5.473	2.130	3.608
3	−1	+1	2.690	4.855	5.628	7.587	2.982	6.408
			2.654	4.809	5.576	7.531	3.091	6.606
4	+1	+1	1.383	3.137	3.500	4.183	1.983	3.428
			1.379	3.130	3.493	4.174	1.948	3.364
5	−1.414	0	3.171	6.256	7.303	10.132	3.184	7.108
			3.154	6.231	7.267	10.085	3.155	7.109
6	+1.414	0	1.187	3.280	3.642	4.285	1.879	3.085
			1.170	3.255	3.608	4.240	1.856	3.035
7	0	−1.414	1.785	5.222	5.957	7.514	2.696	5.018
			1.786	5.223	5.956	7.513	2.707	5.000
8	0	+1.414	1.850	3.563	4.056	5.122	2.445	4.636
			1.819	3.523	4.015	5.061	2.449	4.619
9	0	0	1.850	4.275	4.869	6.129	2.573	4.843
10	0	0	1.841	4.262	4.857	6.123	2.579	4.839
11	0	0	1.838	4.257	4.844	6.101	2.574	4.871
12	0	0	1.822	4.233	4.824	6.088	2.554	4.815

[a]Randomized

determine all the terms in the cubic model. In this work, therefore, the following quadratic model was used to describe the response surface:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \qquad (2)$$

where Y is the single response (k, t_R, or R_S) to be modelled, β is the regression coefficient, and X_1 and X_2 represent factors A and B, respectively.

To obtain a simple and yet a realistic model, insignificant terms (i.e. terms having a probability (P) value >0.05) were eliminated from the model by a 'backward elimination' process. The statistical data obtained from ANOVA for the reduced models are given in Table II. Because the correlation coefficient (R^2) always decreases when a regressor variable is eliminated from a regression model, in statistical modelling the adjusted R^2 which

Table II

Reduced response models and statistical data obtained from ANOVA (after backward elimination)

Response	Reduced response models[a]	Adjusted R^2	Model P value	RSD (%)	Adequate precision
k_1	$1.83 - 0.68A^a + 0.18A^2$	0.997	0.000	1.65	151.98
t_{R1}	$4.26 - 1.03A - 0.61B + 0.16AB + 0.26A^2 + 0.07B^2$	0.999	0.000	0.94	140.39
t_{R2}	$4.85 - 1.27A - 0.70B + 0.19AB + 0.31A^2 + 0.08B^2$	0.999	0.000	0.91	152.91
t_{R3}	$6.11 - 2.02A - 0.89B + 0.29AB + 0.55A^2 + 0.11B^2$	0.999	0.000	1.05	154.64
$R_{S1,2}$	$2.56 - 0.49A - 0.08B$	0.989	0.000	1.86	75.65
$R_{S2,3}$	$4.86 - 1.49A - 0.12B + 0.14A^2$	0.995	0.000	1.97	96.13

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

takes the number of regressor variables into account is usually selected [26]. In this study, the adjusted R^2 were well within acceptable limits of $R^2 \geq 0.80$ [27] which revealed the experimental data were a good fit to the second-order polynomial equations. P values <0.05 were obtained for all the reduced models, implying the models were significant. The value of the adequate precision is a measure of the 'signal (response) to noise (deviation) ratio'. A ratio >4 is desirable [28]. In this study the ratio was in the range 26.24 to 125.14, which indicates the signal is adequate and, therefore, the model is significant for the separation process. The relative standard deviation (RSD) is a measure of the reproducibility of the model and, as a general rule, a model can be regarded as reasonably reproducible if the RSD is <10% [28]. RSD was <10% for all the models. Further, examination

of diagnostic plots [29] such as the normal probability plot of residuals and a plot of residuals vs. predicted values revealed that the assumptions of normality and constant variance of the residuals were satisfied for all the fitted models.

As is apparent from Table II, the interaction term with the largest absolute coefficients among the fitted models is AB (+0.29) of the t_{R3} model. The positive interaction between A and B is statistically significant ($P < 0.0001$) for t_{R3}. The study reveals that changing the fraction of MeCN from low to high results in a rapid decrease in t_{R3} at both low and high flow rate. Further, at the low level of factor A an increase in the flow rate results in a marginal decrease in the retention time. Therefore, when the MeCN concentration is set at its lowest level, the flow rate must be at its highest level to reduce the analysis time. This interaction, especially, is synergistic, because it led to a decrease in analysis time. The existence of such interactions emphasizes the need to perform active multifactor experiments for optimization of chromatographic separations.

Partial Desirability (d_i) and Global Desirability (D) Functions

The procedure followed in this work for simultaneous optimization of the six responses is a modification of the method developed by Derringer and Suich [30]. The method involves transformation of each predicted response, $\hat{y}$, to a dimensionless partial desirability function, d_i, which includes the researcher's priorities and desires when building the optimization procedure. One or two-sided functions are used, depending on whether each of the n responses has to be maximized or minimized, or has an allotted target value. If the response i is to be maximized the quantity d_i is defined as:

$$\begin{aligned} d_i &= \left(\frac{\hat{y} - A}{B - A}\right)^{wi}, \quad A \le \hat{y} \le B \\ d_i &= 1, \qquad\qquad \hat{y} > B \\ d_i &= 0, \qquad\qquad \hat{y} < A \end{aligned} \tag{3}$$

Likewise, d_i can be defined when the response is to be minimized or if there is a target value for the response. In Eq. (3), A and B are, respectively, the lowest and the highest values obtained for the response i, and wi is the weight. d_i ranges between 0, for a completely undesired response, and 1, for a fully desired response. In both cases, d_i will vary non-linearly while approaching the desired value. But with a weight of 1,

d_i varies linearly. In this work we chose weights equal to 1 for all six responses.

The partial desirability functions are then combined into a single composite response, the so-called global desirability function D, defined as the geometric mean of the different d_i-values:

$$D = \left[d_1^{p1} \times d_2^{p2} \times d_3^{p3} \times \cdots \times d_n^{pn} \right]^{1/n} \quad (4)$$

A value of D different from 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 optimum, so the response values are near the target values. In Eq. (4), *pi* is the relative importance assigned to the response *i*. The relative importance *pi* is a comparative scale for weighting each of the resulting d_i in the overall desirability product and it varies from the least important (*pi* = 1) to the most important (*pi* = 5). It is noteworthy that the outcome of the overall desirability D depends on the *pi* value that offers users flexibility in the definition of desirability functions.

Optimum Conditions for Formulation Samples

The criteria for the optimization of individual response are shown in Table III. Criteria I have been proposed for selecting the optimum experimental conditions for analysis of routine quality-control samples. The

Table III

Optimization of the individual responses for analysis of quality-control samples (criteria I) and plasma samples (criteria II)

Response	Lower limit	Upper limit	Weight	Criteria I		Criteria II	
				Goal	Relative importance	Goal	Relative importance
k_1	1.17	3.17	1	Target = 1.2	3	Target = 2	5
t_{R1}	3.13	6.41	1	Range	1	Range	1
t_{R2}	3.49	7.43	1	Range	1	Range	1
t_{R3}	4.17	10.13	1	Minimize	5	Minimize	3
$R_{S1,2}$	1.86	3.20	1	Target = 2	3	Target = 2	3
$R_{S2,3}$	3.04	7.11	1	Minimize	2	Minimize	2

first requirement was to achieve baseline resolution of all the peaks; for chiral resolution $R_{S1,2}$, especially, the target value was set at 2.0. The target

value for k_1 was 1.2, to enable resolution of the *R* enantiomer of ketoprofen from the dead volume. As short analysis time is usually preferred for routine analysis, the response t_{R3} was minimized and a high importantce value of 5 was assigned. The optimization procedure was conducted under these conditions and restrictions. The partial desirability functions (d_i) of each of the responses, and the calculated geometric mean as the maximum global desirability function ($D = 0.969$), are presented in Fig. 2, in which d_i varies from 0 to 1, depending on the closeness of the response to its target

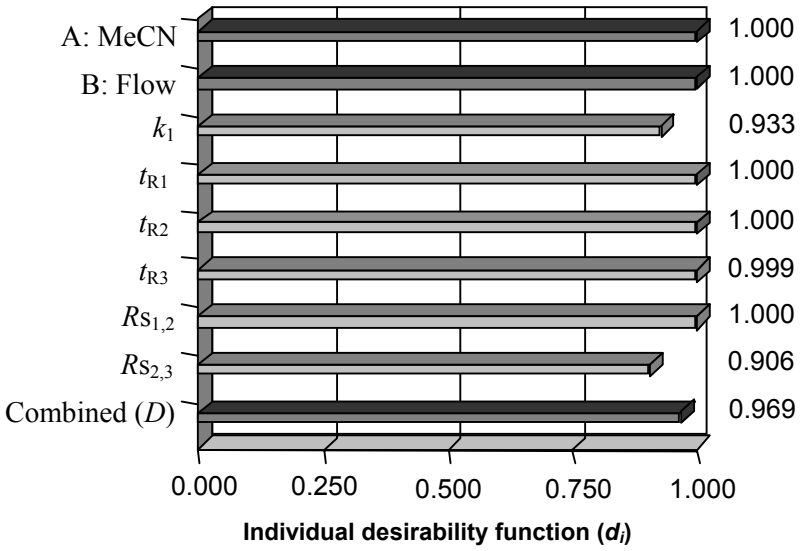


Fig. 2

Bar graph showing individual desirability values (d_i) of various objective responses and their association as a geometric mean (D) corresponding to formulation samples

value. The response-surface plot corresponding to this *D* value is depicted in Fig. 3, where the best compromise is obtained at the top of the graph, *D*. The coordinates of *D* represent the optimum conditions; the corresponding predicted responses are shown in Table IV. When these optimum conditions were used the enantiomers of ketoprofen were separated to baseline and the analysis time was approximately 4.2 min (Fig. 4B), which is approximately 68% lower than that previously reported for an optimized method [14]. This short analysis time makes the method viable for routine quality-control analysis in a pharmaceutical laboratory.

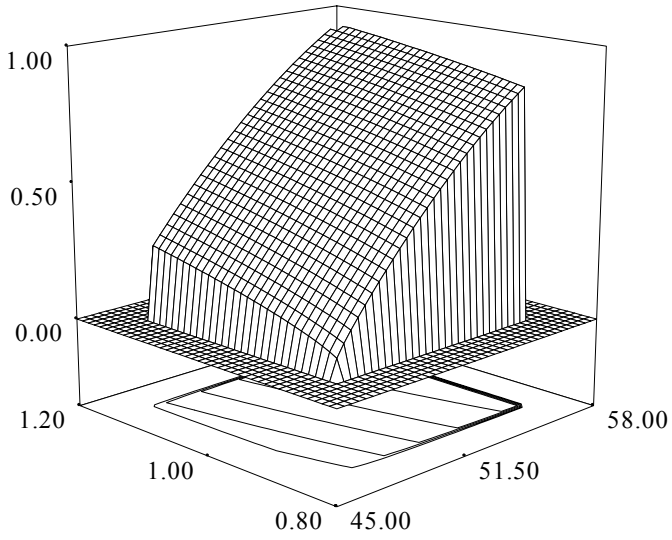


Fig. 3

Graphical representation of the maximum global desirability function corresponding to formulation samples. The best compromise is obtained at the top of the graph, $D = 0.969$

Table IV

Comparison of experimental and predicted values of different objective functions under the optimum conditions

Optimum conditions	MeCN (% *v*/*v*)	Flow rate (mL min^{-1})	k_1	t_{R1} (min)	t_{R2} (min)	t_{R3} (min)	$R_{S1,2}$	$R_{S2,3}$
I	Desirability value (D) = 0.969							
	56.01	1.13						
	Predicted		1.33	3.13	3.50	4.18	2.00	3.42
	Experimental		1.39	3.17	3.52	4.20	1.94	3.40
II	Desirability value (D) = 0.714							
	50.96	1.14						
	Predicted		1.91	3.83	4.37	5.54	2.54	4.92
	Experimental		1.88	3.79	4.27	5.41	2.50	4.74
Average error			2.78%	1.29%			2.21%	

Optimal Conditions for Plasma Samples

To find separate, optimum conditions for analysis of plasma samples, criteria II were established by varying the response goals and their im-

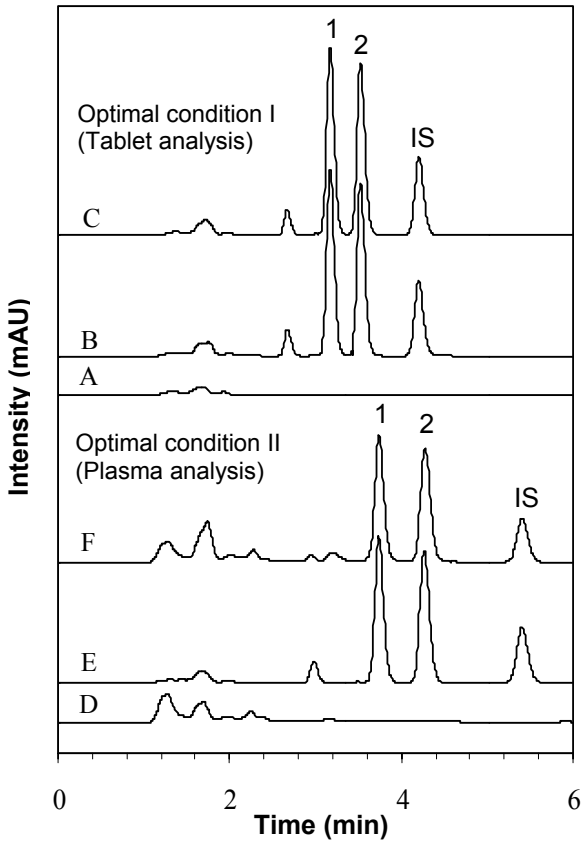


Fig. 4

A, B, C: chromatograms obtained under optimum conditions I (56.01% MeCN, flow rate 1.13 mL min^{-1}) after derivatization of (A) a placebo solution, (B) a synthetic mixture containing 10 μg mL^{-1} (*R*,*S*)-ketoprofen, and (C) a real sample of Rhofenid tablets containing (*R*,*S*)-ketoprofen. D, E, F: chromatograms obtained under optimum conditions II (50.96% MeCN, flow rate 1.14 mL min^{-1}), after extraction and derivatization of (D) blank human plasma (E) a synthetic mixture containing 10 μg mL^{-1} (*R*,*S*)-ketoprofen, and (F) human plasma spiked with 10 μg mL^{-1} (*R*,*S*)-ketoprofen. Peaks: 1, *R*-ketoprofen; 2, *S*-ketoprofen; IS, probenecid

portance values (Table III). For example, a large value of k_1 must be selected for separation of *R*-ketoprofen from the initial disturbances of the plasma components. Therefore, k_1 was targeted at 2.0 with a high importance value of 5. The optimization procedure was then conducted under these conditions and restrictions. The desirability function was maximized at an overall desirability of approximately $D = 0.714$, which provides optimum conditions II (Table IV) for analysis of plasma samples. The chromatogram

obtained under these optimum conditions showed the retention times of *R* and *S*-ketoprofen were 3.79 and 4.27 min, respectively, and analysis time was 5.41 min (Fig. 4E). Predicted and experimental responses were in good agreement [31], within a difference of 3%. This approach offers flexibility to the chromatographer to alter k_1 values depending on the environment of the analyte under consideration.

A chromatogram obtained from drug-spiked plasma under optimum conditions II is shown in Fig. 4F. Under these optimized chromatographic conditions, specificity was apparent from the absence of any endogenous interference at the retention times of the ketoprofen enantiomers and the IS. When the reproducibility of the proposed extraction procedure was tested experimentally ($n = 6$), recovery (mean ± %*RSD*) of the *R* and *S* enantiomers of ketoprofen was found to be 88.45 ± 2.1% and 87.98 ± 2.7%, respectively. These low *RSD* values also confirm that the reproducibility of the extraction procedure makes it reliable for enantioselective analysis of ketoprofen in human plasma.

Validation of the Method

Linearity was established for five levels over the concentration range 1.0–10.0 μg mL^{-1} for each enantiomer. Peak areas (y) for each enantiomer were plotted against the respective concentrations (x) and linear regression analysis was performed on the resulting calibration plots ($n = 6$). Typical mean regression equations were $y = 0.396x + 0.004$ for *R*-ketoprofen and $y = 0.394x - 0.004$ for *S*-ketoprofen. Correlation coefficients were >0.999 for both enantiomers. Because correlation coefficients are not good indicators of the linearity of an analytical procedure [32], one-way ANOVA was performed. For both enantiomers, the calculated *F*-value (F_{Calc}) was less than the theoretical *F*-value (F_{Crit}) at the 5% significance level, indicating there was no significant difference between replicate determinations for each concentration (Table V). When homoscedasticity for the calibration plots was verified by applying Cochran's test there was no statistical difference ($P > 0.05$) between the variances [33,34].

The limits of detection (*LOD*) and quantitation (*LOQ*) for each enantiomer were determined in accordance with ICH guideline Q2B [19]. *LOD* was defined as $3.3\sigma/S$ and *LOQ* as $10\sigma/S$, where σ is the residual standard deviation and S the slope of the calibration plot constructed for five levels in the range 0.05 to 1.0% of the target enantiomer concentration [35]. Using these equations, *LOD* and *LOQ* were estimated as 1.83 and 5.54 ng mL^{-1}, respectively, for *R*-ketoprofen, and 2.03 and 6.14 ng mL^{-1} for *S*-ketoprofen.

Table V

Results from one-way ANOVA testing of the linearity of the method

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	*F*-value	
				F_{Calc}	F_{Crit}[a]
R-Ketoprofen					
Between group	5	1.23×10^{-3}	2.47×10^{-4}	0.0001	2.6207
Within group	24	45.77	1.91		
Total	29	45.77			
S-Ketoprofen					
Between group	5	1.11×10^{-3}	2.22×10^{-4}	0.0001	2.6207
Within group	24	45.56	1.90		
Total	29	45.56			

[a]Theoretical value of $F(5,24)$ at $P = 0.05$ level of significance

The optimized method for assay of formulations is specific for the tablet excipients and derivatization reagents used in this study (Fig. 4C).

Both intra-day and inter-day ($n = 6$) accuracy and precision were determined for 1.0, 5.0, and 10.0 μg mL^{-1} of each enantiomer. Accuracy was expressed as bias, i.e. the difference between the results obtained and the reference value. Assay precision was expressed as *RSD* (%). Intra-day accuracy ranged from −2.00 to 3.00% and precision from 0.565 to 0.784%. Inter-day accuracy ranged from −4.00 to 4.20% and precision from 1.449 to 1.979%. Intra-day and inter-day accuracy were within acceptability criteria for bias, ±5% [36]. Intra and inter-assay precision were also acceptable because *RSD* (%) values were well within the target criteria of ≤2 and ≤3, respectively [37].

The robustness of the method to small deliberate variation of the experimental conditions was assessed to provide an indication of method reliability during normal use. Variation of MeCN concentration (56 ± 0.5%), pH (6.5 ± 0.2), and buffer concentration (10 ± 2.0 mM) did not affect results for either enantiomer by more than 1% and it was, therefore, concluded that the method is robust.

Application to a Pharmaceutical Product

The method was used for quantitative analysis of the enantiomers of ketoprofen in Rhofenid tablets containing racemic ketoprofen. Representative chromatograms are depicted in Fig. 4C. Mean recovery (±*SD*, %) was 100.20 ± 0.96 (0.96) for *R*-ketoprofen and 99.21 ± 0.97 (0.98) for *S*-ke-

toprofen (the values in parentheses are *RSD* (%) for six replicates). *RSD* values <2% indicate the method is precise.

Mean recovery of each enantiomer was also tested for significance by use of Student's *t*-test to assess whether the value was different from the label claim for the tablets. The values of t_{Calc} for *R*-ketoprofen (0.511) and *S*-ketoprofen (2.007) were less than $t_{Crit} = 2.571$ at the 5% significance level, suggesting there was no significant difference between mean recovery of the enantiomers and the label claim of the product.

CONCLUSIONS

Reversed-phase HPLC methods for separation of the enantiomers of ketoprofen have been developed and optimized by using a CCD. The CCD provides a better insight into the effects on the separation of chromatographic conditions and their interactions. The objective responses chiral resolution and analysis time were optimized simultaneously by use of Derringer's desirability function. The optimized conditions obtained by use of this chemometric approach enabled baseline resolution of the enantiomers in a reasonable analysis time. Chromatographic techniques coupled with chemometrics tools can provide a complete picture of a separation process, making this combined technique a powerful and convenient analytical tool.

The methods were validated. The validation study supported the conditions selected by confirming the assays were specific, accurate, linear, precise, and robust. Application of the methods for determination of the enantiomers of ketoprofen in a pharmaceutical formulation and in plasma samples was demonstrated. The methods can also be used in enantioselective release studies to evaluate stereoselective interactions between ketoprofen and chiral excipients during stereoselective formulation development. These rapid and sensitive methods can also be used for assessment of the enantiomeric purity of single-enantiomer products.

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