Research Article

Development and Validation of HPLC Method for Estimation of Etodolac in Rat Plasma

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Abstract

Etodolac is 1, 8-diethyl-1, 3, 4, 8- tetrahydropyrano [3,4-b] indole-1-acetic acid, used for osteoarthritis. This study was designed to develop and validate high performance liquid chromatography method of etodolac in rat plasma. The samples were analyzed by HiQ Sil C_{18} HS (250 × 4.6 mm, 5μ.) columns, using Acetonitrile: 0.02M potassium dihydrogen orthophosphate (65: 35 v/v) as a mobile phase. The method showed linearity (r^2= 0.9964) over a concentration range of (1-25μg/ml). The method showed good mean recovery (97.53%) for etodolac. The method was found to be accurate, precise, linear, specific, sensitive and stable.

Keywords: Etodolac; HPLC; Rat plasma; Analytical method; Validation

Abbreviations

HPLC: High Performance Liquid Chromatography; ETDO: Etodolac; THIO: Thiocolchicoside; NSAID: Non-steroidal Anti-inflammatory Drug; RP-HPLC: Reverse Phase High Performance Liquid Chromatography; GC-MS: Gas Chromatography Mass Spectroscopy; ICH: International Conference on Harmonization; Rpm: Revolution per minute; ISTD: Internal Standard; ACN: Acetonitrile; DMSO: Dimethyl Sulfoxide; LLOQ: Lower Limit of Quantification; CV: Coefficient of Variation; LQC: Low Quality Control; MQC: Middle Quality Control; HQC: High Quality Control

Introduction

Etodolac is 1, 8-diethyl-1, 3, 4, 8 - tetrahydropyrano [3, 4-b] indole-1-acetic acid. Etodolac contains not less than 98.0% and not more than 102.0% of $C_{17}H_{21}NO_3$, calculated on the anhydrous basis [1]. Etodolac is a nonsteriodal anti-inflammatory drug (NSAID) that exhibits anti-inflammatory, analgesic, and antipyretic activities. The mechanism of action of etodolac, like that of other NSAIDs, is not completely understood, but may be related to prostaglandin synthetase inhibition [2]. Well absorbed following oral administration; bioavailability is about 80%. Peak plasma concentration usually attained within about 1.4 hours (conventional capsules and tablets) or 6.7 hours (extended-release tablets) [3]. Etodolac is white or almost white crystalline powder [1]. Soluble in water (<1 mg/ml), methanol (9.80-10.20mg/ml), chloroform, DMSO (58mg/ml), and ethanol (58mg/ml) [4-6]. Several spectroscopic and chromatographic methods are available in literature to determine concentration of Etodolac, individually or in combination with other drugs or metabolites as UV [7-8], HPLC- UV [9], RP-HPLC [10-11], GC-MS [12]. The present article describes a simple HPLC method for estimation of Etodolac and validation of the method as per the guidelines of ICH [13].

Materials and Methods

Reagents and chemicals

Etodolac (Ipca Laboratories, Mumbai), Acetonitrile HPLC grade, Methanol HPLC grade. (Merck Laboratories, Mumbai), Double distilled water, Glacial Acetic Acid, Potassium dihydrogen orthophosphate.

Selection of mobile phase

Different mobile phases like methanol with Phosphate buffer of different molarities at various pH, acetonitrile and Phosphate buffer, methanol and water were tried in different ratio in order to find the best conditions for Etodolac (ETO). After several trials Acetonitrile: 0.02M potassium dihydrogen orthophosphate (65: 35 v/v) was chosen as the mobile phase for analysis in which optimum system suitability parameters were obtained.

Preparation of mobile phase

130 ml of HPLC grade Acetonitrile was added in 70ml of 0.02M potassium dihydrogen orthophosphate i.e. in 65: 35 v/v proportions. The solution was further filtered through 0.45μ membrane filter and sonicated in sonicator bath for 10min.

Preparation of standard stock solutions of Etodolac (100μg/ml)

10 mg of Etodolac was dissolved in 10ml of Acetonitrile and 1ml of this solution was diluted with Acetonitrile to final volume of 10ml in volumetric flask to get concentration 100μg/ml (stock I).

Preparation of intermediate stock solution by using stock solutions of Etodolac (100μg/ml) for plasma calibration curve
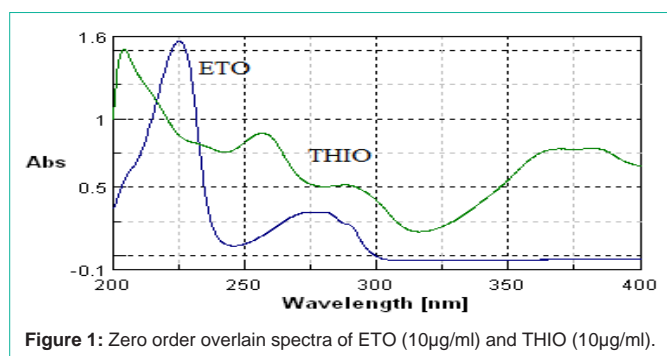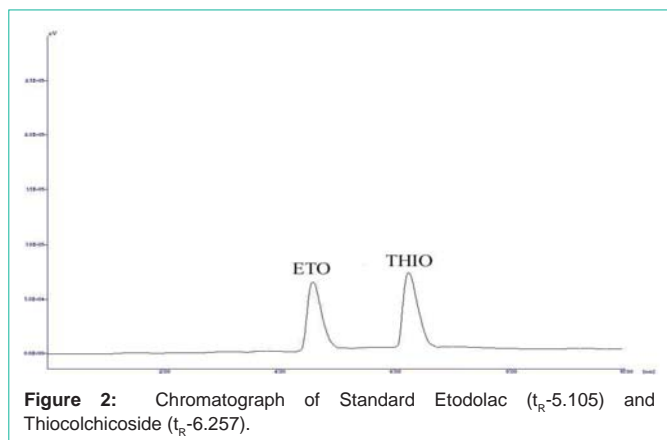
Using a calibrated pipette 0.4, 1, 2, 4, 6 and 8 ml of stock solution I was transferred to separate volumetric flasks and then diluted to 10ml with acetonitrile to get concentrations 4, 10, 20, 40, 60 and 80μg/ml.

Preparation of internal standard stock solution of Thiocolchicoside (100μg/ml)

10 mg of Thiocolchicoside was dissolved in 10 ml of Acetonitrile and 1 ml of this solution was diluted with Acetonitrile to final volume of 10 ml in volumetric flask to get concentration 100 μg/ml (stock I). Using a calibrated pipette, 4 ml of ISTD stock solution (100μg/ml) was pipette into a 10.0 ml volumetric flask and made up the volume with the mobile phase to get concentration of 40μg/ml.

Table 1: Plasma sample preparation.

Rat Plasma	Stock solution of ETO (0.5 ml)	Stock solution of THIO (0.5 ml)	ACN	Conc. of ETO (µg/ml)	Conc. of THIO (µg/ml)
0.5 ml	Mobile phase	Mobile phase	0.5 ml	Blank	Blank
0.5 ml	4 µg/ml	40 µg/ml	0.5 ml	1	10
0.5 ml	10 µg/ml	40 µg/ml	0.5 ml	2.5	10
0.5 ml	20 µg/ml	40 µg/ml	0.5 ml	5	10
0.5 ml	40 µg/ml	40 µg/ml	0.5 ml	10	10
0.5 ml	60 µg/ml	40 µg/ml	0.5 ml	15	10
0.5 ml	80 µg/ml	40 µg/ml	0.5 ml	20	10
0.5 ml	100 µg/ml	40 µg/ml	0.5 ml	25	10

Figure 1: Zero order overlain spectra of ETO (10µg/ml) and THIO (10µg/ml).

Figure 3: Chromatograph of Blank Plasma.

Figure 2: Chromatograph of Standard Etodolac (t_R-5.105) and Thiocolchicoside (t_R-6.257).

Figure 4: Chromatograph of Plasma spiked with Etodolac (t_R-5.128) and Thiocolchicoside (t_R-6.262).
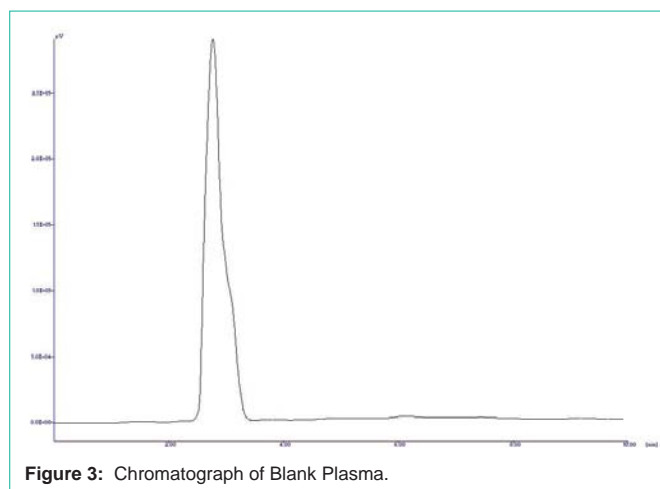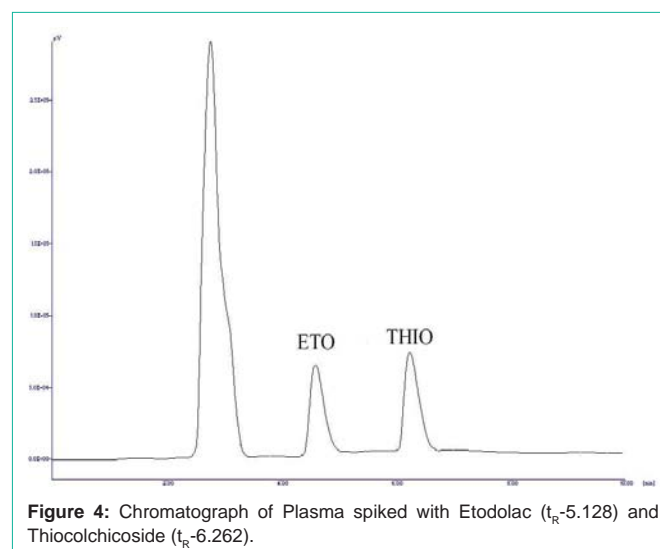
Preparation of plasma sample solution

To 0.5ml of rat plasma, 0.5ml of stock solution of Etodolac (Concentrations: 4, 10, 20, 40, 60, 80 and 100µg/ml), 0.5ml of an internal standard solution (Thiocolchicoside, 40µg/ml) and 0.5 ml Acetonitrile were added in a glass tubes (Table 1). Each sample tube was vortex mixed for 10min and centrifuged (3000rpm for 10min). After centrifugation 20µl aliquots of supernatant of each concentration were injected into the HPLC system.

Selection of analytical wavelength

From the standard stock solution further dilutions were done using mobile phase and scanned over the range of 200-400nm and the spectra were overlain (Figure 1).

Summary of chromatographic parameters selected

1.   Column : HiQ Sil C_{18} HS (250 × 4.6mm, 5µ.)

2.   Mobile phase : Acetonitrile : 0.02M potassium dihydrogen orthophosphate (65: 35 v/v)

3.   Flow rate : 1.00 ml/min

4.   Detection Wavelength : 225nm

5.   Sample injector : 20µl loop

6.   Temperature : Ambient

7.   Internal standard : Thiocolchicoside

Table 2: Results for selectivity.

Replicate No.	Nominal Conc. (LLOQ) (0.2 µg/ml)
	Calculated Conc. (µg/ml)
1	1.00
2	1.10
3	1.06
4	0.99
5	1.07
6	1.02
Mean	1.04
SD	0.04249
% CV	4.083
% Mean Accuracy	104.08

Acceptance Criteria: At least 67 % (4 out of 6) sample should be within 80.00-120.00 %. The % Mean accuracy should be within 80.00-120.00 %. The % CV should be ≤ 20.00 %.

Table 3: Observation table for Linearity of ETO plain sample.

Concentration (µg/ml) (n=3)	Response Factor*
1	0.6833
2.5	0.9959
5	1.7539
10	3.6666
15	5.2310
20	6.6110
25	8.4985

*Average of three determinations.

Table 4: Observation table for Linearity of ETO in plasma sample.

| Concentration (µg/ml) (n=3)	Response Factor*
1	0.6853
2.5	0.9859
5	1.7486
10	3.6510
15	5.4339
20	6.8026
25	8.1686

*Average of three determinations.

Method validation

Selectivity/Specificity: Selectivity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. The selectivity of the method was evaluated by analyzing 6 replicates of plasma samples spiked at LLOQ (Lower Limit of Quantification - 1µg/ml).

Linearity: Linearity was tested for the range of concentrations 1 - 25 µg/ml. Each standard in three replicates were analyzed and peak areas were recorded. The response factors were plotted against the corresponding concentrations to obtain the calibration graphs.

Accuracy: The accuracy of the assay was calculated as the absolute value of the ratio of the calculated mean values of the quality control samples to their respective nominal values, expressed as percentage.
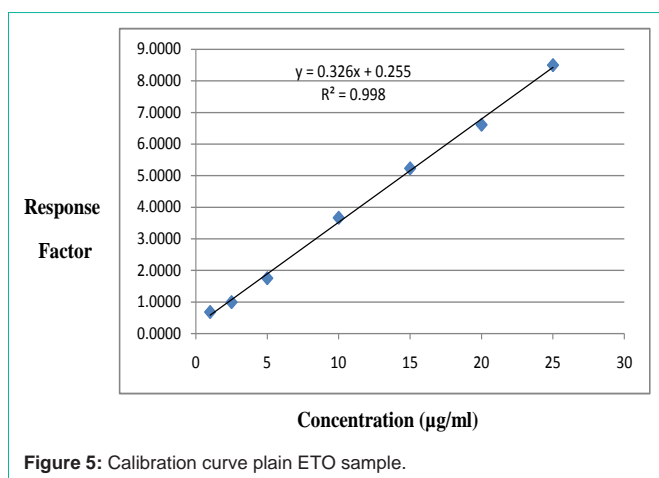
Figure 5: Calibration curve plain ETO sample.

$$y = 0.326x + 0.255$$
$$R^2 = 0.998$$
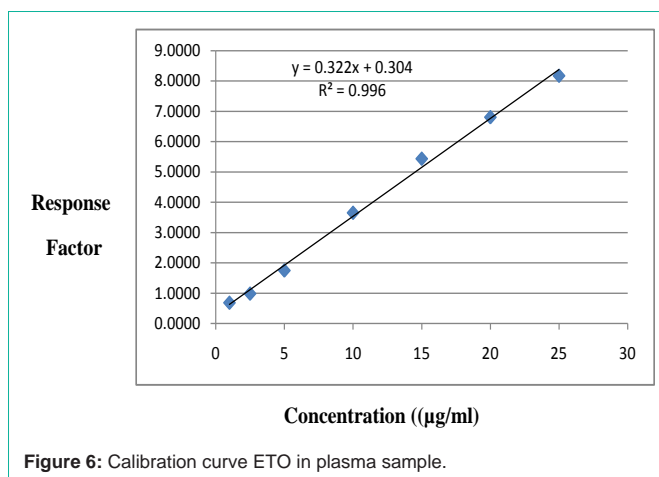
Figure 6: Calibration curve ETO in plasma sample.

$$y = 0.322x + 0.304$$
$$R^2 = 0.996$$

Accuracy should be measured using minimum five determinations per three concentrations (2.5, 10, 20µg/ml). 20-µl aliquots of supernatant of each concentration were injected in to the HPLC system.

Precision: The precision of this method was evaluated by the % CV at different concentration levels corresponding to LQC, MQC and HQC during the course of validation.

Interday precision (Reproducibility): The % CV of calculated concentrations for all quality control samples of LQC, MQC and HQC concentration levels are ranged from 2.24 to 7.15 %, which is within the acceptance limit of 15.00%. The reproducibility (inter-assay precision) was evaluated in three replicates for three different concentrations of ETO (2.5, 10, 20µg/ml) on three consecutive days (fresh samples were prepared every day).

Intraday precision (Repeatability): The repeatability (intra-assay precision) of the method was evaluated in five replicates on the same day for three different concentrations of ETO (5, 15, 20µg/ml). The results, expressed as mean amount of drug found in plasma and summarized.

Recovery: The % mean recoveries were determined by measuring the responses of the extracted plasma quality control samples against unextracted quality control samples at HQC, MQC and LQC levels. Recovery from human plasma samples was evaluated in triplicate for each three concentrations of ETO (2.5, 10, 20µg/ml). 20-µl aliquots

Table 5: Results for Accuracy.

Replicate No.	LQC	MQC	HQC
	Nominal concentration (µg/ml)		
	2.5	10	20
	Calculated Concentration (µg/ml)		
1	2.246	9.429	16.821
2	2.366	10.290	20.402
3	2.561	9.781	18.966
4	2.649	10.467	20.117
5	2.263	9.810	19.777
Mean	2.42	9.96	19.22
SD	0.180	0.419	1.443
%CV	7.46	4.21	7.51
% Mean Accuracy	96.69	99.56	96.08

Acceptance Criteria: The % Mean Accuracy for HQC, MQC, and LQC sample should be within 85.00-115.00 %.

Table 6: Observation table for Inter Day Precision.

A	DAY 1	DAY 2	DAY 3
	Nominal concentration 2.5 µg/ml (LQC)		
	Calculated concentration (µg/ml)		
1	2.355	2.430	2.474
2	2.420	2.331	2.355
3	2.298	2.341	2.328
Mean	2.358	2.367	2.385
SD	0.0611	0.0545	0.0777
% CV	2.59	2.30	3.26
B	Nominal concentration 10 µg/ml (MQC)		
	Calculated concentrations (µg/ml)		
1	10.733	10.139	10.046
2	9.677	10.102	11.310
3	10.375	9.435	10.731
Mean	10.262	9.892	10.696
SD	0.5372	0.3963	0.6327
% CV	5.23	4.01	5.92
C	Nominal concentration 20 µg/ml (HQC)		
	Calculated concentrations (µg/ml)		
1	20.266	20.422	17.994
2	19.439	19.689	18.657
3	20.148	19.318	20.619
Mean	19.951	19.810	19.090
SD	0.4476	0.5617	1.3646
% CV	2.24	2.84	7.15

Acceptance Criteria: The % CV for LQC, MQC and HQC samples should be within 15.00 %.

of supernatant of each concentration were injected in to the HPLC system.

Stability: Drug stability in a biological fluid was a function of the storage conditions, the chemical properties of the drug, the matrix,

Table 7: Observation table for Intra Day Precision.

Replicate No.	Nominal concentrations		
	(LQC) (5 µg/ml)	(MQC) (15 µg/ml)	(HQC) (20 µg/ml)
	Calculated concentrations (µg/ml)		
1	4.743	15.164	19.210
2	4.432	15.715	20.352
3	4.364	16.778	20.228
4	4.460	15.785	18.914
5	4.615	15.842	19.371
Mean	4.523	15.857	19.615
SD	0.1535	0.5817	0.6391
% CV	3.39	3.67	3.26

Acceptance Criteria: The % CV for HQC, MQC, and LQC samples should be within 15.00 %.

and the container system. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage conditions.

Freeze and Thaw stability: Freeze thaw stability of the spiked quality control samples was determined after three freeze thaw cycles stored at -5°C ± 0°C. Comparing them against the freshly spiked quality control samples assessed stability.

Bench top stability: Bench top stability of the spiked quality control samples was determined for a period of 5 hours 30min stored at room temperature. Comparing them against the freshly spiked quality control samples assessed stability.

Stock solution stability: Stock solution stability of the HQC and LQC was determined for a period of 5hours 30min stored at room temperature. Comparing them against the freshly weighed stock solution assessed for stability.

Result and Discussion

Selection of analytical wavelength

It was observed that both drugs showed considerable absorbance at 257nm (Figure 1). No endogenous interferences are noted at the retention time of the drugs as shown in Figure 2.

Method validation

Selectivity/Specificity: The precision and accuracy for at LLOQ level are found to be 4.083% (as % CV) and 104.08% (as % recovery), respectively. The results are summarized in Table 2. No endogenous interferences are noted at the retention time of the drugs as shown in Figure 3 and 4.

Linearity: All the calibration curves analyzed during the course of validation were found to be linear for the standards concentration ranging from 1-25µg/ml (Table 3 and 4) and best fitted by a linear equation y = mx + c, the coefficient of determination for plain ETO (R^2) is 0.998 and plasma containing ETO (R^2) is 0.996. An averaged calibration curves are shown in Figure 5 and 6.

Accuracy: The % mean accuracy of calculated concentrations for all quality control samples at LQC, MQC and HQC concentration levels are ranged from 96.08% to 99.56%, which is within acceptance limit 85.00 - 115.00 % (Table 5).

Table 8: Observation table for Recovery.

Replicate No.	LQC (2.5 µg/ml)		MQC (10 µg/ml)		HQC (20 µg/ml)	
	Plain sample	Plasma sample	Plain sample	Plasma sample	Plain sample	Plasma sample
	Calculated concentrations (µg/ml)					
1	2.307	2.090	9.114	9.588	20.583	19.633
2	2.338	2.295	9.739	9.340	20.704	19.181
3	2.152	2.265	9.414	9.310	19.501	18.853
Mean	**2.265**	**2.217**	**9.423**	**9.412**	**20.263**	**19.222**
SD	**0.0997**	**0.1108**	**0.3126**	**0.1526**	**0.6623**	**0.3917**
% CV	**4.40**	**5.00**	**3.32**	**1.62**	**3.27**	**2.04**
% Mean Recovery	97.85		99.89		94.86	
% Overall Mean Recovery	97.53					
Overall SD	2.53					
Overall % CV	2.59					

Acceptance Criteria: The % CV of recovery at each QC levels should be ≤ 15.00 %. The overall mean recovery for all QC levels should be ≤ 20.00 %.

Table 9: Results for Freeze and Thaw stability Study.

Replicate No.	Nominal concentrations			
	LQC (2.5 µg/ml)		HQC (20 µg/ml)	
	Comparison Sample	Stability Sample	Comparison sample	Stability sample
	Calculated concentrations (µg/ml)			
1	2.153	2.091	20.521	18.829
2	2.408	2.086	20.445	17.120
3	2.204	2.214	21.249	17.666
Mean	**2.255**	**2.130**	**20.738**	**17.872**
SD	**0.1354**	**0.0725**	**0.4440**	**0.8730**
% CV	**6.00**	**3.40**	**2.14**	**4.88**
% Mean stability	94.47		86.18	

Acceptance Criteria: The % CV for LQC and HQC should be sample should be ≤ 15.00. The % mean stability of LQC and HQC sample should be within 85.00-115.00 %.

Table 10: Results for Bench top Stability Study.

Replicate No.	Nominal concentrations			
	LQC (2.5 µg/ml)		HQC (20 µg/ml)	
	Comparison sample	Stability Sample	Comparison sample	Stability Sample
	Calculated concentrations (µg/ml)			
1	2.374	2.154	20.422	17.783
2	2.265	2.241	19.234	17.774
3	2.287	2.107	21.811	18.935
Mean	**2.309**	**2.167**	**20.489**	**18.164**
SD	**0.0575**	**0.0679**	**1.2899**	**0.6679**
% CV	**2.49**	**3.14**	**6.30**	**3.68**
% Mean Stability	93.86		88.65	

Acceptance Criteria: The % CV for LQC and HQC should be sample should be ≤ 15.00. The % mean stability of LQC and HQC sample should be within 85.00-115.00 %.

Precision: The precision of this method was evaluated by the % CV at different concentration levels corresponding to LQC, MQC and HQC during the course of validation.

A] Inter Day Precision (Reproducibility): The % CV of calculated concentrations for all quality control samples of LQC, MQC and HQC concentration levels are ranged from 2.24 to 7.15 %, which is within the acceptance limit of 15.00%. The reproducibility (inter-assay precision) was evaluated in three replicates for three different concentrations of ETO (2.5, 10, 20 µg/ml) on three consecutive days

Table 11: Results for Stock solution Stability Study.

Replicate No.	Nominal concentrations			
	LQC (0.5 µg/ml)		HQC (4 µg/ml)	
	Comparison sample	Stability sample	Comparison sample	Stability sample
	Calculated concentrations (µg/ml)			
1	2.315	2.285	18.790	18.583
2	2.212	2.167	19.047	18.179
3	2.231	2.046	19.755	17.417
Mean	**2.253**	**2.166**	**19.197**	**18.059**
SD	**0.0549**	**0.1196**	**0.5000**	**0.5916**
% CV	**2.44**	**5.52**	**2.60**	**3.28**
% Mean stability	**96.14**		**94.07**	

Acceptance Criteria: The % mean solution stability for drug should be within range 90.00-110.00 %.

(fresh samples were prepared every day). The results, expressed as mean amounts of drug found in plasma and summarized in the Table 6.

B] Intra Day Precision (Repeatability): The % CV of calculated concentrations for all quality control samples at LQC, MQC and HQC concentration levels are ranged from 3.26 to 3.67 %, which is within acceptance limit 15.00 % as shown in Table 7.

Recovery: The % mean recovery for ETO at HQC, MQC and LQC levels are found to be 97.85%, 99.89% and 94.86% respectively. Over all % CV at all QC levels is 2.59%, which is within the acceptance limit of 15.00% and % over all mean recovery is 97.53%, which is within the acceptance limit of 20.00%. The results are summarized in the Table 8.

Stability

Freeze and Thaw stability: The % mean stability for HQC (20µg/ml) and LQC (2.5µg/ml) are found to be 86.18% and 94.47% respectively, which is within the acceptance limit of 85.00 - 115.00 %. The results are summarized in the Table 9.

Bench top stability: The % mean stability for HQC (20µg/ml) and LQC (2.5µg/ml) are found to be 88.65% and 93.86% respectively, which is within the acceptance limit of 85.00 - 115.00 %. The results are summarized in the Table 10.

Stock solution stability: The % mean stability for HQC (20µg/ml) and LQC (2.5µg/ml) are found to be 94.07% and 96.14% respectively, which is within the acceptance limit of 90.00 - 110.00 %. The results are summarized in the Table 11.

Conclusion

This study presents a simple and validated HPLC method for estimation of Etodolac from rat plasma.

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