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Optimization of Mobile Phase of High Performance Liquid Chromatography Using Full Factorial Design for Simultaneous Estimation of Thiocolchicoside and Dexketoprofen Trometamol in Tablets

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ABSTRACT

The present study was aimed to develop and validate a high performace liquid chromatography method for determination of thiocolchicoside and dexketoprofen trometamol in combined dosage form. The proposed HPLC method utilizes an Agilent Eclipse C-8 column (5 µm, 250×4.6 mm) at ambient temperature. A 2^3 full factorial design was performed for optimization of mobile phase for separation of two drugs. For the factorial design, the dependent variables chosen were % of acetonitrile, % of 0.1% o-phosphoric acid and pH. The optimum mobile phase consisted of acetonitrile: 0.1% o-phosphoric acid in water (41.9:58.1; pH 2.6). The flow rate was kept 1 mL/min with UV detection at 254 nm. The run time was found to be 1.41 min and 7.58 min for thiocolchicoside and dexketoprofen trometamol, respectively. The method was validated as per ICH guidelines. The calibration plots were linear in the range of 1-5 µg/mL with r^2=0.998 for thiocolchicoside and 6-30 µg/mL with r^2 = 0.995 for dexketoprofen trometamol. The % recovery values were found to be 98.52 ± 1.21 and 98.16 ± 1.45 for thiocolchicoside and dexketoprofen trometamol, respectively. The minimum detectable and minimum quantifiable amounts were found to be 0.11 and 0.35 µg/mL, respectively for thiocolchicoside and 1.41 and 4.38 µg/mL, respectively for dexketoprofen trometamol. The developed method could be utilized successfully in simultaneously analyzing the drugs in bulk as well as combined dosage form.

KEYWORDS: Thiocolchicoside; Dexketoprofen Trometamol; High Performance Liquid Chromatography; Full Factorial Design.

1. INTRODUCTION

Thiocolchicoside (TC) Fig. 1a, (2-Demethoxy-2-glucosidoxythiocolchicine) is a colchicin derivative, Competitive $GABA_A$ receptor antagonist and also inhibits glycine receptors [1-3]. It has muscle relaxant effect alongwith anti-inflammatory & analgesic properties [4-7]. Both drugs are official in Indian Pharmacopoeia (IP) 2010 [8-10]. Dexketoprofen trometamol (DKT) Fig. 1b, (amino-2-(hydroxymethyl) propane-1, 3-diol; 2-(3-benzoylphenyl) propanoic acid) is an NSAID propionic acid derivative. It causes reduction of prostaglandin synthesis by the inhibition of cyclooxygenase pathway. It is used in symptomatic treatment of pain of mild to moderate intensity, such as musculoskeletal pain, Dysmenorrhoea, Dental pain [11-12].

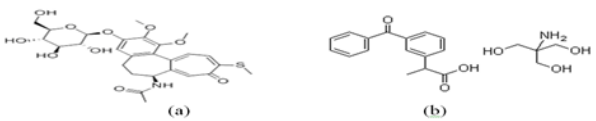

Fig. 1. Structure of (a) TC and (b) DKT.

The traditional approach for optimization of experiments is time consuming, involves a large number of runs and does not allow establishing the multiple interacting parameters. All above mentioned hurdles in developing robust method could be overcome by use of experimental design. When applying experimental design methodologies, it is advisable to keep the number of variables as low as possible in order to avoid very complex response models and large variability. However, if the number of influencing factors is up to four, full factorial design (FFD) should be used [13-16].

The literature review revealed that there are few HPLC, HPTLC and UV spectrophotometric methods available for estimation of TC either in combination or alone [17-37]. For estimation of DKT, few HPLC and UV spectrophotometry methods are also available either in combination with other drug or alone [38-42]. However, for estimation of combination of TC and DKT one UV spectrophotometry and one HPLC method are available which lacks any statastical

treatment of data [43-44]. Hence, it was thought to develop and optimize a mobile phase for separation of TC and DKT in their combined dosage form using a factorial design; a more scientific approach.

Factorial designs are constructed to obtain maximum information from the least amount of experimental runs. In general, factorial designs involve the study of two or more factors or variables, where each factor is assigned discrete values or levels and each possible factor-level variation is tested over multiple experimental trials or runs.

2. EXPERIMENTAL

2.1. Reagents and Standard

Standards for TC and DKT were obtained from Troikaa pharmaceuticals, Ahmedabad and Lincoln pharmaceuticals, Ahmedabad, respectively. Acetonitrile-HPLC grade (Merck Specialitites Pvt Ltd., Mumbai), Ortho-phosphoric acid (Fisher scientific India Pvt. Ltd, Mumbai) and HPLC grade water (Milli-Q, Q-Gard and Made in France). Tablets containing TC (4 mg) and DKT (25 mg) were purchased from local market.

2.2. Chromatographic Conditions and Instrumentation

The LC system consisted of HPLC (JASCO PU-2080) equipped with Intelligent HPLC pumps UV-2075, Intelligent UV / VIS Detector and processed by Borwin software. Separation was carried out on a Agilent Eclipse XDB C-8 column (150x4.60 mm i.d., 5µ particle size).

The mobile phase consisted of 0.1% Ortho-phosporic acid & acetonitile (42+65 v/v). The flow rate was 1 mL/min. UV detection was performed at 254 nm. The LC system was operated at room temperature (25 ± 0.5^{o}C). The injection volume was 20µL for all standards and samples.

2.3. Preparation of mobile Phase

0.1% Ortho-Phosphoric acid in water was prepared by dissolving 0.1 ml OPA in 100 ml water. A mobile phase was prepared by mixing 40 volumes of ACN, 60 volumes of water with 0.1% OPA was prepared and sonicated for 10 minutes. The mobile phase was filtered through nylon 0.45 µm membrane filter and was degassed for 20 min before use.

2.4. Preparation of standard solutions

10 mg of TC and 10 mg of DKT were weighed accurately, transferred in 10 ml volumetric flask separately, dissolved and diluted up to the mark with HPLC grade water to get final concentration of 1000 µg/mL. 1ml of above solution was taken and diluted to 10 ml with water (100 µg/mL).

From this working stock solution 1 mL was taken and diluted to 10 ml with mobile phase to get final concentration of 10 µg/mL for both drugs. The linear response of TC and DKT was determined by analyzing five independent levels in the range of 1-5 µg/ml (i.e.1, 2, 3, 4 and 5 µg/mL) and 6-30 µg/mL (i.e. 6, 12, 18, 24 and 30 µg/mL), respectively.

2.5. Preparation of test solution

Twenty tablets were weighed accurately and powdered. A tablet powder equivalent to 2 mg of TC and 125 mg of DKT was transferred into 10 ml volumetric flask and dissolved in sufficient amount of water (HPLC Grade). The solution was sonicated for 15 min. The solution was filtered through whatman filter paper No 41. From the above solution, 0.1 ml aliquot was taken and diluted to 10 ml with mobile phase and injected to HPLC.

2.6. Experimental design

An 8-run, 2^3 factorial design consisting of 3 factors at 2 levels was set up to standardize the chromatographic conditions which are likely to be employed in optimization of chromatographic method. Proportion of acetonitrile in organic phase (X_1), proportion of 0.1% OPA in water in mobile phase (X_2) and pH (X_3) were selected as 3 factors. By varying the proportion of X_1, X_2 and X_3 a total of 8 runs were performed. The higher and lower values of factors were selected as mentioned in Table 1. Evaluation of four different responses were considered. Retention time and asymmetry of TC were taken as responses 1(Y_1) and 3(Y_3). Retention time and asymmetry of DKT were taken as responses 2(Y_2) and 4 (Y_4).

Table 1. The variables and their coded values used in full factorial design (FFD)

Variables	Abbreviation	Coded variable levels	
		+1	-1
Proportion of acetonitrile	X_1	70	40
Proportion of 0.1% o-phosphoric acid ,% v/v	X_2	50	80
pH	X_3	2.5	4.0

2.7. Method validation

The linear response of TC and DKT was determined by analyzing five independent levels in the range of 1-5 µg/ml (i.e.1, 2, 3, 4 and 5 µg/ml) and 6-30 µg/ml (i.e. 6, 12, 18, 24 and 30 µg/ml), respectively. Each solution was analyzed in triplicate. An overlay chromatogram of linearity range was recorded. Peak area was recorded and calibration curve was constructed by plotting peak area versus concentration at 254 nm for both drugs.

Method precision was performed by analyzing test solution of TC and DKT (2 and 12.5 µg/ml, n=6) from tablet and analyzed using the proposed method. Variation of results within same day is called Intra-day precision and it was determined for test solution of TC and DKT (2 and 12.5 µg/mL, n=6) on the same day. Variation of results amongst different days called Inter-day precision. The Inter-day precision was determined for test solution of TC and DKT (2 and 12.5 µg/mL)

for three days. Specificity of the method was determined by comparing the spectra of placebo, standard and sample solution. Accuracy can be expressed as % recovery by the assay of known, added amount of standard of drugs. The recovery experiments were carried out in triplicate by spiking previously analyzed samples of the TC and DKT tablets (2 and 12.5 µg/ml, i.e.100%) with three different concentrations of standards at 80%, 100% and 120% respectively. The lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions; limit of detection (LOD) can be calculated using following equation:

$$LOD = 3.3 \times N/S$$

The lowest concentration of analyte in a sample that can be determined with the acceptable precision and accuracy under stated experimental conditions; limit of quantification (LOQ) was calculated using following equation:

$$LOQ = 10 \times N/S$$

In both equations N = Standard deviation of the peak areas of the drug and S = Slope of the corresponding calibration curve.

Robustness of the method was studied by making small changes in the mobile phase composition, pH and flow rate. System suitability parameters were also established during validation of method.

3. RESULTS AND DISCUSSIONS

Initially, overlay spectra of the both drugs was recorded in methanol (Fig. 2) using UV spectrophotometer and 254 nm was selected as the detection wavelength as both drugs showed good absorbance.

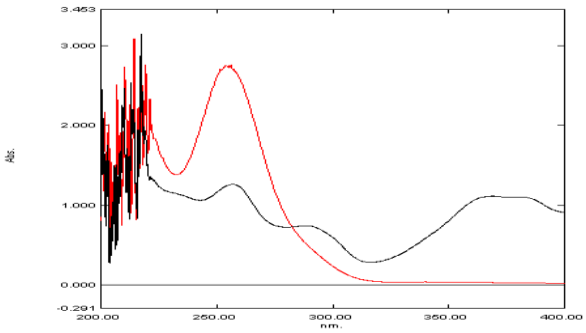

Fig. 2. Overlay spectra of TC and DKT.

3.1. Optimization of chromatographic conditions

The factors generally selected to optimize the chromatographic separation of ionizing compounds are pH, type of stationary phase and the content of organic solvent of the mobile phase. For the development of mobile phase for HPLC, several mobile phases composed of plain solvents such as acetonitrile, methanol, water followed by combination of organic solvents with buffers were tried. The results of trials with different mobile phase are as shown in Table 2. Ultimately mobile phase consisting acetonitrile and 0.1% v/v OPA showed reasonable separation of TC and DKT. Further to refine the composition of mobile phase along with change in pH, the full factorial design was used. For selection of suitable stationary phase, C-18 and C-8 columns were tried. When the trials were conducted using C-18 column it did not give good peak shape for DKT. Hence, C-8 column was used for separation of components which gave good peak shape for both drugs. To further refine the chromatographic conditions a full factorial design was employed.

Table 2. List of mobile phases tried

Sr. No.	Mobile phase composition	Results
1.	MeOH (100 %)	Both drugs were not separated
2.	Acetonitrile (100 %)	Both drugs were not separated
3.	Water (100 %)	Both drugs were not separated
4.	Acetonitrile : MeOH (50:50 v/v)	Both drugs were not separated
5.	MeOH : water(70:30 v/v)	Both drugs were not separated
6.	Acetonitrile : MeOH (30:70 v/v)	Both drugs were not separated
7.	Acetonitrile : MeOH : water (75: 10: 15)	Both drugs were not separated
8.	MeOH : Ammonium acetate buffer (90:10) pH 6.4	No separation
9.	MeOH : Ammonium acetate buffer (90: 10)	Broad peaks
10.	MeOH : Ammonium acetate buffer (70: 30) pH-3	Only one drug elutes
11.	MeOH : Ammonium acetate buffer (80: 20) pH-3	Broad asymmetric peaks with long retention time
12.	MeOH : Sodium acetate buffer (80:20) pH- 5	Asymmetric peaks
13.	MeOH : Sodium acetate buffer (80:20) pH- 4.3	Prolonged retention time and asymmetric peaks
14.	MeOH : Sodium acetate buffer (70:30) pH- 5	No separation
15.	MeOH : Sodium acetate buffer (60:40) pH- 4	Splitting of peaks and Second drug poorly resolved
16.	MeOH : Sodium acetate buffer (80:20) pH- 5	Splitting of peaks and Second drug poorly resolved
17.	MeOH : Sodium acetate buffer (70:30) pH- 4.5	Splitting of peak
18.	MeOH : Sodium acetate buffer (60:35) pH- 4.5	Only one peak
19.	MeOH: Diammonium hydrogen phosphate buffer (65:35) pH 3	Splitting of peak with poor separation
20.	Acetonitrile : 0.1% O-Phosphoric Acid (OPA) in water (60:40)	Only one peak with broadening of second peak
21.	Acetonitrile : 0.1% OPA in water (40:60) in C-18 column	Both peaks separated with significant asymmetry in second peak
22.	Acetonitrile : 0.1% OPA in water (40:60) on C-8 column	Separation of both drugs with total run time of less than 10 min.

Further to refine the separation conditions with respect to the change in pH of the mobile phase acetonitrile: 0.1% OPA in water. The upper and lower limits for acetonitrile were selected on following basis: The minimum amount of organic solvent required was 40% as seen during trials for desirable separation of both drugs. Hence, lower limit was kept at 40% and above 60% a broad peak was observed with tailing of peak of DKT. Hence, upper limit was kept at 70% of organic solvent in mobile phase. Similarly, the upper and lower limits for 0.1% OPA were selected to be 50%, as it is the least amount required for resolution of peaks for DKT and TC and 80% as above this value the run time for DKT was increased more than 10 min. As pH is an important factor which affects the separation of the molecules due to change in the ionization states of the molecule. For optimization of mobile phase it is advisable to set the pH of mobile phase pKa of drug ±2. The pKa values were 2.27 and 4.45 for TC and DKT, respectively. Hence, lower limit for pH was selected as 2.5 and upper limit was selected as 4 (see Table 3.).

Table 3. FFD matrix and results obtained

Run	Proportion of acetonitrile **(**X_1)	Proportion of 0.1% o-phosphoric acid ,% v/v (X_2)	pH **(**X_3)	Retention time of TC (Y_1)	Retention time of DKT (Y_2)	Asymmetry for TC (Y_3)	Asymmetry for DKT (Y_4)
1	70	50	4.0	1.37	4.91	1.21	1.07
2	40	50	4.0	1.48	5.94	1.36	1.19
3	70	80	2.5	1.41	6.94	1.24	1.12
4	40	80	4.0	1.56	11.95	1.46	1.35
5	70	50	2.5	1.29	3.93	1.12	1.05
6	40	50	2.5	1.34	5.21	1.22	1.06
7	70	80	4.0	1.53	8.67	1.29	1.16
8	40	80	2.5	1.44	10.96	1.31	1.19

Table 4. Summary of each factor and its p-value for response Y_1, Y_2, Y_3 and Y_4.

Response	Factor	Factor effect	*P-value*
Y_1	A	-0.028	0.0295
	B	+0.057	0.0023
Y_2	C	+0.058	0.0023
	A	-1.20	0.0016
	B	+2.32	0.0002
	C	+0.55	0.0144
Y_3	AB	-0.62	0.0103
	A	-0.061	0.0007
	B	+0.049	0.0014
	C	+0.054	0.0011
	AC	-0.019	0.0219
Y_4	A	-0.049	0.0168
	B	+0.056	0.0114
	C	+0.044	0.0226
	AC	-0.029	0.0650

Table 5. Validation of 2^3 full factorial design (FFD)

Variables	Values	Responses	Observed values	Predicted values	% Error
X_1	41.92	Y_1	1.41	1.40	-0.71
X_2	58.08	Y_2	7.59	7.94	+4.61
X_3	2.63	Y_3	1.31	1.27	-3.06
		Y_4	1.08	1.13	+4.62

Using the above experimental design (shown in Tables 4 and 5), the effects of all three factors were determined on all four responses. Individual contour plot and 3D graphs for all the four responses are shown below

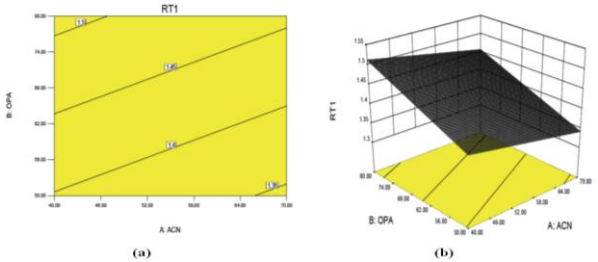

Fig. 3. (a): Contour plot of effect of ACN and OPA on retention time of TC (b) : 3D graph of effect of ACN and OPA on retention time of TC. (Y_1)

Fig. 3 (a) and 3 (b) shows the response surface plots (contour plot and 3D surface plot, respectively) of the effect of ACN (X_1) and OPA (X_2) on retention time of TC. It is clear that the retention increases as the concentration of OPA (buffer) increases as buffers have a pronounced effect on separation of peaks and retention time decreases with increase in ACN as organic phase ionizes the drug faster.

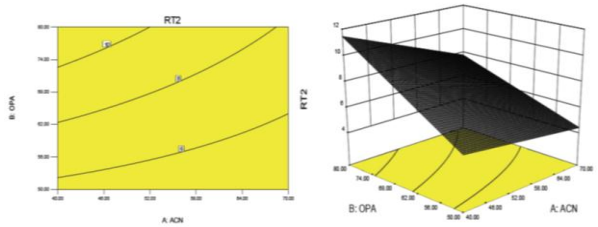

Fig. 4. (a) : Contour plot of effect of ACN and OPA on retention time of DKT. (b) : 3D graph of effect of ACN and OPA on retention time of DKT (Y_2).

Fig. 4 (a) and 4 (b) shows the response surface plots (contour plot and 3D surface plot, respectively) of the effect of ACN (X_1) and OPA (X_2) on retention time of DKT. It is clear that the retention increases as the concentration of OPA (buffer) increases as buffers have a pronounced effect on separation of peaks and retention time decreases with increase in ACN as organic phase ionizes the drug faster.

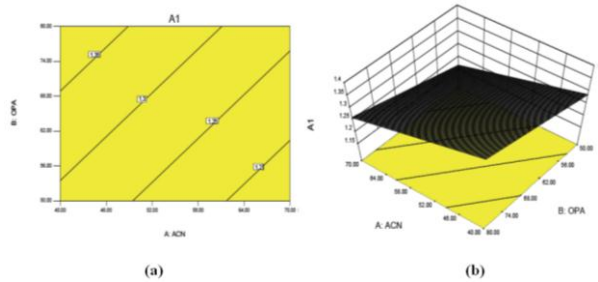

Fig. 5. (a) : Contour plot of effect of ACN and OPA on asymmetry of TC. (b) : 3D graph of effect of ACN and OPA on asymmetry of TC (Y_3).

Fig. 5 (a) and 5 (b) shows the response surface plots (contour plot and 3D surface plot, respectively) of the effect of ACN (X_1) and OPA (X_2) on asymmetry of thiocolchicoside. It is clear that asymmetry increases as the concentration of OPA (buffer) increases as buffers have a pronounced effect on separation of peaks and asymmetry decreases with increase in ACN as organic phase ionizes the drug faster and gives a sharp peak.

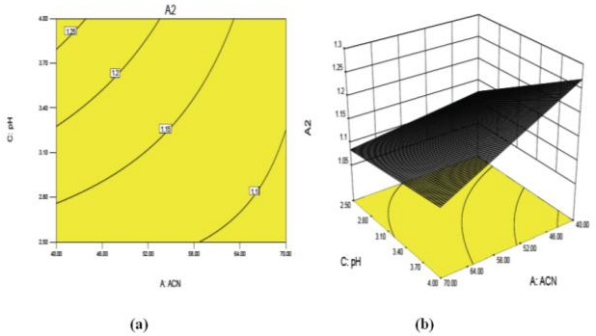

Fig. 6. (a): Contour plot of effect of ACN and OPA on asymmetry of DKT. (b): 3D graph of effect of ACN and OPA on asymmetry of DKT (Y_4).

Fig. 6 (a) and 6 (b) shows the response surface plots (contour plot and 3D surface plot, respectively) of the effect of ACN (X_1) and OPA (X_2) on asymmetry of DKT. It is clear that the asymmetry increases as the concentration of OPA (buffer) increases as buffers have a pronounced effect on separation of peaks and asymmetry decreases with increase in ACN as organic phase ionizes the drug faster.

3.2. Desirability Plot of Mobile Phase

Fig. 7 shows a desirability plot of 2^3 FFD. The red area indicates the desired value of all four responses affected by ACN (X_1), OPA (X_2) and pH (X_3).

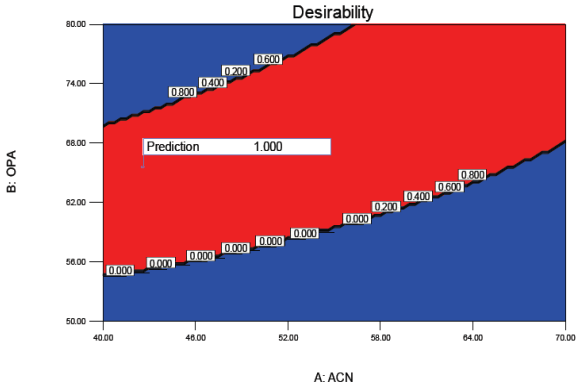

Fig. 7. Desirability graph.

3.3. Overlay Plot of Responses

Fig. 8 indicates the overlay plot which comprises of contour plot from each response laid on top of each other. From this it can be concluded that the optimized mobile phase ratio of ACN: 0.1% OPA in water (~ 42:65) at pH 2.63 will show all the desirable values of four responses. Thus the above mobile phase was selected for further studies and method validation.

Table 6. Validation parameters

Sr. No.	Parameters	TC	DKT
1	Linearity Range (µg/mL)	1-5	6-30
2	Correlation Co-efficient (R^2)	0.998	0.995
3	Precision (% RSD):		
	Intraday precision (n=6)	0.67	1.43
	Interday precision (n=3)	0.46	0.96
4	% Recovery	99.94%	100.3%
5	Limit of Detection (LOD) (µg/mL)	0.11	1.41
6	Limit of Quantification (LOQ) (µg/mL)	0.35	4.28

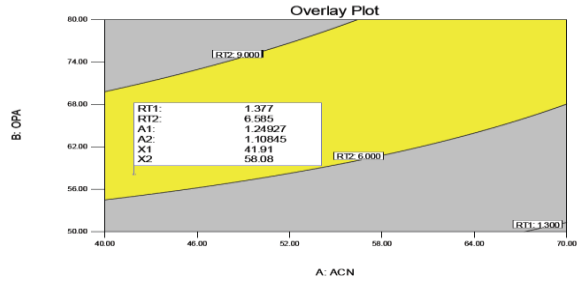

Fig. 8. overlay plot of all responses.

The selected mobile phase was used and the chosen full factorial design model was validated as shown in Table 5.The chosen mobile phase was run through HPLC system and the responses values were recorded and compared with the values predicted by software. The % error for the obtained results were calculated and was found to be less than 10% suggesting that the developed model is best fit for the separation of these two drugs.

3.4. Validation

Linear correlation was obtained between absorbances and concentrations of TC and DKT in the concentration ranges of 1-5 µg/mL and 6-30 µg/mL, respectively. The linearity of the calibration curve was validated by the high values of correlation coefficient of regression. The low values of relative standard deviation (less than 2 %) indicate that the proposed method is repeatable and precise. LOD and LOQ data show that proposed method is sensitive for the determination of TC and DKT. (Table 6)

3.5. System suitability parameters

The parameters calculated for system suitability are as under:

Table 7. System suitability parameters.

Parameters	TC	DKT
Retention time (min.)	1.41	7.59
Theoretical plates	1834.74	11782.04
Tailing factor	1.40	1.08
Resolution	15.08	

3.6. Application of proposed method to the pharmaceutical dosage form

No interference of the excipients with the peaks of interest appeared; hence the proposed method is applicable for the routine estimation of TC and DKT in combined pharmaceutical dosage forms. Results obtained are shown in Table 8.

Table 8. Assay of pharmaceutical dosage form by HPLC method

Formulation	Drug	Amt. Taken (µg/ml)	Amt. Found (µg/ml)	%Amt found (n=3)
Tablet	TC	2	1.97	98.5
	DKT	12.5	12.2	98.4

4. CONCLUSIONS

The HPLC method developed with the aid of All these factors lead to the conclusion that the proposed method is accurate, precise, simple, sensitive, selective and robust and can be applied successfully for the estimation of TC and DKT in bulk drug and in tablet without inference.

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