Original Article

Chemometric Assisted Ion-Pair Chromatography of Metaxolone and Diclofenac in Binary Mixture: A Mechanistic Study

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ABSTRACT

A reversed- phase high-performance liquid chromatographic (RP-HPLC) method based on ion pair formation is demonstrated for the simultaneous determination of Metaxalone (MTX) and Diclofenac potassium (DCP) in commercial formulations. MTX (pKa = 12.24) and DCP (pKa = 4.00) are hydrophilic ionic substances that make the separation critical due to distinct pKa values. Addition of ionic additives (ion-pairing reagents or chaotropic agents) to the mobile phase allowed a significant improvement in retention of the ionic analytes. However, finding the suitable condition of the *Ion-Pair Chromatography* (IPC) to achieve desirable separation was challenging and often rebellious to influential chromatographic parameters. The judicious selection of eluent pH, flow rate, acidic modifiers and organic modifier, detector wavelength was performed with the aid of Plackett–Burman design and Box–Behnken design. Desired separation of MTX and DCP was achieved using an Inertsil ODS2 (250x4.6mm; 5μm) column equilibrated with 10mM phosphate buffer (pH-5.0): acetonitrile (42: 58%v/v) containing 0.4% triethylamine and 0.5% tetrabutyl ammonium hydroxide as an eluent at a flow rate of 1.20 mL/min. Under optimal condition, the detection limits are 0.306μg/mL and 0.807μg/mL and limits of quantification 0.927μg/mL and 2.44μg/mL for MTX and DCP respectively. Subsequently, the plausible retention behavior of both analytes was predicted in the ion interaction condition.

Key words: Amphiphilicity, Chemometrics, Diclofenac, *Ion-Pair* formation, Metaxalone, RP-HPLC.

INTRODUCTION

Metaxalone (MTX), a 2-oxazolidinone derivative possesses skeletal muscle relaxant property.[1,2] Diclofenac potassium (DCP) is an established non-steroidal anti-inflammatory drug used in combination with MTX as an adjunct therapy for acute and painful musculoskeletal conditions. A large number of analytical methods have been reported for determination of MTX and DCP individually. Several methods, including RP-HPLC[3,4,5] LC–MS/MS[6,7,8] and UV spectroscopy.[9,10] were reported to determine MTX. Several analytical methods employing spectrophotometry.[11,12,13,14] RP-HPLC.[15,16,17,18,19,20,21] Gravimetry[22] and diffuse reflectance photometry[23] have been established for the quantitative determination of DCP in single or multiple component mixtures. Both the drugs in combined dosage forms had been simultaneously estimated by spectrophotometry[24,25] HPLC[26,27] and HPTLC.[28] These methods more or less are based on univariate approach (changing one variable at a time, whilst keeping the others constant) and routine assay procedures available in the literature.

No ion-interaction liquid chromatographic methods have been reported yet. Nevertheless, ion suppression mode and

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classic ion pairing as a function of ion-interaction mechanism is most effective to achieve competent separations of the closely related ions including impurities and metabolites. Use of ionic additives (ion-pairing reagents) in the mobile phase allow significant improvement in retention of the hydrophilic analytes over conventional RPLC, but it is still challenging to find suitable conditions that would separate all the components in the mixtures studied. However, *chemometric* approach on the basis of design of experiments (DoE) discover and screen the probable sources of variability that could impair the RP-HPLC method performance; and facilitate to find the optimum combination of factors and their levels.

In this work, an *Ion-Pair* RP-HPLC method was developed for the first time to determine DCP and MTX in bulk and combined dosage form simultaneously. All the influential HPLC parameters have been optimized to facilitate a rapid and selective determination of those drugs with the aid of DoE. In addition, we attempted to cover the probable retention mechanism for separation of both drugs to increase the scope of *Ion-Pair Chromatography* (IPC) as a mature and valuable separation strategy.

EXPERIMENTAL

Instrumentation and software's

A binary HPLC system equipped with two LC-20AD pumps, a SPD-M20A diode array detector with a manual injector (all from Shimadzu, Kyoto, Japan) was used to execute the experiments. An Inertsil ODS2 (250 × 4.6 mm; 5µm) analytical column was used for chromatographic separation of the analytes. The chromatographic analysis and data integration were recorded on a computer system using LC-Solution data acquiring software (Shimadzu, Kyoto, Japan). The choice of the experimental design and runs was done by the software package Design-Expert 9.0.3 trial version for Windows (Stat-Ease Inc.).

Chemicals and reagents

Tetrabutyl ammonium hydroxide used as *Ion-Pair* reagent was obtained from Sigma- Aldrich. All the analytical grade chemicals and reagents (potassium dihydrogen phosphate, triethylamine, ortho-phosphoric acid) were purchased from S.D. Fine Chem. (Mumbai, India). HPLC grade acetonitrile and water was purchased from Merck (Mumbai, India).

Preparation of analytical solutions

Standard solutions

Pure samples of MTX and DCP (99% purity) were procured from a local manufacturing unit in Hyderabad, India. Stock standard solutions (1mg/mL) were freshly prepared by dissolving 25 mg of each drug in 25 mL of methanol (HPLC grade). Working standard solutions were made by diluting the stock standard solution with phosphate buffer (pH-5.0) for use in development and optimization of the RP-HPLC method.

Sample solution

Twenty Tablets [Flexura-D, (Sun Pharma)] were weighed, finely powdered and an accurately weighed sample equivalent to 50mg of MTX and 12.5 mg of DCP was extracted with Methanol in a 25 mL volumetric flask using ultrasonicator. This solution was filtered and further diluted with methanol to obtain suitable concentrations within the linearity range.

Chromatographic conditions

An Inertsil ODS2 (250 x 4.6 mm; 5µm) column was used for the separation of the analytes. An ion pair mobile phase system consisting of potassium dihydrogen phosphate buffer (10mM; containing 0.4% triethylamine and 0.5% tetrabutyl ammonium hydroxide, adjusted to pH 5.0 using ortho-phosphoric acid) and acetonitrile (41.89: 58.11%v/v) was filtered through 0.25 µm membrane filter and degassed in an ultrasonic bath. The mobile phase was pumped at 1.2 mL/ min flow rate in an isocratic mode. The UV detection of eluents was performed at 272 nm, at room temperature.

Development and optimization of the *Ion-Pair* RP-HPLC method

In IPC, analytes' retention is significantly affected by numerous experimental factors, including type and concentration of the *Ion-Pair* reagent; composition, organic modifier concentration, pH, and flow of mobile phase. Hence, several variables were chosen from the operating procedure and screened in an experimental design to attain the optimum condition. Acetonitrile was preferred as the organic phase instead of methanol owing to its higher eluting capacity. An acidic buffer (potassium dihydrogen phosphate) was used as the aqueous phase to maintain pH 4.5 to 7.0. The resultant unacceptable asymmetric and tailing factors were partly resolved using triethylamine as an organic modifier. However, the chromatographic conditions were markedly improved by the addition of Bu_4NOH as an *Ion-Pair* reagent.

Screening of potential factors

Since a huge number of factors are responsible to influence the separation process, few of them do not have significant effect on it. Hence, the primary objective of employing experimental design in HPLC is to screen out the most influential factors.[29] Plackett–Burman

design (PBD), a two-level factorial design is used for the screening purpose that can determine the most important factors and their interactions effects with fewer runs.[30,31] The five factors with extreme levels of low (-) and high (+) were studied in the PBD for 11 factors requiring 12 experiments (Table 1). Dummy factors are entered in the spare columns of the design. These variables are imaginary and do not result a physical change in the method when changed from one level to the other. Each of the 12 experimental design runs was performed in triplicate. The significance of factors' effects (Eq 1) was interpreted by analysis of variance (ANOVA) of resultant design values for the responses.

$$E_X = \frac{\Sigma Y(+1)}{n} - \frac{\Sigma Y(-1)}{n} \quad (1)$$

Where, E_X is the effect of factor *X;* $\sum Y(+1)$ *and* $\sum Y(-1)$ are the sums of responses at factor level (+1) and (−1) respectively; and *n* is half of the number of experimental runs from the design.

Method optimization by Box–Behnken design

From the PBD, only significant factors were selected with an aim that such factors must be more strictly controlled during the execution of the method. The impact of these factors on the separation selectivity with respect to desirability of responses[32] and other performance criteria was examined by a response surface methodology (RSM) based Box–Behnken experimental design (BBD).[33] The method facilitates the development of polynomial models to assess the statistical significance of the variable influences being studied including the interaction and quadratic terms for all the responses using following multiple linear regression (MLR) equation.[34]

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{33}X_3^2 \quad (2)$$

Where, b_0 is the intercept representing the quantitative results of all the experimental runs; b_1 to b_{33} are the regression coefficients figured out from the predicted response values of Y; X_1, X_2 and X_3 are the independent variables selected from the PBD; and the terms X_1X_2, X_1X_3 and X_2X_3 represent the interaction of variables; and X_i^2 (i= 1 to 3) are quadratic terms.

Method validation

The validation parameters like linearity, sensitivity, accuracy, precision, robustness and the recovery of the assay method were studied in accordance to the ICH guidelines. The linearity and range was established by analyzing suitable dilutions from the standard mixture solution in the concentration range of 1 to 300µg/mL. The resultant peak areas were plotted against the corresponding concentrations to obtain the calibration curve. The linearity of the method is indicated by the linear regression equation and correlation coefficient for both the drugs. Intra- and inter-day precision and accuracy study was performed by analyzing multiple replications of three QC samples (20, 100 and 250 µg/mL) and percent recovery was determined. To study the robustness of the method, the test solutions were injected with

Table 1: Plackett–Burman design (-1= low factor level, +1= high factor level).

Runs	Variables										
	pH	Dum1	%ACN	Dum 2	Flow rate	Dum 3	%TEA	Dum 4	Dum 5	Wave length	Dum 6
1	4.5	1	50	-1	0.8	-1	0	-1	1	252	-1
2	7	1	70	-1	0.8	1	0	1	1	252	1
3	7	1	50	1	1.2	-1	0.4	1	1	252	-1
4	4.5	1	70	-1	1.2	1	0.4	-1	-1	252	1
5	4.5	-1	50	1	0.8	1	0.4	1	-1	252	-1
6	7	1	50	1	1.2	-1	0.4	1	-1	272	1
7	7	-1	50	-1	0.8	1	0	1	1	272	-1
8	7	-1	70	-1	1.2	-1	0	-1	-1	252	-1
9	7	-1	70	1	0.8	1	0.4	-1	1	272	1
10	4.5	-1	50	-1	1.2	-1	0	1	-1	272	1
11	4.5	1	70	1	1.2	1	0	-1	-1	272	-1
12	4.5	-1	70	1	0.8	-1	0.4	-1	1	272	1

deliberate variations in method parameters like flow rate, detector wave length and % organic phase. Sensitivity of the method was confirmed by calculating the limits of detection (LOD) and quantification (LOQ) on the basis of standard deviation of the response and the slope of the calibration curve. Tablets (marketed formulation) containing both MTX and DCP were analyzed to evaluate the method's reproducibility and % assay by calculating the % drug recovery and % coefficient of variance (%CV).

RESULTS AND DISCUSSION

The influence of addition of *Ion-Pair* reagents on the retention of the analytes

Based on their pKa values, MTX (pKa, 12.24) is characterized as a strongly basic compound, while DCP (pKa, 4.00) as an acidic compound. When such compounds undergo ionization, the charged species become much more polar or hydrophilic. Necessarily, their retention factor (*k'*) in reversed-phase column (RPC) can be reduced 10 times or more, and they are less retained. The main feature of the proposed *Ion-Pair* chromatographic separation is based on the fact that oppositely charged ions in the liquid phase tend to attract one another. The dielectric constant of the mobile phase and degree of solvation of individual ions govern the strength of this attraction.[35]

Selecting a mobile phase for separating both the drugs with distinct pKa values is challenging. Especially it is difficult to set the pH of the mobile phase for separation of the MTX due to one or more of the following reasons: (i) the elution of the protonated MTX may become closer to the void volume, or (ii) longer analysis time to suppress MTX in neutral form, and (iii) peak tailing as well. In order to suppress the MTX in its neutral form, the pH of the mobile phase must be adjusted two units above the MTX pKa, i.e. around pH 14.00; which is beyond the withstand capacity of the stationary phase materials.[36,37] These limitations can be conquered by substituting mobile phase pH, column, and organic modifier or by using "chaotropic agents (small inorganic liophilic ions such as BF_4^-, CF_3COO^-, ClO_4^-, and $H_2PO_4^-$ etc.)".[36,38,39] The pH of mobile phase not only affects the protonation of basic analytes but also the ionization of the acidic modifier. Potassium dihydrogen phosphate (acidic modifier) undergoes ionization in aqueous phase to release $H_2PO_4^-$ ions, which are liophilic in nature. The presence of such liophilic counteranions (chaotropic ions) in aqueous mobile phase either disrupt the MTX solvation shell thereby increasing its apparent hydrophobicity or involve in classic ion paring with the protonated MTX ion and increase its retention [Figure 1(A-B)]. Moreover, it is observed that retention of the protonated analyte is increased with increase of $H_2PO_4^-$ ion (acidic modifier) concentration or with decrease in pH.

Addition of ion pair reagents [ionic additives (amphiphilic ions)] to the mobile phase can affect the retention of ionizable analytes. *Ion-Pair* agents can be helpful for the retention of both acidic and basic analytes, whilst chaotropic ions are useful for improving the retention of only basic compounds. Selecting the suitable type of *Ion-Pair* agents (i.e., cationic and anionic) varies

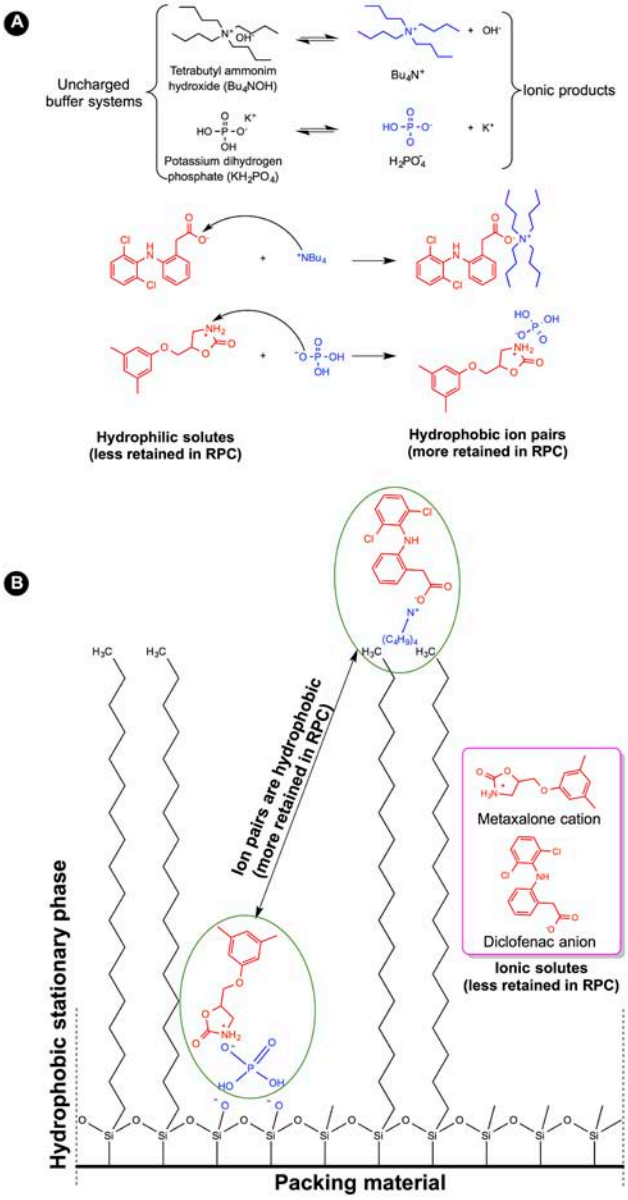

Figure: 1 (A) Formation of hydrophobic ion-pairs because of the interaction of *Ion-Pair* reagent (Bu_4NOH) and acid modifier (KH_2PO_4) with the ionised solutes to render them more retained in RPC. (B) Separation mechanism of the ion pair RP-HPLC.

with nature of the analyte. Effects of amphiphilic ions remain independent for liophilic ions (chaotropic ions) and vice versa. Since, DCP is an acidic compound, a cationic *Ion-Pair* reagent [tetrabutyl ammonium hydroxide (Bu_4NOH)] has been chosen. Upon ionization, the amphiphilic cations (Bu_4N^+) [typically possess a long alkyl chain and a charged group at one end] possess highly localized charges and gathered at the interface between the hydrophobic stationary phase and mobile phase. The orientation of such molecules at the interface is in such a fashion that the alkyl chain (hydrophobic part) is adsorbed on the alkyl chain of bonded phase (stationary phase) and the charged part of the molecule remains in the eluent. Such interaction formed a positively charged surface, which permits the pairing of the DCP anions [Figure1(A-B)]. Since, the chaotropicity of $H_2PO_4^-$ is weaker, retention of MTX cation is greatly influenced on the positively charged surface.[38,39]

Method optimization

Screening of potential factors

The investigated responses from the PBD screening design of the ion pair RP-HPLC method for selection of influential factors are summarized in Table 2. The represented chromatograms obtained from the 12 experiments are shown in Figure 2. In contrast to the calculated effects of all the factors, %TEA and wavelength were observed to have negligible influence on the responses. Therefore, pH, %ACN and flow rate were chosen as the most significant factors for further optimization of the chromatographic process.

Optimization using Box–Behnken design

BBD is employed to determine the critical conditions for optimizing the separation criteria of MTX and DCP in the chromatographic method. Three factors (pH, %ACN and flow rate) each at three levels were selected as input factors. The effects of these factors on the investigated responses were evaluated for the desirability of responses such as *k'*, Asf (MTX), *Rs* and t_R (DCP) and total 17 runs were executed in this study (Table 3). The selected chromatograms resulted from the BBD experimental trials were revealed in Figure 3.

Data analysis and interpretation

Interpretation of factorial effects by analysis of variance (ANOVA)

To identify significant effects of factor on various responses an analysis of variance Table was created. The *P*-value (probability) provides an indication of the significance of an effect. As the *P*-values for the factors are below the considered level of confidence α ($P<0.05$ to 0.1), the effects are considered to be statistically significant and valid for each of the responses. The effects of the factors on various responses and their *P* values are summarized in Table 4. The positive or negative sign of the effects indicates a synergistic or antagonistic influence respectively.

Evaluation of response sensitiveness by perturbation plots

Perturbation plots (Figure 4) were created to identify the sensitiveness of a particular response towards the effect of an independent factor while, remaining factors held constant at a reference point. A steepest slope or curvature states that the response is extremely susceptible to the specific factor. Figure 4 (b) demonstrates that tailing factor (MTX) was greatly influenced by % ACN. A raise in level of % ACN results in increased tailing factor. From Figure 4 (a, c, d), it is noticed that pH of the mobile phase has remarkable effect on capacity factor, resolution and total analysis time. In addition, it is notable that flow rate also have intermediate contribution to affect all the responses.

Formation and interpretation of 3D response surface plots

Three-dimensional (3D) response surface plots were formed to identify the significance of the polynomial model through graphical visual interpretation. These plots are valuable to asses; (i) the relationship of factors and their interactions with the responses, and (ii) the changes in response surface. Since, the number of factors is more than two, one of the factors was held constant for each plot.

3D plots [Figure 5] showing the interaction effects of (i): pH and %ACN on the responses (a), *k'*; (b), Asf (MTX); (c), *Rs* and (ii): %ACN and flow rate on (d) t_R (DCP). Since, the majority of the response surfaces formed large curvatures; it can be observed that all the factors do not have sole contribution towards the analytes' separation. Most of the responses vary in a curvilinear order attributable to the interaction of factors. Minimum 3D response curves were obtained for response *k'*, Rs and tR (DCP); while a maximum curve for Asf (MTX). Figure 5(a) exhibits increased *k'* at extreme levels [low (-) and high (+)] of buffer pH, while an apparently constant effect by all concentrations of ACN. In Figure 5(b) it was observed that Asf (MTX) varies in a non-linearly ascending order with increase in %ACN and buffer pH. With increasing ACN concentration, Rs varied in a non-linearly ascending fashion. Increased Rs was noticed at extreme levels of buffer pH alike *k'*. Total analysis time [t_R (DCP)] was evidenced to

Table 2: Results of the experiments of Plackett–Burman design.								
Runs	Responses*							
	N(MTX)	N(DCP)	k'	Asf(MTX)	Asf(DCP)	R_s	t_R(DCP)	RRT
1	112.203	2843.14	2.516	1.263	1.627	7.846	8.665	3.516
2	4064.89	5933.33	0.478	2.046	1.71	6.854	4.934	1.478
3	1855.86	3228.48	0.537	1.089	1.012	5.338	5.967	1.537
4	4152.41	4512.22	0.394	1.795	1.794	5.435	4.61	1.394
5	523.391	4845.71	0.531	1.497	1.704	4.042	8.591	1.531
6	3173.29	3684.5	0.515	1.454	1.04	6.024	6.01	1.515
7	1857.72	2644.64	0.924	1.967	1.88	7.62	8.637	1.924
8	1896.88	4138.47	0.493	1.947	1.843	5.339	3.301	1.493
9	3218.44	3943.88	0.322	2.532	2.704	4.159	4.913	1.322
10	2220.69	1837.85	0.693	1.279	0.913	5.71	10.054	1.693
11	5070.66	4835.73	0.336	2.171	1.689	5.057	6.064	1.336
12	5478.7	4300.06	0.396	1.909	1.261	5.684	6.853	1.396

***Abbreviations:** N(MTX), number of theoretical plates of metaxalone; N(DCP), number of theoretical plates of diclofenac potassium; *k'*, capacity factor of diclofenac potassium; Asf(MTX), tailing factor of metaxalone; Asf(DCP), tailing factor of diclofenac potassium; *Rs*, resolution between DCP and MTX; t_R(DCP), retention time of diclofenac potassium or total analysis time; RRT, relative retention time.

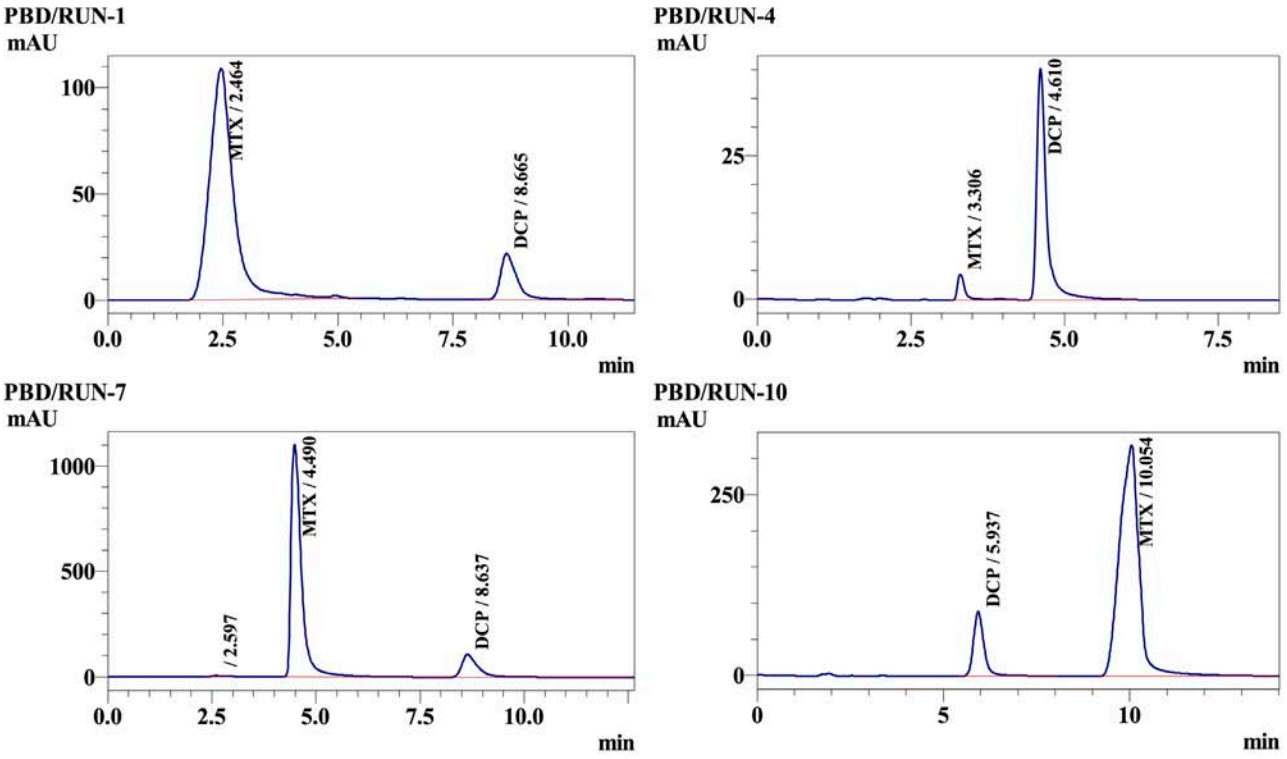

Figure 2: Chromatograms of the standard solution containing MTX and DCP under various method conditions as per Plackett-Burman design.

vary in a non-linearly descending manner with increasing flow rate and %ACN.

Optimization of the fitted model

Optimization of the model was accomplished based on the following desired separation criteria: (i) maximum capacity factor, (ii) good tailing factor, (iii) maximum resolution between the peaks, and (iv) Minimum analysis time. Five check point solutions obtained from the model were experimented and the observed values were compared with the predicted values to verify a close

Table 3: Box–Behnken experimental design of three variables and the observed responses.

Runs	Factors				Responses			
	pH	% ACN	Flow rate		k'	Asf(MTX)	R_s	t_R(DCP)
1	6.00	70.00	0.80		0.301	2.284	5.366	6.207
2	5.00	60.00	0.80		1.045	1.736	14.436	17.813
3	6.00	50.00	1.20		0.508	1.662	4.681	10.765
4	5.00	50.00	1.00		0.968	1.47	4.587	12.85
5	6.00	70.00	1.20		0.29	2.269	4.622	4.189
6	6.00	50.00	0.80		0.482	2.228	4.644	15.938
7	6.00	60.00	1.00		0.413	2.18	6.083	7.144
8	6.00	60.00	1.00		0.417	2.226	6.078	7.149
9	6.00	60.00	1.00		0.418	2.183	6.049	7.135
10	7.00	60.00	0.80		0.7	1.722	9.914	8.919
11	5.00	70.00	1.00		0.681	1.919	10.817	8.409
12	7.00	50.00	1.00		0.854	1.72	7.454	12.443
13	7.00	70.00	1.00		0.496	1.937	7.829	4.979
14	6.00	60.00	1.00		0.418	2.245	6.089	7.16
15	6.00	60.00	1.00		0.413	2.158	6.175	7.131
16	5.00	60.00	1.20		1.048	1.768	12.126	12.029
17	7.00	60.00	1.20		0.715	1.748	6.837	5.878

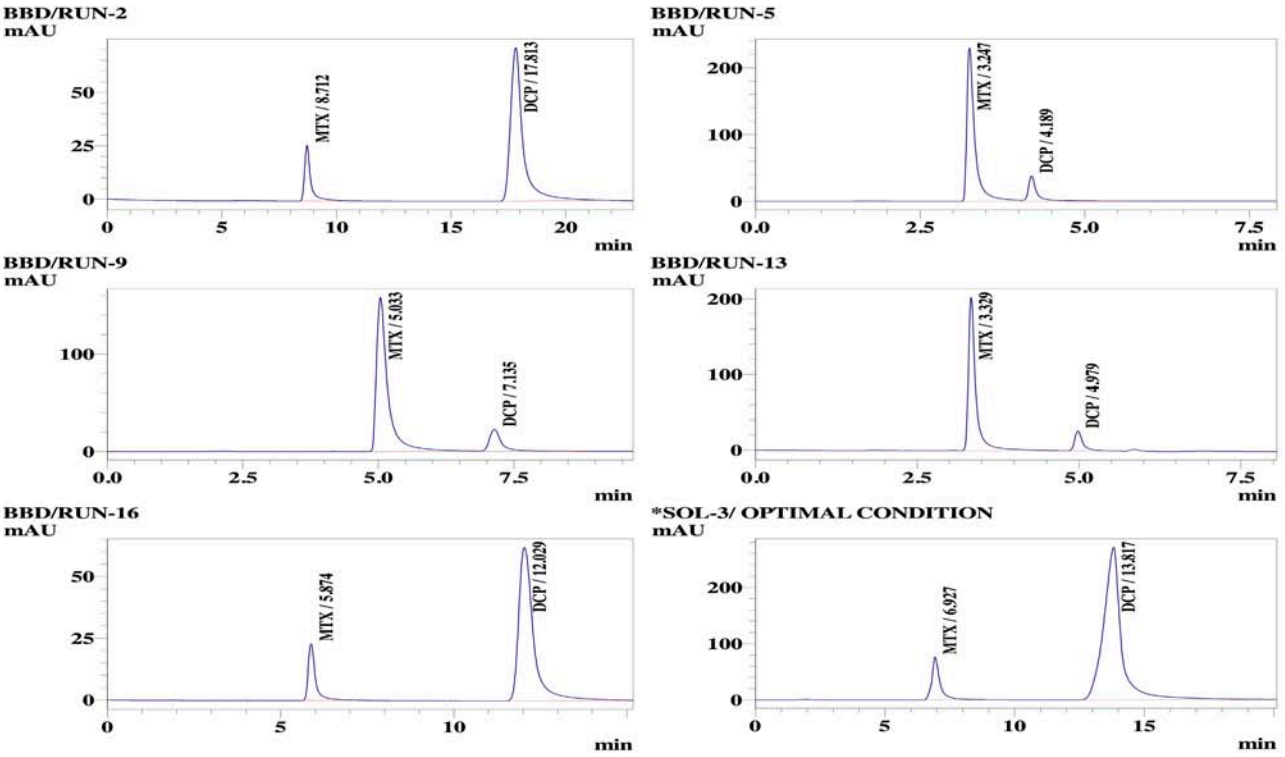

Figure 3: Chromatograms of the standard solution containing MTX and DCP under various method conditions as stated by the Box–Behnken design.

Table 4(a): Factorial effects on the different responses, (b) *P* values obtained for these effects.				
Factors	**(a) Effects on different responses**			
	k'	**Asf(MTX)**	R_s	t_R**(DCP)**
Model	+0.42	+2.20	+6.09	+7.14
A-pH	-0.12	+0.029	-1.24	-2.36
B-% ACN	-0.13	+0.17	+0.91	-3.53
C-Flow rate	+0.00413	-0.065	-0.76	-2.00
AB	-0.018	-0.058	-1.46	-0.76
AC	+0.003	-0.0015	-0.19	+0.69
BC	-0.00925	+0.14	-0.20	+0.79
A^2	+0.41	-0.40	+3.79	+2.21
B^2	-0.074	-0.035	-2.21	+0.32
C^2	+0.053	-0.053	+0.94	+1.81
	(b) *P* values obtained for these effects			
Model	*< 0.0001	*0.0016	**0.0266	*0.0039
A-pH	*0.0007	0.4276	**0.0699	*0.0045
B-% ACN	*0.0005	*0.0020	0.1618	*0.0005
C-Flow rate	0.8523	0.1019	0.2311	**0.0102
AB	0.5752	0.2763	0.1180	0.3829
AC	0.9237	0.9765	0.8221	0.4263
BC	0.7683	**0.0264	0.8189	0.3637
A^2	*< 0.0001	*< 0.0001	*0.0021	**0.0270
B^2	**0.0404	0.4907	**0.0280	0.6974
C^2	0.1129	0.3064	0.2765	**0.0560

**Significance at α=0.10 level, *Significance at α=0.05 level.

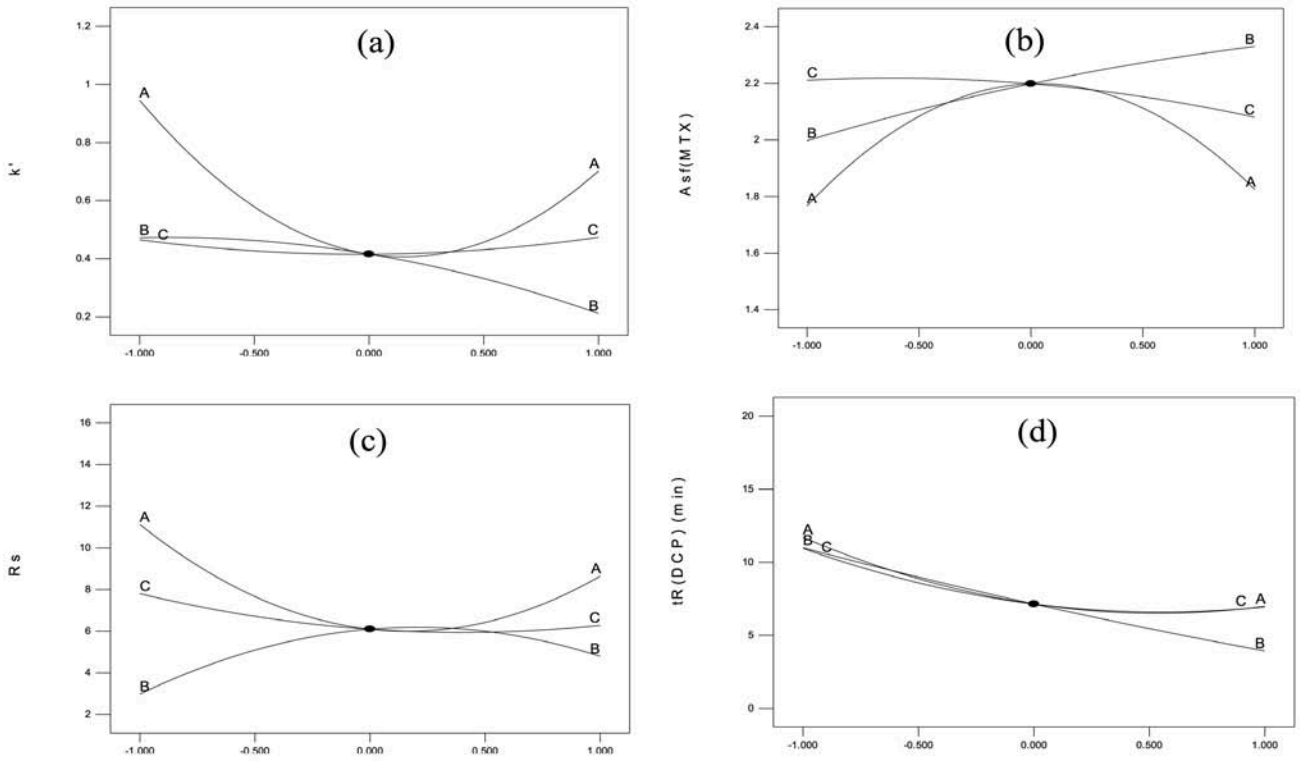

Figure 4: Perturbation plots showing the effects of pH (variable-A), % ACN (variable-B) and flow rate (variable-C) on the responses: (a) *k'*, (b) Asf(MTX), (c) R_s, and (d) t_R(DCP)

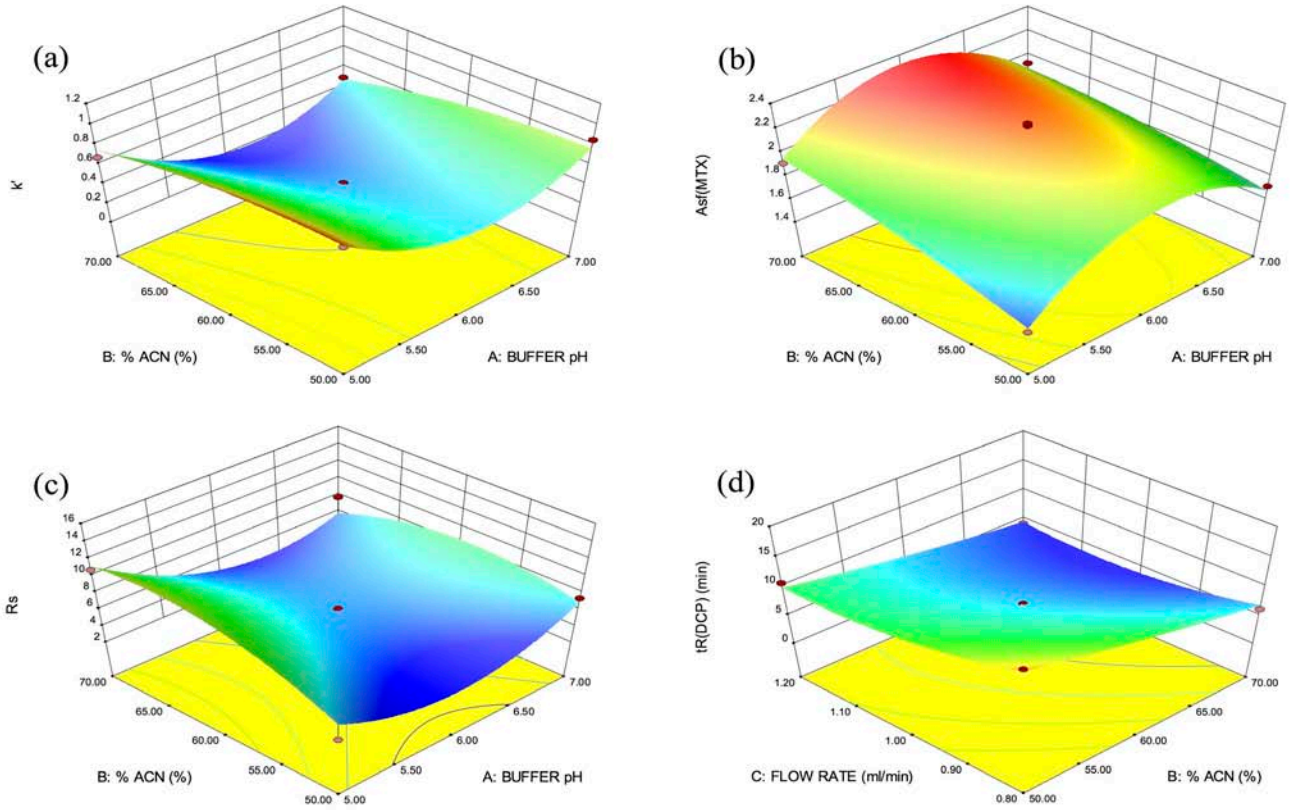

Figure 5: 3D response surface plots showing the interaction effects of (i): % ACN (variable-B) with buffer pH (variable-A) on the responses: (a), *k'*; (b), Asf(MTX); (c), R_s and (ii): %ACN (variable-B) and flow rate (variable-C) on (d), t_R(DCP).

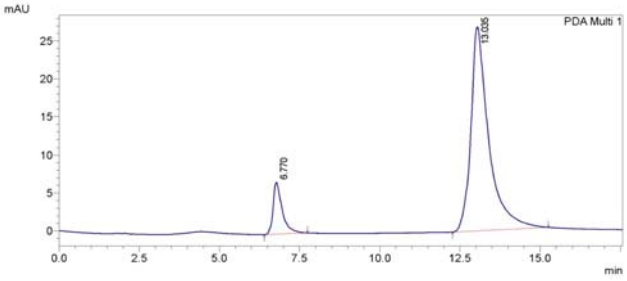

Figure 6: Chromatogram obtained for commercial formulation [t_R(MTX): 6.770min; and t_R(DCP): 13.035 min].

agreement between them. The predictability of the HPLC method was calculated by the following equation (4).

$$\text{Percentage prediction error (P.E.)} = \frac{\text{Observed} - \text{Predicted}}{\text{Predicted}} \times 100 \quad (4)$$

Mobile phase consisting of buffer (pH 5.00) and acetonitrile (42: 58 %v/v) at a flow rate of 1.20 mL/min was established as the optimal HPLC condition to achieve desired responses based on least P.E. value (*SOL-3 of Figure 3). At this condition, *k'* of 0.995, Asf (MTX) of 1.526, *Rs* of 8.511 and t_R (DCP) of 13.817 min were observed.

METHOD VALIDATION

The validation was executed by examining the linearity, intra and inter-day precision, recovery (accuracy), limit of detection (LOD) and limit of quantification (LOQ) of the method. When a series of dilutions were analyzed, the concentrations from 1 to 300µg/mL and 10 to 300µg/mL were found to be the linear range to construct the calibration curve for MTX and DCP respectively. The linear regression equations were y = 2122.4x - 6195.3; (R^2 = 0.9992) and y = 397.03x + 5333.7; (R^2 = 0.9905) for MTX and DCP respectively. Intra and inter-day precision (Table 5) of the method were studied over a period of 3 consecutive days using three different concentrations, i.e. 20, 100 and 250 µg/mL. Statistical evaluation revealed that %CVs of both drugs at different concentration levels were <1.281. During accuracy study good recoveries were obtained with the mean recovery of 99.933% (Table 5). The mean recovery from commercial tablet formulation was found to be >99.8% and the represented chromatogram is shown in Figure 6. LOD and LOQ for MTX, 0.306µg/mL and 0.927µg/mL; and for DCP, 0.807µg/mL and 2.445µg/mL were found respectively. The robustness study of the

Table 5: Intra and inter-day precision and accuracy of DCP and MTX.				
	Intra-day		Inter-day	
	MTX	DCP	MTX	DCP
Precision (n=6)				
Actual concentration (µg/mL)	Mean peak area mean ± SD; %RSD			
20	21254±17.058; 0.08	36409±12.767; 0.035	21241.67±9.073; 0.042	36417.67±5.507; 0.015
100	42209.67±31.659; 0.075	200058.3±94.796; 0.047	42153±64.645; 0.153	199533±469.277; 0.235
250	108398.3±693.341; 0.639	515691.7±5651.114; 1.096	106864.3±799.5; 0.748	512691.7±6570.091; 1.281
Accuracy (n=3)				
%Spiked formulation conc.	Mean percent recovery ± SD; %RSD			
80%	100.033±1.463; 1.462	99.866±0.225; 0.225	100.126±0.368; 0.367	99.988±0.078; 0.078
100%	100.005±0.027; 0.027	100.008±0.026; 0.026	99.636±0.490; 0.492	99.676±0.505; 0.506
120%	99.854±0.449; 0.449	99.737±0.2; 0.201	100.254±0.196; 0.195	100.014±0.365; 0.366

method didn't show any significant change when the critical parameters were deliberately modified. The tailing factor for both the drugs was always less than 2.0 and the components were well separated under all the altered conditions. Considering the modifications in the system suitability parameters and the specificity of the method, as well as carrying the experiment at room temperature may conclude that the method conditions were robust. The system suitability parameters of the method are found to be within the specified limits.

CONCLUSION

A chemometric-assisted ion pair RP-HPLC method for simultaneous determination of MTX and DCP was developed and validated in this study. The significant factors responsible for the chromatographic performance were identified and controlled with the aid of suitable experimental designs so as to achieve the optimal condition. The probable retention mechanism was stated and the use of Bu_4NOH as *Ion-Pair* reagent was justified. Addition of triethylamine could improve the peaks' shape, but was ineffective to govern the retention behavior of the ions. The method was found to be linear, precise, accurate, selective and robust. The method can be proposed for routine analysis of MTX and DCP in APIs (Active Pharmaceutical Ingredients), formulations and biologic and other matrices.

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CONFLICTS OF INTEREST

All the authors of the manuscript declare that there are no conflicts of interest.

ABBREVIATIONS

pH: pH of the buffer; **Dum (1-6):** dummy variables; **%ACN:** percentage acetonitrile in mobile phase; **flow rate:** flow of the mobile phase; **%TEA:** percentage triethylamine in the mobile phase as organic modifier; **wave length:** detection wavelength.

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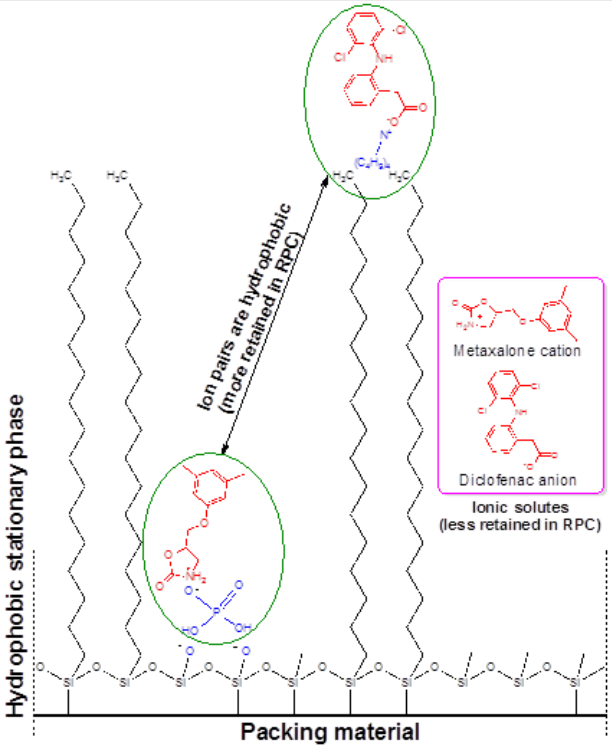
SUMMARY

- A first of its kind ion-pair RP-HPLC method for simultaneous determination of diclofenac and metaxalone in combined dosage has been developed.
- Both are hydrophilic ionic substances and troublesome to separate by RP-HPLC.
- Addition of ion-pairing reagent to the mobile phase markedly improved their retention and separation.
- Influential HPLC parameters have been screened and optimized to facilitate a rapid and selective determination of analytes with the aid of experimental designs.
- The probable retention behavior of both drugs was predicted in the ion interaction condition.

PICTORIAL ABSTRACT



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