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A novel validated RP-HPLC-DAD method for the simultaneous estimation of Phenylephrine and Ketorolac in bulk and pharmaceutical dosage form with forced degradation studies

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Abstract : A novel approach was used todevelop and validate a rapid isocratic Reversed Phase-High Performance Liquid Chromatographic method for the simultaneous estimation of Phenylephrine and Ketorolac in bulk and pharmaceutical dosage form with forced degradation studies. The separation was performed by BDS C_{18} (150mm×4.6 mm, 5µm particle size) column, Waters Alliance e2695 HPLC system with 2998 PDA detector and mobile phase contained a mixture of 0.01M Ammonium acetate (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile (30:70, v/v). The flow rate was set to 1ml/min with responses measured at 259nm. The retention time of Phenylephrine and Ketorolac was 2.291min and 3.827min respectively with resolution of 11.11. Linearity was established in the range of 20-120µg/ml for Phenylephrine and 6-36µg/ml for Ketorolac with correlation coefficients (r^2=0.999). The percentage recoveries were between (100.30-101.03%) and (99.93-100.65%) for Phenylephrine and Ketorolac respectively. Validation parameters were evaluated according to the International Conference on Harmonization (ICH) Q2 R1 guidelines. The forced degradation studies were performed by using HCl, NaOH, H_2O_2, thermal, UV radiation and water. Phenylephrine and Ketorolac are more sensitive towards alkaline hydrolysis degradation condition. The developed method was successfully applied for the quantification and hyphenated instrumental analysis.
Key words: Phenylephrine, Ketorolac, PDA detector, Hyphenated, ICH.

Introduction

Phenylephrine and Ketorolac combined dosage form is used to prevent intraoperative miosis and reduce postoperative ocular pain. Phenylephrine is a selective α_1-adrenergic receptor agonist of the phenethylamine class used primarily as a decongestant, as an agent to dilate the pupil, and to increase blood pressure[1]. Phenylephrine is chemically known as (R)-3-[-1-hydroxy-2-(methylamino)ethyl] phenol were shown in (Figure 1). Ketorolac is a non-steroidal anti-inflammatory drug (NSAID) in the family of heterocyclic acetic acid derivatives, used as an analgesic and a non-selective COX inhibitor. The primary mechanism of action is anti-inflammatory, antipyretic and analgesic effects by the inhibition of

prostaglandin synthesis by competitive blocking of the enzyme cyclooxygenase (COX)[2]. Ketorolac is chemically known as 5-benzoyl-2, 3-dihydro-1H-pyrrolizine-1-carboxylic acid was shown in (Figure 2). Literature survey reveals that many analytical methods are reported for determination of Phenylephrine and Ketorolac individually and with other combinations which include ultra performance liquid chromatography (UPLC)[3], high performance liquid chromatography (HPLC)[4-23] and UV-Spectrophotometry[24,25] methods. However, no method is reported for simultaneous estimation of Phenylephrine and Ketorolac in combined dosage form by reverse phase HPLC with forced degradation studies. The present study was aimed to develop a novel and validated method for the simultaneous estimation of Phenylephrine and Ketorolac in bulk and pharmaceutical dosage form with forced degradation studies according to ICH guidelines[26].

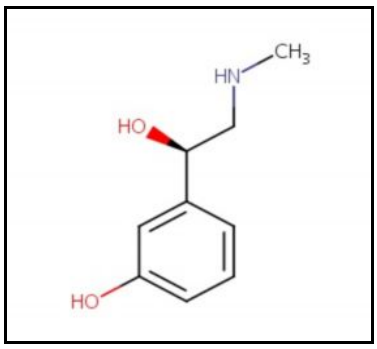


Figure 1: Chemical structure of Phenylephrine

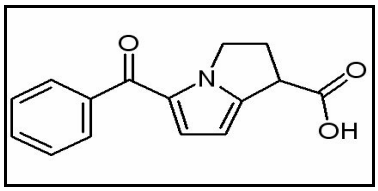


Figure 2: Chemical structure of Ketorolac

Materials and methods

Chemicals and reagents

Phenylephrine (API) was obtained from Spansules Pharmatech Pvt. Ltd., Hyderabad, India and Ketorolac (API) was obtained from Fugen Laboratories, Hyderabad, India. HPLC grade of Ammonium Acetate was obtained from Rankem Ltd., India and HPLC grade of Acetonitrile was obtained from Merck Specialities Private Limited, India. HPLC grade of Water and Ortho phosphoric acid was obtained from Rankem Ltd., India. Omidria (Phenylephrine and Ketorolac injection, 1% / 0.3%) contains Phenylephrine 40mg and Ketorolac 12mg per 4ml vial were kindly supplied by Omeros Pharmaceuticals, Inc.

Instrumentation

The analysis was performed by using a chromatographic system from Waters Alliance e2695 HPLC system with 2998 PDA detector. The HPLC system was equipped with Empower 2 software. Semi-micro analytical balance (India), Ultrasonic bath sonicator (Frontline FS 4, Mumbai, India), Digital pH meter (Systronics model 802) and Whatmann filter paper No. 41 (Whatmann International Ltd., England) were used in the study.

Chromatographic conditions

Phenylephrine and Ketorolac was analyzed in BDS C_{18} (150mm×4.6mm, 5µm particle size) column for the chromatographic separation. The mobile phase was composed of 0.01M Ammonium acetate (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile (30:70, v/v). Filtered through 0.45µm nylon membrane filter under vacuum filtration and pumped at ambient temperature, at a flow rate of 1 ml/min with

UV detection wavelength at 259nm. Injection volume was 20µl. The run time was 8 min and the retention time of Phenylephrine and Ketorolac was found to be 2.291min and 3.827min respectively with resolution of 11.11.

Chromatographic Parameters:

Equipment	: Waters Alliance e2695 HPLC system with 2998 PDA detector
Column	: BDS C_{18} column (150mm×4.6 mm, 5µm particle size)
Flow rate	: 1ml/min
Wavelength	: 259 nm
Injection volume	: 20 µl
Column oven	: Ambient
Run time	: 8 Minutes

Solutions and sample preparation

Preparation of Ammonium acetate buffer

A 0.01M Ammonium acetate buffer was prepared by dissolving 0.77gm of Ammonium acetate in 1000ml of HPLC grade water and pH was adjusted to 3.5 with orthophosphoric acid. The buffer was filtered through 0.45µm nylon membrane filter to remove all fine particles and gases.

Preparation of mobile phase

The above prepared 0.01M Ammonium acetate buffer and Acetonitrile HPLC grade were mixed in the proportion of 30:70, v/v and was filtered through 0.45µm nylon membrane filter and degassed by sonication.

Preparation of diluent

Mobile phase was used as diluent.

Preparation of standard stock solutions of Phenylephrine and Ketorolac

Standard stock solutions of Phenylephrine and Ketorolac were prepared by dissolving 40mg of Phenylephrine (API) and 12mg of Ketorolac (API) in 100ml of diluent into a 100ml clean dry volumetric flask and the standard solutions was filtered through 0.45 µm nylon membrane filter and degassed by sonicator to get the concentration of 400µg/ml of Phenylephrine and 120µg/ml of Ketorolac.

Preparation of standard solutions of Phenylephrine and Ketorolac for assay

From the above standard stock solution of 400µg/ml of Phenylephrine and 120µg/ml of Ketorolac further pipette 2ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac.

Preparation of sample solutions of Phenylephrine and Ketorolac

Omidria (Phenylephrine and Ketorolac injection, 1% / 0.3%) contains equivalent amount of Phenylephrine 40mg and Ketorolac 12mg per 4ml vial were taken into 100ml clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same diluent. Further pipette out 2ml from the above Phenylephrine and Ketorolac sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac. 20µl from standard and sample solution were injected into the chromatographic system and the peak areas were measured for Phenylephrine and Ketorolac which was shown in (Figure 6 and 7) and the % assay was calculated by comparing the peak area of standard and sample chromatogram by using the formula given below and the assay results was shown in Table 1.

Table 1: Assay of marketed formulation of Phenylephrine and Ketorolac.

Drug	Omidria (injection, 1% / 0.3%) Label Claim (mg)	Amount Found (mg) (n=6)	% Label Claim ± % RSD (n=6)
Phenylephrine	40	39.73	99.33±0.29
Ketorolac	12	12.003	100.03±0.47

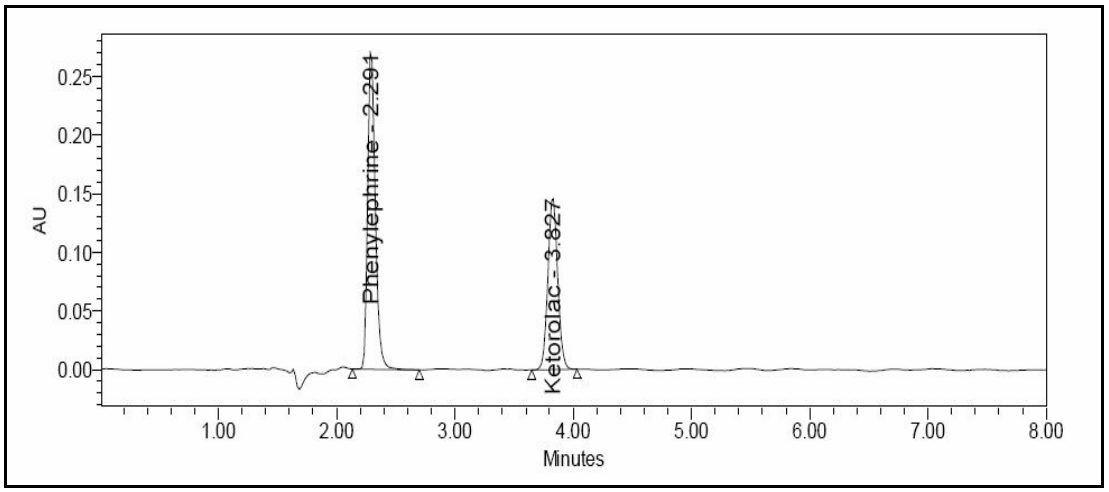


Figure 6: Standard chromatogram of Phenylephrine and Ketorolac

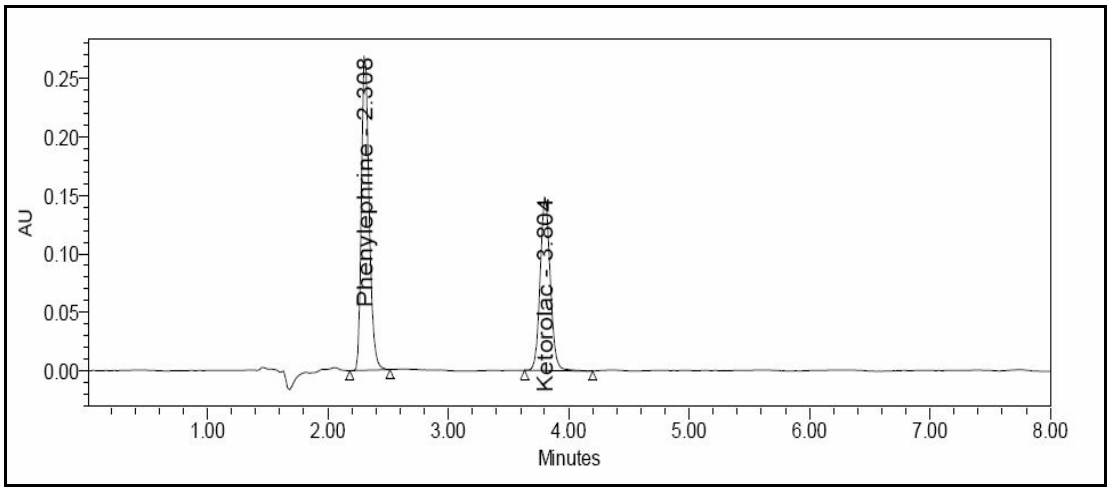


Figure 7: Sample chromatogram of Phenylephrine and Ketorolac

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = Average peak area of sample preparation
AS= Average peak area of standard preparation
WS = Weight of standard taken in mg
WT=Weight of sample taken in mg
P = Percentage purity of working standard
DS= Dilution factor for standard preparation
DT=Dilution factor for sample preparation

Selection of wavelength

In simultaneous estimation of Phenylephrine and Ketorolac isosbestic wavelength is used. Standard stock solutions of Phenylephrine and Ketorolac were prepared by dissolving 40mg of Phenylephrine and 12mg of Ketorolac in 100ml of diluent into a 100ml clean dry volumetric flask and the standard solutions was filtered through 0.45µm nylon membrane filter and degassed by sonicator to get the concentration of 400µg/ml of Phenylephrine and 120µg/ml of Ketorolac. From the above standard stock solution of 400µg/ml of Phenylephrine and 120µg/ml of Ketorolac further pipette 2ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac. The wavelength of maximum absorption (λmax) of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against mobile phase as blank. The isosbestic wavelength (λmax) was found to be 259nm for the combination shown in (Figure 3).

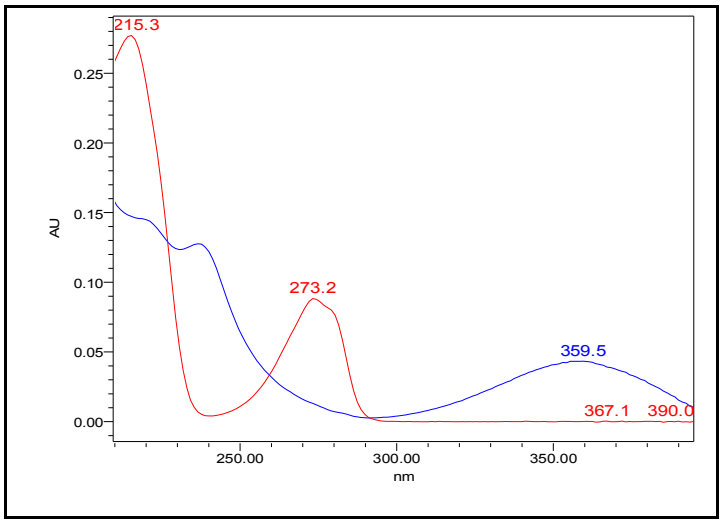


Figure 3: Isosbestic point of Phenylephrine and Ketorolac at 259nm

Results and discussion

Method Development

To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Phenylephrine and Ketorolac were obtained with a mobile phase containing a mixture of 0.01M Ammonium acetate (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile (30:70, v/v) was delivered at a flow rate of 1ml/min to get better reproducibility and repeatability. Quantification was achieved with PDA detection at 259nm based on peak area. The retention time of Phenylephrine and Ketorolac was found to be 2.291min and 3.827min respectively with resolution of 11.11. Linearity was established for Phenylephrine and Ketorolac in the range of 20-120µg/ml for Phenylephrine and 6-36µg/ml for Ketorolac with correlation coefficients (r^2=0.999) and the percentage recoveries were between 100.30-101.03% and 99.93-100.65% for Phenylephrine and Ketorolac respectively, which indicate accuracy of the proposed method. The % RSD values of accuracy for Phenylephrine and Ketorolac were found to be < 2 %. The % RSD values of method precision are 0.29% and 0.47% for Phenylephrine and Ketorolac respectively and % RSD values of system precision are 0.98% and 0.37% for Phenylephrine and Ketorolac. The % RSD values of reproducibility are 0.33% and 0.22% for Phenylephrine and Ketorolac respectively, reveal that the proposed method is precise. LOD values for Phenylephrine and Ketorolac were found to be 0.03µg/ml and 0.005µg/ml respectively and LOQ values for Phenylephrine and Ketorolac were found to be 0.09µg/ml and 0.015µg/ml respectively. The % RSD values of robustness studies were found to be < 2% reveal that the method is robust enough. These data show that the proposed method is specific and sensitive for the determination of Phenylephrine and Ketorolac.

Method validation

The developed method for the simultaneous estimation of Phenylephrine and Ketorolac was validated as per the ICH guidelines for the parameters like system suitability, specificity, linearity, accuracy, precision, ruggedness, robustness, limit of detection (LOD) and limit of quantitation (LOQ) [26].

System suitability

At first the HPLC system was optimized as per the chromatographic conditions. One blank followed by six replicates of a single calibration standard solution of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac was injected to check the system suitability. To ascertain the system suitability for the proposed method, the parameters such as retention time, theoretical plates, peak asymmetry and resolution were taken and results were presented in Table 2.

Table 2: System suitability parameters for Phenylephrine and Ketorolac.

Parameter (n=6)	Phenylephrine	Ketorolac
Retention Time (Mins)	2.291	3.827
Theoretical plates	6386	10047
Tailing factor	1.26	1.07
Resolution		11.11

Specificity

The effect of excipients and other additives usually present in the combined dosage form of Phenylephrine and Ketorolac in the determination under optimum conditions was investigated. The specificity of the RP-HPLC method was established by injecting the blank and placebo solution into the HPLC system. The representative chromatogram of blank and placebo was shown in (Figure 4 and 5).

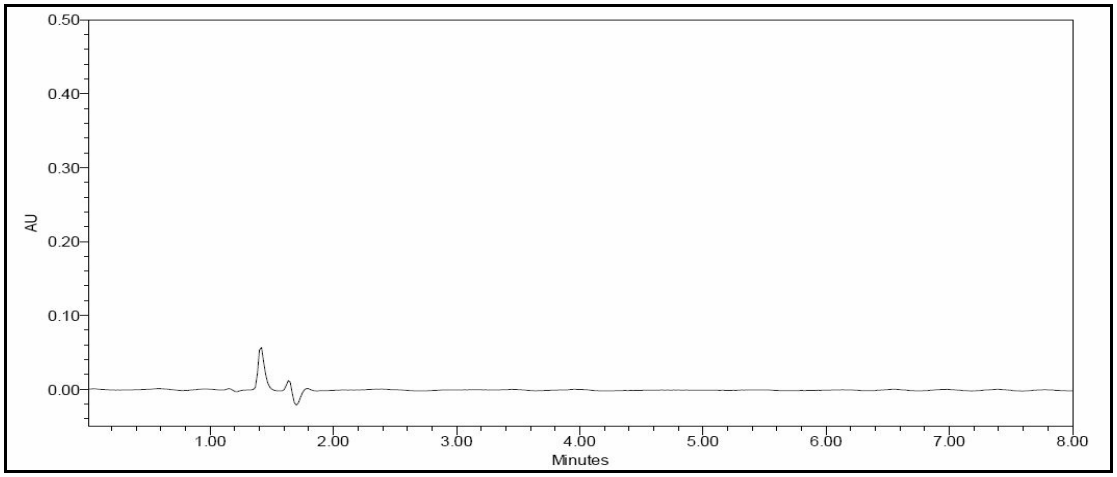


Figure 4: Chromatogram of blank

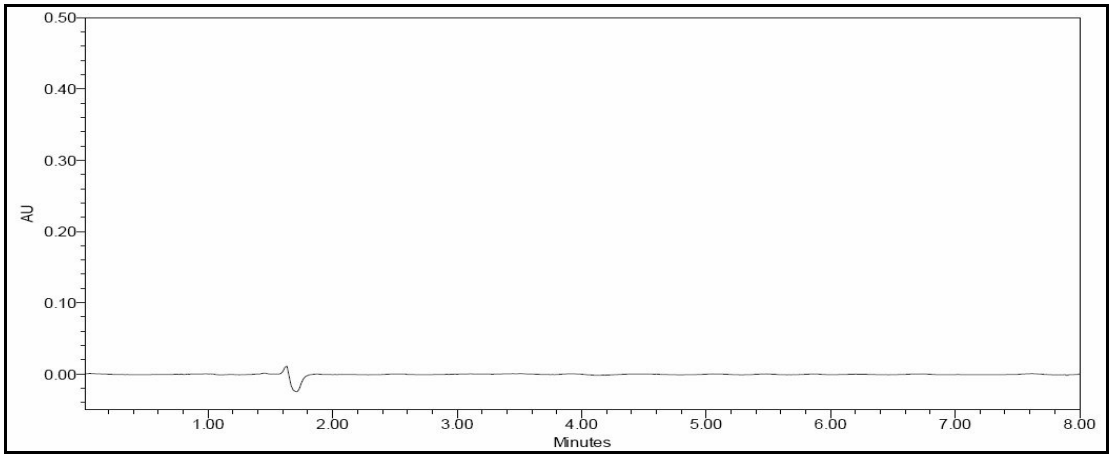
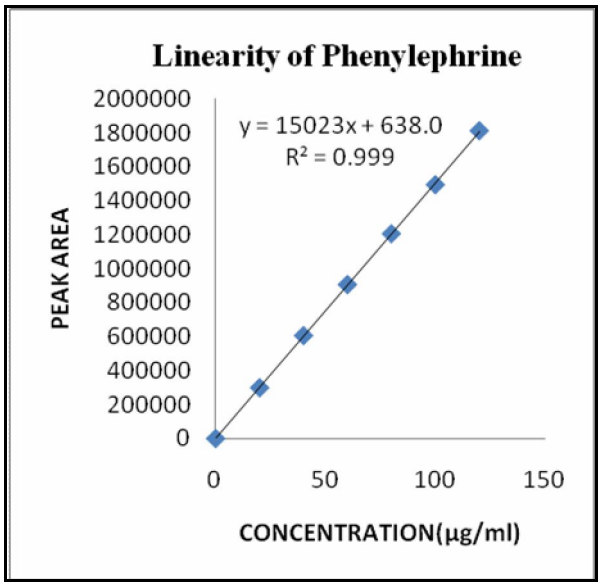


Figure 5: Chromatogram of placebo

Linearity and range for Phenylephrine and Ketorolac

Aliquots of 0.5, 1, 1.5, 2, 2.5 and 3ml of mixed standard working solutions of Phenylephrine and Ketorolac was pipette out from the standard stock solution of 400µg/ml of Phenylephrine and 120µg/ml of Ketorolac and transferred into a series of 10ml clean dry volumetric flask and make volume up to the mark with the same diluent to get the concentration of 20, 40, 60, 80, 100 and 120µg/ml of Phenylephrine and 6, 12, 18, 24, 30 and 36µg/ml of Ketorolac. The calibration standard solutions of Phenylephrine and Ketorolac were injected using a 20µl Hamilton Rheodyne injector and the chromatograms were recorded at 259nm and a calibration graph was obtained by plotting peak area versus concentration of Phenylephrine and Ketorolac respectively. The linearity data is presented in (Figure 8 and 9) and Table 3.



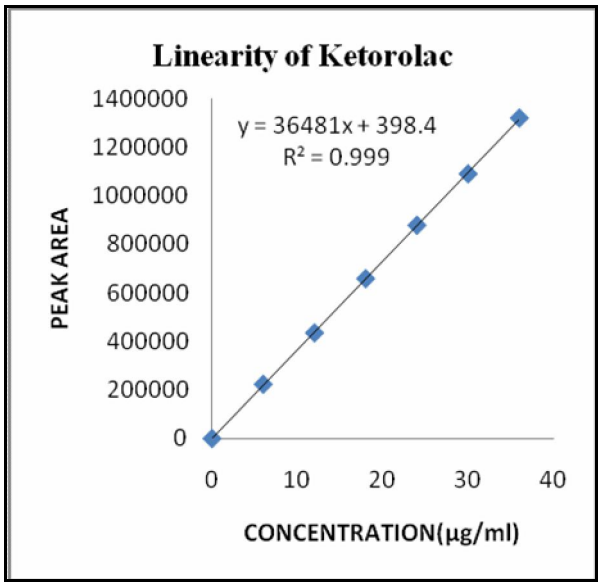


Figure 8: Linearity graph of Phenylephrine **Figure 9: Linearity graph of Ketorolac**

Acceptance Criteria: Correlation coefficient should be not less than 0.999

Table 3: Linearity data for Phenylephrine and Ketorolac.

Linearity of Phenylephrine		Linearity of Ketorolac	
Concentration (µg/ml)	**Peak Area**	**Concentration (µg/ml)**	**Peak Area**
20	298866	6	223020
40	605148	12	434494
60	904930	18	657547
80	1203706	24	877408
100	1492196	30	1088779
120	1809173	36	1318108

Table 4: Recovery study data of Phenylephrine and Ketorolac.

Recovery study data of Phenylephrine				
Sample name	Amount added (µg/ml)	Amount found (µg/ml)	%Recovery	Statistical Analysis
S_1:50%	40	40.73	101.83	Mean=101.03%(n=3) S.D=0.80 %RSD=0.79
S_2:50%	40	40.41	101.03	
S_3:50%	40	40.09	100.23	
S_4:100%	80	81.22	101.53	Mean=100.68%(n=3) S.D=0.76 %RSD=0.75
S_5:100%	80	80.06	100.08	
S_6:100%	80	80.34	100.43	
S_7:150%	120	119.64	99.70	Mean=100.30%(n=3) S.D=0.54 %RSD=0.54
S_8:150%	120	120.51	100.43	
S_9 :150%	120	120.9	100.75	
Recovery study data of Ketorolac				
Sample name	Amount added (µg/ml)	Amount found (µg/ml)	%Recovery	Statistical Analysis
S_1:50%	12	11.99	99.92	Mean=100.06%(n=3) S.D=0.24 %RSD=0.24
S_2:50%	12	12.04	100.33	
S_3:50%	12	11.99	99.92	
S_4:100%	24	24.01	100.04	Mean=100.65%(n=3) S.D=0.65 %RSD=0.64
S_5:100%	24	24.14	100.58	
S_6:100%	24	24.32	101.33	
S_7:150%	36	35.96	99.89	Mean=99.93%(n=3) S.D=0.17 %RSD=0.17
S_8:150%	36	36.04	100.11	
S_9 :150%	36	35.92	99.78	

Accuracy studies for Phenylephrine and Ketorolac

The accuracy of the method was determined by calculating recovery of Phenylephrine and Ketorolac by the method of standard addition. Known amount of standard solution of Phenylephrine and Ketorolac at 50%, 100% and 150% was added to a pre quantified sample solution and injected into the HPLC system. The mean percentage recovery of Phenylephrine and Ketorolac at each level was calculated and the results were presented in Table 4.

Preparation of pre quantified sample solution for accuracy studies

Omidria (Phenylephrine and Ketorolac injection, 1% / 0.3%) contains equivalent amount of Phenylephrine 40mg and Ketorolac 12mg per 4ml vial were taken into 100ml clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same diluent. Further pipette out 2ml from the above Phenylephrine and Ketorolac sample stock solution

into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac.

Preparation of standard solution of Phenylephrine and Ketorolac for accuracy studies

Standard stock solutions of Phenylephrine and Ketorolac were prepared by dissolving 40mg of Phenylephrine and 12mg of Ketorolac in 100ml of diluent into a 100ml clean dry volumetric flask and the standard solutions was filtered through 0.45 µm nylon membrane filter and degassed by sonicator to get the concentration of 400µg/ml of Phenylephrine and 120µg/ml of Ketorolac.

a.) Preparation of 50% standard solution

From the standard stock solution of 400µg/ml of Phenylephrine and 120µg/ml of Ketorolac further pipette 1ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 40µg/ml of Phenylephrine and 12µg/ml of Ketorolac.

b.) Preparation of 100% standard solution

From the standard stock solution of 400µg/ml of Phenylephrine and 120µg/ml of Ketorolac further pipette 2ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac.

c.) Preparation of 150% standard solution

From the standard stock solution of 400µg/ml of Phenylephrine and 120µg/ml of Ketorolac further pipette 3ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 120µg/ml of Phenylephrine and 36µg/ml of Ketorolac.

Acceptance Criteria**:** The % Recovery for each level should be between 98.0 to 102.0%.

Precision studies for Phenylephrine and Ketorolac

Method precision (Repeatability)

Omidria (Phenylephrine and Ketorolac injection, 1% / 0.3%) contains equivalent amount of Phenylephrine 40mg and Ketorolac 12mg per 4ml vial were taken into 100ml clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same diluent. Further pipette out 2ml from the above Phenylephrine and Ketorolac sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac. A homogenous sample of a single batch is analyzed six times and was checked whether the method is giving consistent results. The %RSD for the assay of six replicate injections was calculated as mentioned in Table 5.

Acceptance Criteria: The % RSD for the assay of six sample injections should not be more than 2%.

Table 5: Method precision data for Phenylephrine and Ketorolac.

	Phenylephrine				**Ketorolac**			
S.No.	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay
1	80	2.308	1211525	99.05	24	3.777	879659	100.98
2	80	2.315	1219734	99.72	24	3.783	869675	99.84
3	80	2.316	1211240	99.02	24	3.786	868725	99.73
4	80	2.318	1218324	99.60	24	3.797	870299	99.91
5	80	2.32	1214662	99.30	24	3.804	870508	99.93
6	80	2.323	1213138	99.18	24	3.806	869247	99.79
Average		2.317	1214771	99.31	**Average**	3.792	871352	100.03
SD		0.005125	3547.9	0.29	**SD**	0.01189	4122.4	0.47
%RSD		0.22	0.3	0.29	**%RSD**	0.31	0.5	0.47

Table 6: System precision data for Phenylephrine and Ketorolac.

	Phenylephrine			Ketorolac		
S.No.	Conc. (µg/ml)	Retention time (min)	Peak Area	Conc. (µg/ml)	Retention time (min)	Peak Area
1	80	2.282	1195614	24	3.814	869305
2	80	2.291	1215794	24	3.821	867006
3	80	2.299	1229048	24	3.827	864544
4	80	2.301	1220791	24	3.845	863351
5	80	2.303	1225869	24	3.846	871736
6	80	2.304	1222527	24	3.851	869685
Average		2.297	1218274	**Average**	3.834	867605
SD		0.008548	11982	**SD**	0.01531	3228.21
%RSD		0.37	0.98	**%RSD**	0.40	0.37

System precision

The system precision was carried out to ensure that the analytical system is working properly. The standard preparation concentration of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac was injected six times into the HPLC system and the %RSD for the area of six replicate injections was calculated as mentioned in Table 6.

Acceptance Criteria: The % RSD for the peak area of six standard injections should not be more than 2%.

Intermediate precision/ruggedness

The intermediate precision (also known as Ruggedness) of the method was evaluated by performing precision on different laboratories by different analysts and different days. The sample preparation concentration of 80µg/ml of Phenylephrine and 24µg/ml of Ketorolac was injected six times into the HPLC system and the %RSD for the assay of six replicate injections was calculated as mentioned in Table 7.

Acceptance Criteria: The % RSD for the assay of six sample injections should not be more than 2%.

Table 7: Ruggedness data for Phenylephrine and Ketorolac.

Ruggedness Data for Phenylephrine								
Laboratory-1 (% Assay)-HPLC-1					Laboratory-2 (% Assay)-HPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
Conc. (µg/ml)	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
80	99.60	99.31	99.51	99.40	100.19	100.19	100.14	99.35
80	99.53	100.27	99.52	99.61	99.90	99.73	99.30	99.93
80	99.24	99.20	99.47	99.30	100.12	100.15	100.19	100.31
80	99.05	100.29	99.53	99.29	99.69	100.39	100.13	100.12
80	99.56	99.16	99.54	99.44	100.15	100.16	100.18	100.17
80	99.32	99.89	99.34	99.48	99.49	99.05	99.28	99.29
Average	99.38	99.69	99.49	99.42	99.92	99.95	99.87	99.86
SD	0.22	0.53	0.075	0.12	0.284	0.489	0.45	0.437
%RSD	0.22	0.53	0.08	0.12	0.28	0.49	0.45	0.44
Intermediate precision within-laboratories variations (n=24)								
Laboratory-1 (% Assay)-HPLC-1					Laboratory-2 (% Assay)-HPLC-2			
Average	99.50				**Average**	99.90		
SD	0.236				**SD**	0.415		
%RSD	0.24				**%RSD**	0.42		

Reproducibility between laboratories (n=48) (% Assay)								
Average	99.70							
SD	0.326							
%RSD	0.33							
Ruggedness Data for Ketorolac								
Laboratory-1 (% Assay)-HPLC-1					Laboratory-2 (% Assay)-HPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
Conc. (µg/ml)	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
24	99.66	99.67	100.02	99.85	99.15	99.68	99.70	99.85
24	99.75	99.16	99.86	99.78	99.90	100.07	99.81	99.82
24	99.78	99.13	99.56	99.60	99.67	99.81	99.69	99.91
24	100.10	100.15	99.75	99.69	99.79	99.21	99.75	99.86
24	100.04	99.91	100.09	100.07	100.14	99.75	99.80	99.92
24	99.83	99.13	99.85	99.97	100.02	99.69	99.81	99.83
Average	99.86	99.53	99.86	99.83	99.78	99.70	99.76	99.87
SD	0.17	0.45	0.19	0.17	0.35	0.28	0.06	0.04
%RSD	0.17	0.45	0.19	0.17	0.35	0.28	0.06	0.04
Intermediate precision within-laboratories variations (n=24)								
Laboratory-1 (% Assay)-HPLC-1					Laboratory-2 (% Assay)-HPLC-2			
Average	99.77				**Average**	99.78		
SD	0.245				**SD**	0.183		
%RSD	0.25				**%RSD**	0.18		
Reproducibility between laboratories (n=48) (% Assay)								
Average	99.78							
SD	0.214							
%RSD	0.22							

Table 8: Summary of validation parameter for Phenylephrine and Ketorolac.

Parameters	**RP-HPLC method**			
	Phenylephrine		**Ketorolac**	
Linearity range (µg/ml)	20-120		6-36	
Slope	15023		36481	
Intercept	638		398.4	
Correlation coefficient	0.999		0.999	
LOD (µg/ml)	0.03		0.005	
LOQ (µg/ml)	0.09		0.015	
Method Precision (% RSD, n=6)	0.29		0.47	
System precision (% RSD, n=6)	0.98		0.37	
	Lab-1	Lab-2	Lab-1	Lab-2
Ruggedness (% RSD, n=24)	0.24	0.42	0.25	0.18
Reproducibility (% RSD, n=48)	0.33		0.22	
% Accuracy	100.30-101.03		99.93-100.65	
	Less Flow rate	More Flow rate	Less Flow rate	More Flow rate
Robustness (% RSD, n=6)	0.9	0.41	1.5	0.31
	Less Organic phase	More Organic phase	Less Organic phase	More Organic phase
	0.76	1.7	1.2	1.5
	Less Temperature	More Temperature	Less Temperature	More Temperature
	0.9	0.5	1.7	0.3

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as 3.3×SD/S and 10×SD/S respectively as per ICH guidelines, Where SD is the standard deviation of the response (Y-intercept) and S is the slope of the calibration curve. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LOD of Phenylephrine and Ketorolac was calculated and shown in Table 8. The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ of Phenylephrine and Ketorolac was calculated and shown in Table 8.

Robustness

As part of the Robustness, deliberate change in the flow rate, mobile phase proportion of ±10% and column temperature was made to evaluate the impact on the method. The results reveal that the method is robust. The results are summarized in Table 9, 10 and 11.

Table 9: Summary of robustness (Change in flow rate) for Phenylephrine and Ketorolac.

Drug	Change in Flow rate (ml/min)	Retention Time (Mins)	Change in flow Rate (0.9 ml/min to 1.1 ml/min)				
			Average peak area (n=6)	SD	% RSD	USP Plate Count	Asymmetry
Phenylephrine	0.9	2.654	1363863	12587.5	0.9	6555	1.25
	1.0	2.297	1218274	11981.8	0.98	6386	1.22
	1.1	2.176	1106826	4561.8	0.41	6069	1.24
Ketorolac	0.9	4.198	978432	14650.2	1.5	10124	1.09
	1.0	3.834	867605	3228.21	0.4	10047	1.07
	1.1	3.463	770974	2411.5	0.31	9351	1.08

Table 10: Summary of robustness (Change in mobile phase) for Phenylephrine and Ketorolac.

Drug	Change in Mobile Phase	Retention Time (Mins)	Change in mobile phase (0.01M Ammonium acetate buffer (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile) (37:63v/v to 23:77v/v)				
			Average peak area (n=6)	SD	% RSD	USP Plate Count	Asymmetry
Phenylephrine	10% less Organic (37:63 v/v)	2.428	1216473	9291.53	0.76	6445	1.26
	Actual (30:70 v/v)	2.297	1218274	11981.8	0.98	6386	1.22
	10% more Organic (23:77v/v)	2.381	1212810	20682.7	1.7	6369	1.26
Ketorolac	10% less Organic (37:63 v/v)	3.649	876593	10218.6	1.2	9758	1.07
	Actual (30:70 v/v)	3.834	867605	3228.21	0.4	10047	1.07
	10% more Organic (23:77v/v)	3.926	849059	12520.7	1.5	10026	1.06

Table 11: Summary of Robustness (Change in Column Temperature) for Phenylephrine and Ketorolac.

Drug	Change in column temperature	Retention Time (Mins)	Change in column temperature (28°C to 32°C)				
			Average peak area (n=6)	SD	% RSD	USP Plate Count	Asymmetry
Phenylephrine	28°C	2.654	1358426	12600.2	0.9	6562	1.24
	Actual temperature (30°C)	2.297	1218274	11981.8	0.98	6386	1.22
	32°C	2.176	1097538	4961.2	0.5	6080	1.23
Ketorolac	28°C	4.198	955328	16590.9	1.7	10185	1.09
	Actual temperature (30°C)	3.834	867605	3228.21	0.4	10047	1.07
	32°C	3.463	769933	1967.6	0.3	9356	1.08

Table 12: Summary of solution stability-effect of P^H of mobile phase (0.01M Ammonium acetate buffer and Acetonitrile (30:70, v/v) (P^H adjusted to 3.5 with Orthophosphoric Acid) for Phenylephrine and Ketorolac for 48 hours at room temperature.

	Solution stability for Phenylephrine					
S.No.	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay	USP Plate Count	Asymmetry
1	80	2.282	1201691	100.11	6314	1.23
2	80	2.291	1196180	99.65	6441	1.25
3	80	2.299	1192638	99.35	6441	1.25
4	80	2.301	1202186	100.15	6463	1.23
5	80	2.303	1190444	99.17	6458	1.25
6	80	2.304	1189681	99.11	6326	1.25
Average		2.297	1194803	99.59	6407	1.24
SD		0.01	6492.28	0.46	68.20	0.01
%RSD		0.4	0.5	0.5	1.1	0.8
Solution stability for Ketorolac						
S.No.	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay	USP Plate Count	Asymmetry
1	24	3.814	843031	99.06	9916	1.06
2	24	3.821	849517	99.82	9993	1.09
3	24	3.827	844544	99.24	10271	1.05
4	24	3.845	852251	100.15	10228	1.06
5	24	3.846	843956	99.17	10070	1.05
6	24	3.851	849526	99.83	10120	1.06
Average		3.834	847138	99.55	10100	1.06
SD		0.02	3774.29	0.45	135.82	0.01
%RSD		0.4	0.4	0.45	1.3	1.4

Stability of solution

The %RSD of the assay of Phenylephrine and Ketorolac from the solution stability and mobile

phase stability experiments was within 2%. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the assays were stable upto 48hours at room temperature was calculated and shown in Table 12.

Forced degradation studies

Acid Degradation Studies

To 1 ml of stock solution of Phenylephrine and Ketorolac, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0C. The resultant solution was diluted to obtain 80µg/ml and 24µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 10) and purity plot of acid degradation for Phenylephrine and Ketorolac was shown in (Figure 11 and 12).

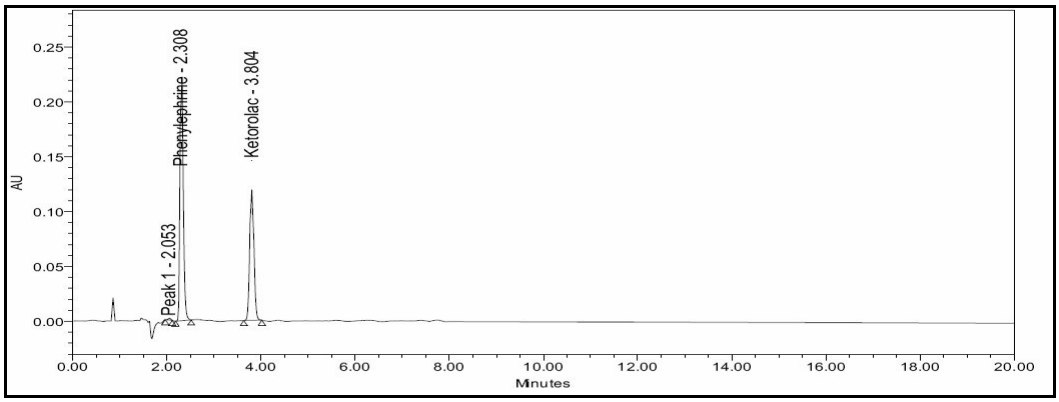


Figure 10: Chromatogram of acid hydrolysis for Phenylephrine and Ketorolac

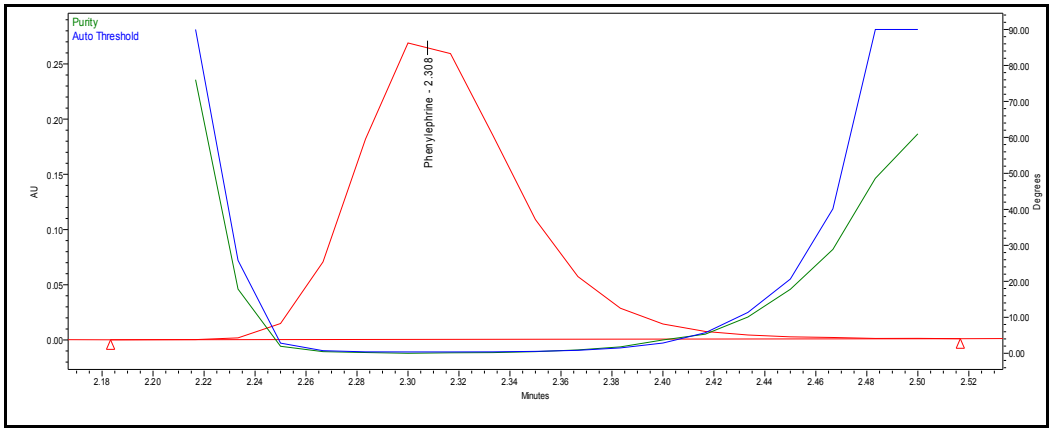


Figure 11: Purity plot of acid hydrolysis for Phenylephrine

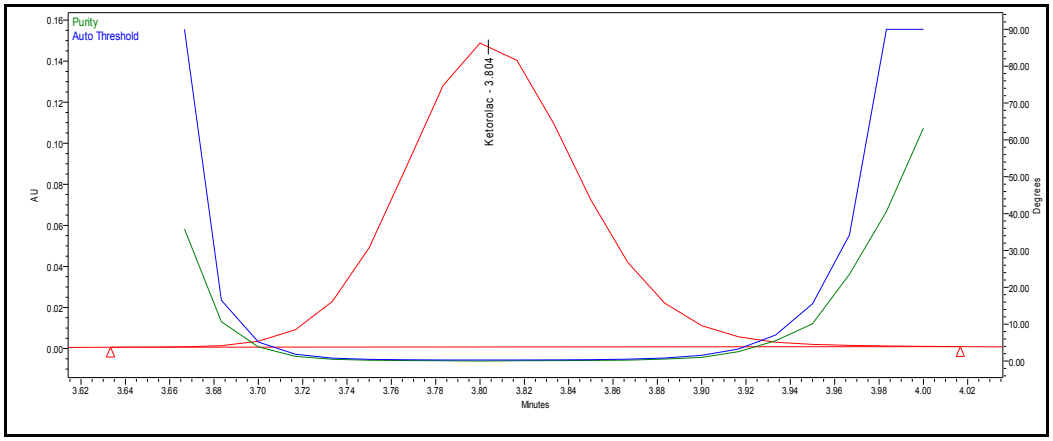


Figure 12: Purity plot of acid hydrolysis for Ketorolac

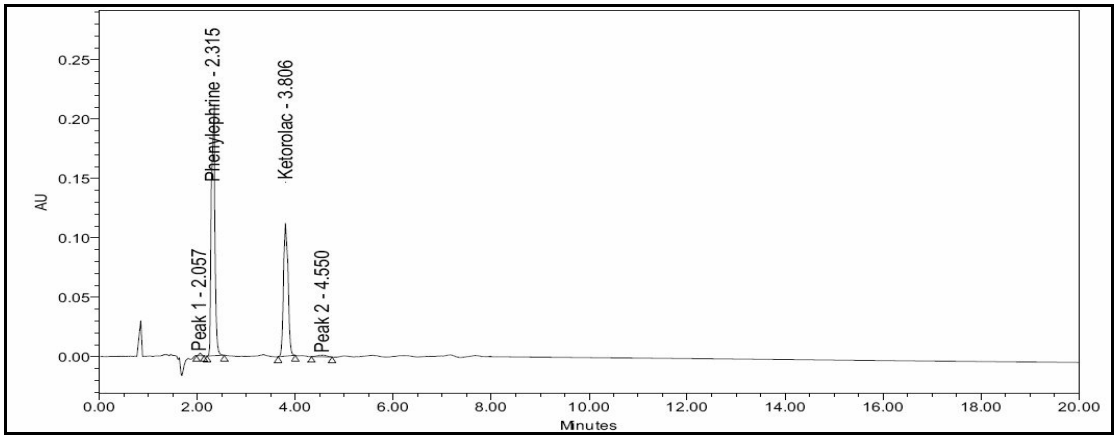


Figure 13: Chromatogram of alkali hydrolysis for Phenylephrine and Ketorolac

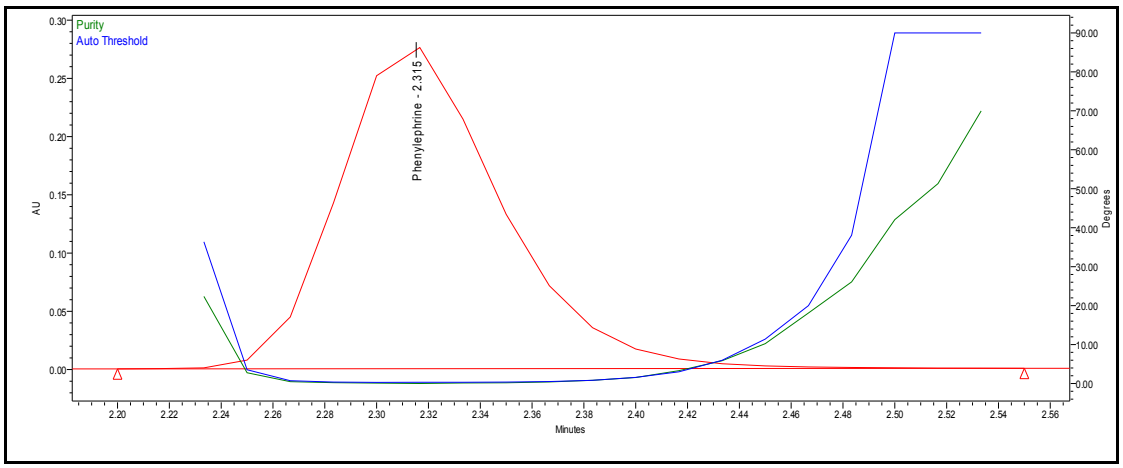


Figure 14: Purity plot of alkali hydrolysis for Phenylephrine

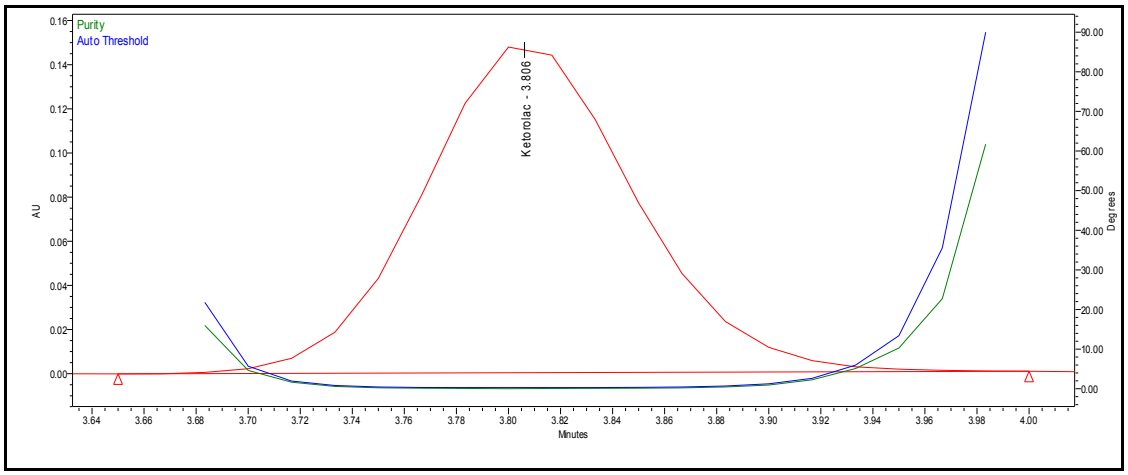


Figure 15: Purity plot of alkali hydrolysis for Ketorolac

Alkali Degradation Studies

To 1ml of stock solution of Phenylephrine and Ketorolac, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0C.The resultant solution was diluted to obtain 80µg/ml and 24µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 13) and purity plot of alkali degradation for Phenylephrine and Ketorolac was shown in (Figure 14 and 15).

Oxidative degradation Studies

To 1ml of stock solution of Phenylephrine and Ketorolac, 1 ml of 3% Hydrogen peroxide (H_2O_2) was added and the solution was kept for 30 mins at 60^0C. For HPLC study, the resultant solution was diluted to obtain 80µg/ml and 24µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 16) and purity plot of oxidative degradation for Phenylephrine and Ketorolac was shown in (Figure 17 and 18).

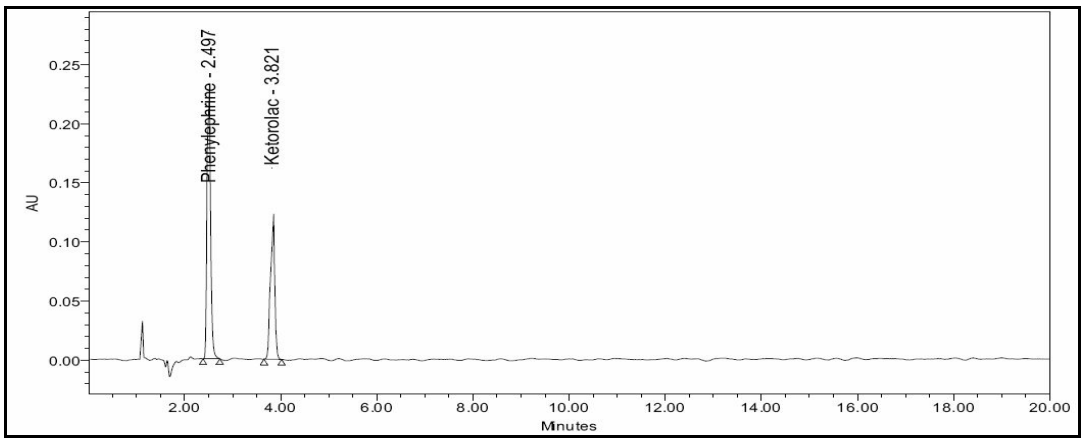


Figure 16: Chromatogram of oxidative degradation for Phenylephrine and Ketorolac

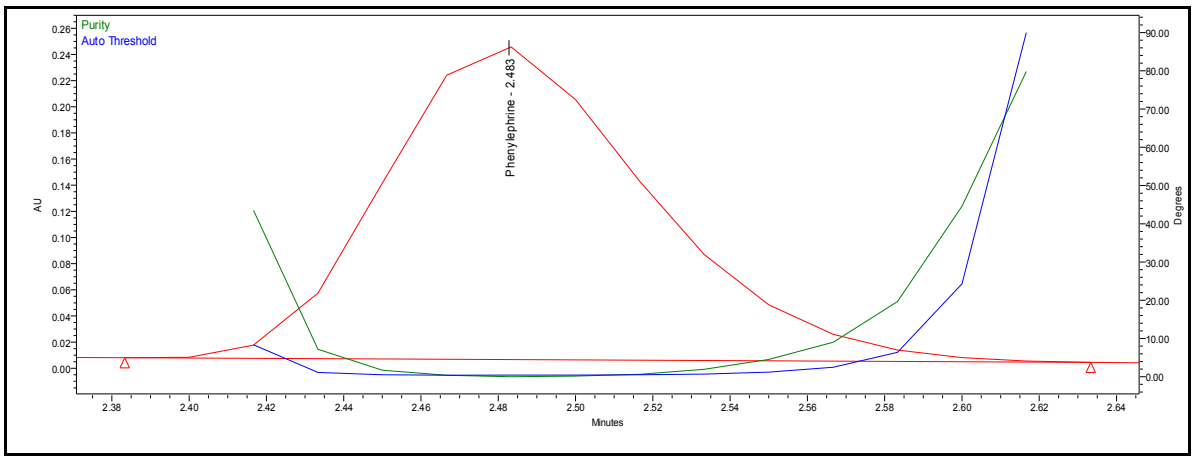


Figure 17: Purity plot of oxidative degradation for Phenylephrine

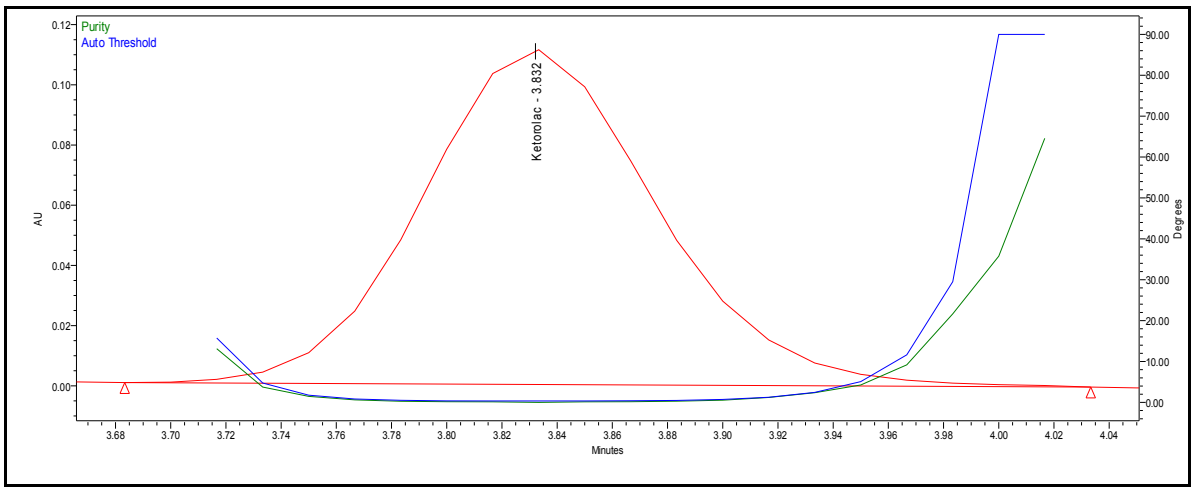


Figure 18: Purity plot of oxidative degradation for Ketorolac

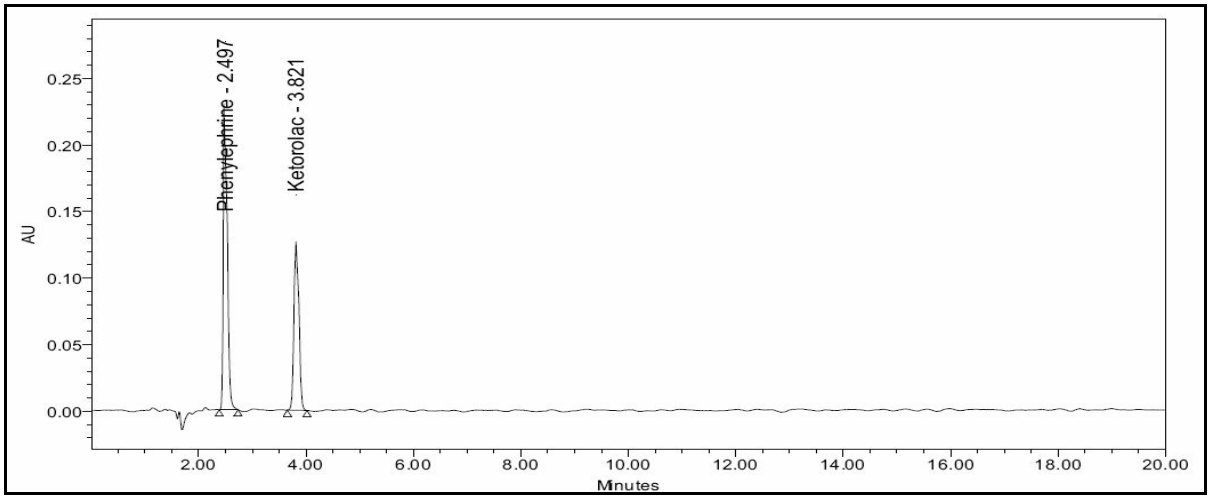


Figure 19: Chromatogram of thermal degradation for Phenylephrine and Ketorolac

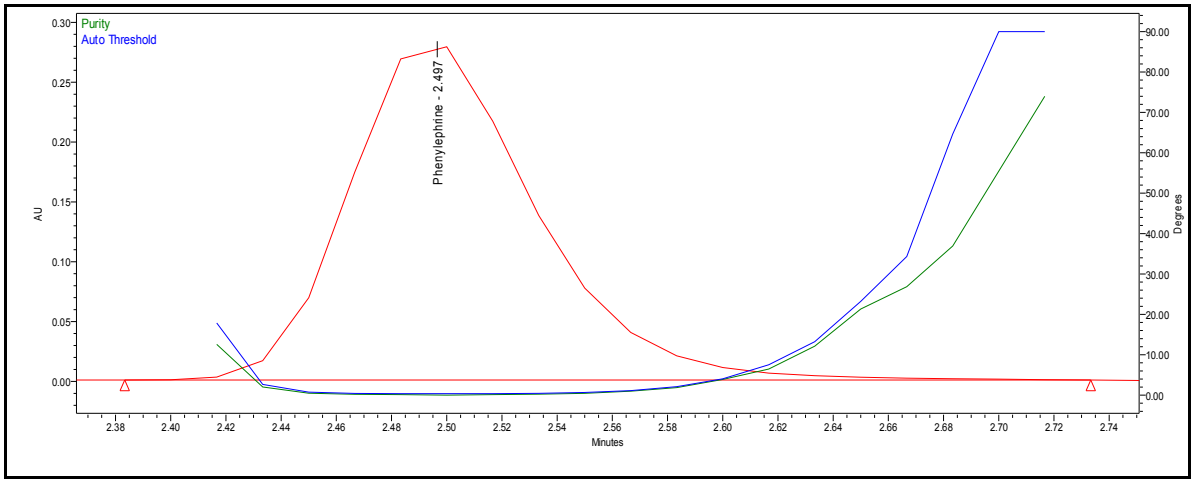


Figure 20: Purity plot of thermal degradation for Phenylephrine

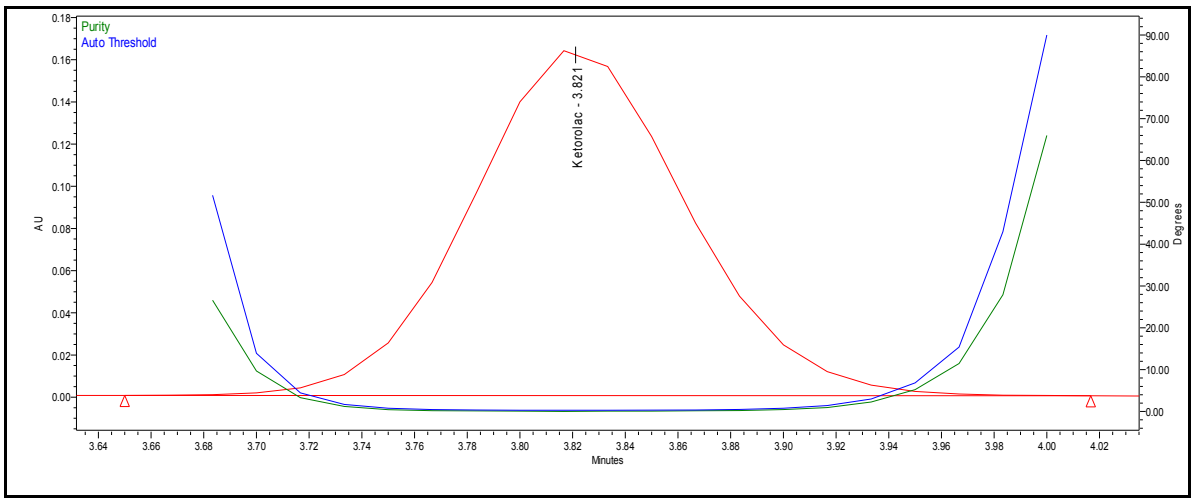


Figure 21: Purity plot of thermal degradation for Ketorolac

Thermal Degradation Studies

The standard drug solution was placed in oven at 105^{0}C for 6hrs to study dry heat degradation. For HPLC study, the resultant solution was diluted to 80µg/ml and 24µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of

sample was shown in (Figure 19) and purity plot of thermal degradation for Phenylephrine and Ketorolac was shown in (Figure 20 and 21).

Photolytic degradation studies

The photochemical stability of the drug was also studied by exposing the drug solution to UV light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 80µg/ml and 24µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 22) and purity plot of photolytic degradation for Phenylephrine and Ketorolac was shown in (Figure 23 and 24).

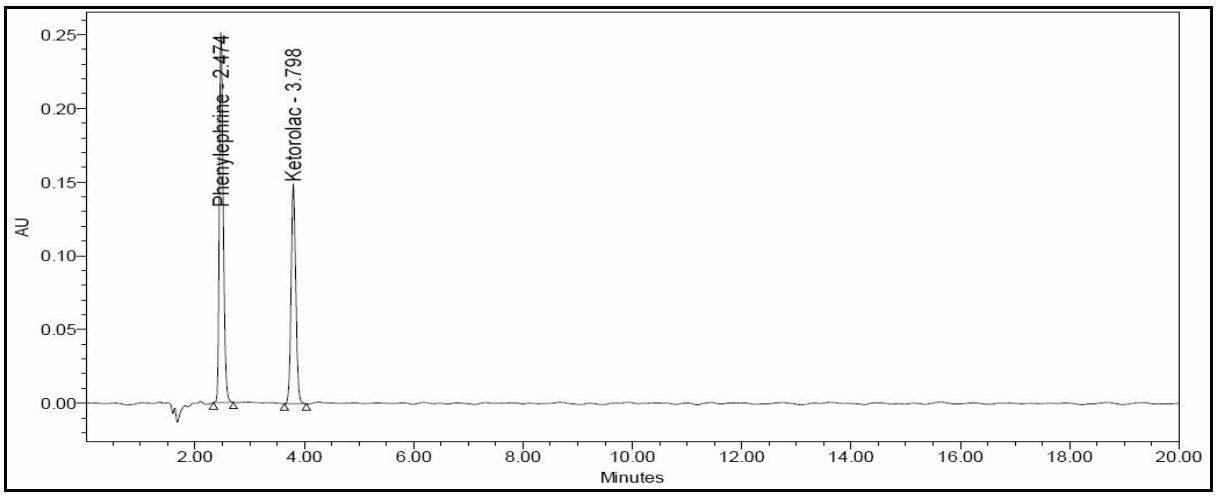


Figure 22: Chromatogram of photolytic degradation for Phenylephrine and Ketorolac

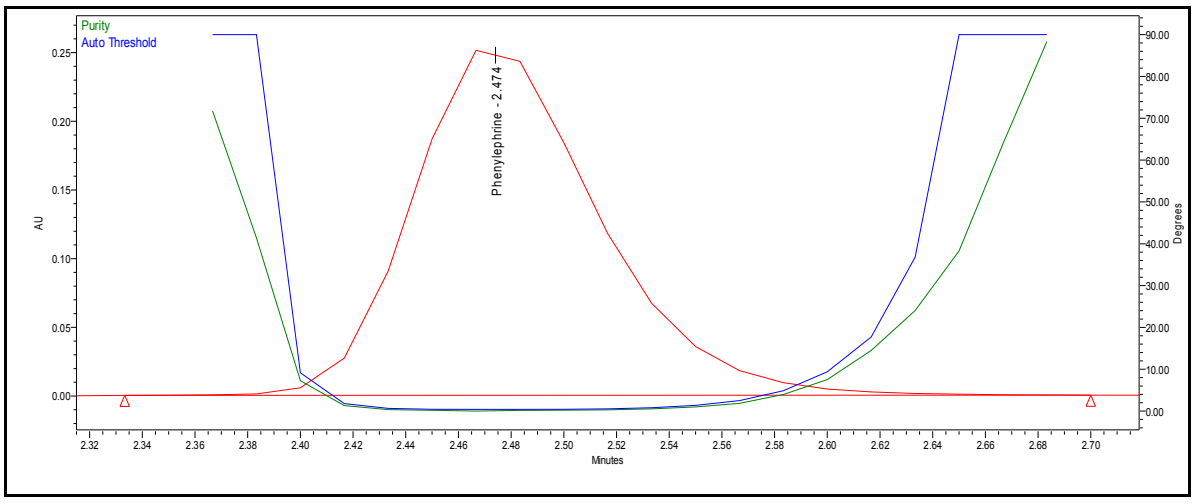


Figure 23: Purity plot of photolytic degradation for Phenylephrine

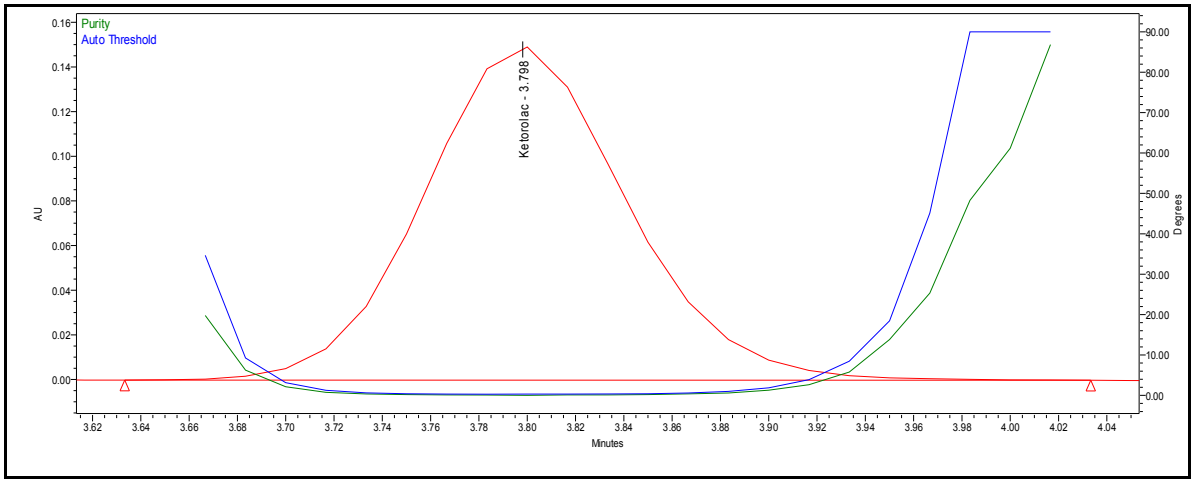


Figure 24: Purity plot of photolytic degradation for Ketorolac

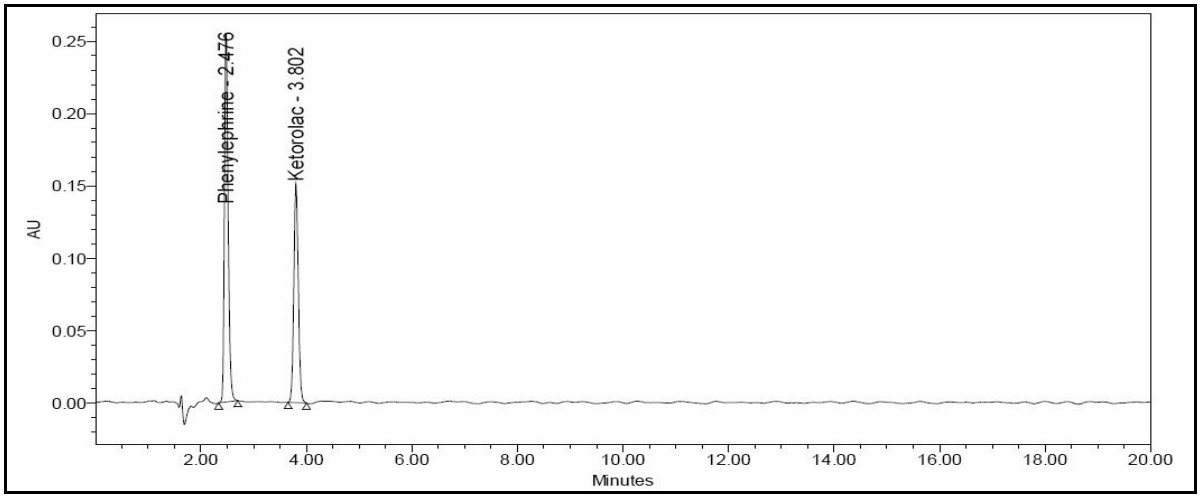


Figure 25: Chromatogram of water degradation for Phenylephrine and Ketorolac

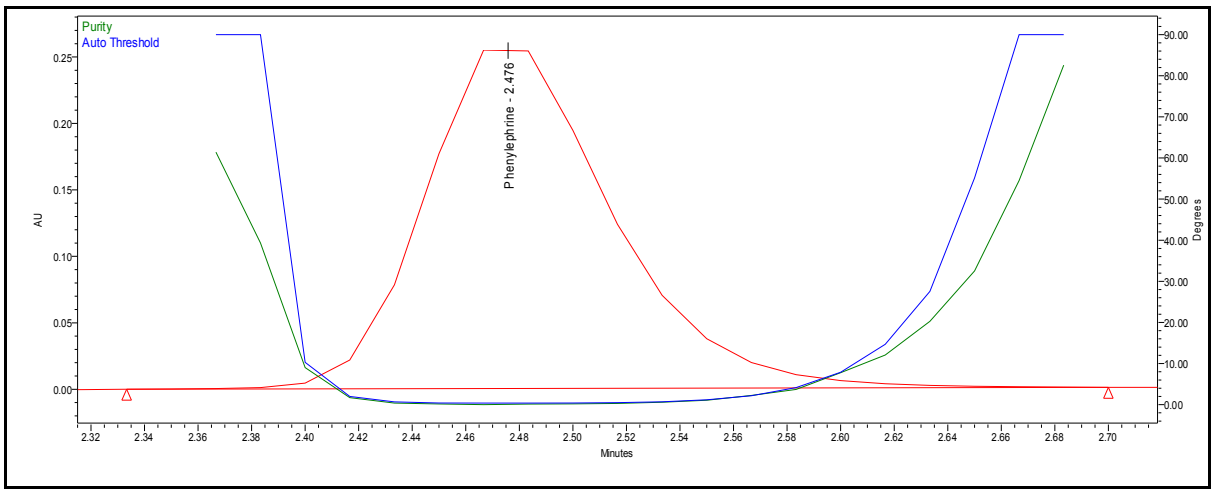


Figure 26: Purity plot of water degradation for Phenylephrine

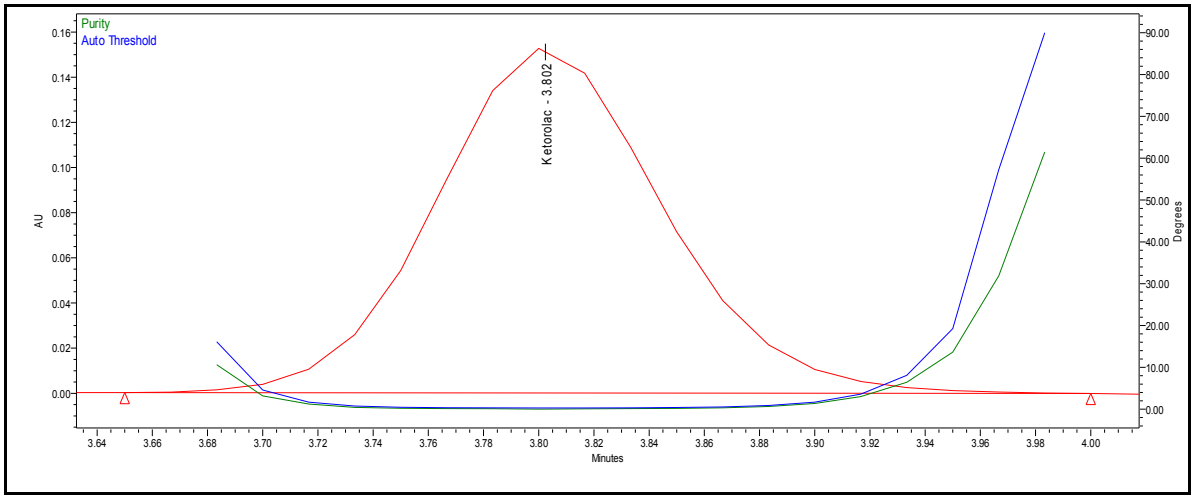


Figure 27: Purity plot of water degradation for Ketorolac

Water Degradation Studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60ºC. For HPLC study, the resultant solution was diluted to 80µg/ml and 24µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 25) and purity plot of water degradation for Phenylephrine and Ketorolac was shown in (Figure 26 and 27).

Conclusion

The present RP-HPLC method for simultaneous estimation of Phenylephrine and Ketorolac in their combine dosage form was established and validated as per the ICH guidelines. Linearity was achieved for Phenylephrine and Ketorolac in the range of 20-120µg/ml for Phenylephrine and 6-36µg/ml for Ketorolac with correlation coefficients (r^2=0.999). The percentage recoveries of Phenylephrine and Ketorolac were achieved in the range of 98-102% which was within the acceptance criteria. The percentage RSD was NMT 2 % which proved the precision of the developed method. The developed method is simple, sensitive, rapid, linear, precise, rugged, accurate, specific, and robust. The forced degradation studies were performed by using HCl, NaOH, H_2O_2, thermal, UV radiation and water. Phenylephrine and Ketorolac are more sensitive towards alkaline hydrolysis degradation condition and moderate degradation towards acidic, oxidative, thermal and photolytic and very much resistant towards water degradation which was shown in Table 13. No interference from any components of pharmaceutical dosage form or degradation products was observed and the method has been successfully used to perform long term and accelerated stability studies of Phenylephrine and Ketorolac formulations. Hence it can be used for the hyphenated instrumental analysis of Phenylephrine and Ketorolac in their bulk and combine dosage form.

Table 13: Forced degradation data of Phenylephrine and Ketorolac in different degradation conditions.

Forced degradation data of Phenylephrine							
Degradation condition	Retention time	Area	% Area	Purity Angle	Purity Threshold	USP Plate Count	Asymmetry
Acid hydrolysis	2.308	1161525	58.43	0.416	0.549	6160	1.3
Alkaline hydrolysis	2.315	1129734	58.18	0.268	0.428	6440	1.2
Oxidative degradation	2.497	1164191	58.38	0.362	0.546	6453	1.2
Thermal degradation	2.497	1184191	58.39	0.369	0.584	6488	1.2
Photolytic degradation	2.474	1196267	58.72	0.396	0.617	5914	1.2
Water degradation	2.476	1206377	58.78	0.386	0.565	6216	1.2
Forced degradation data of Ketorolac							
Degradation condition	Retention time	Area	% Area	Purity Angle	Purity Threshold	USP Plate Count	Asymmetry
Acid hydrolysis	3.804	817790	41.23	0.197	0.408	9948	1.1
Alkaline hydrolysis	3.806	804717	40.63	0.182	0.367	10355	1.1
Oxidative degradation	3.821	833872	41.62	0.181	0.389	9909	1.1
Thermal degradation	3.821	843872	41.61	0.187	0.341	9918	1.1
Photolytic degradation	3.798	846049	41.28	0.205	0.420	9456	1.1
Water degradation	3.802	860940	41.22	0.204	0.403	9684	1.1

Degradation condition	Drug Recovered (%)		Drug Decomposed (%)	
	Phenylephrine	Ketorolac	Phenylephrine	Ketorolac
Standard	100	100	0	0
Acid hydrolysis	94.96	93.88	5.04	6.12
Alkaline hydrolysis	92.36	92.38	7.64	7.62
Oxidative degradation	95.18	95.73	4.82	4.27
Thermal degradation	96.81	96.88	3.19	3.12
Photolytic degradation	97.80	97.13	2.2	2.87
Water degradation	98.63	98.83	1.37	1.17

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