BIO ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF CONCURRENT QUANTIFICATION OF ACECLOFENAC AND THIOCOLCHICOSIDE IN HUMAN PLASMA BY LC-MS/MS

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ABSTRACT

A simple, fast, selective, and precise solid-liquid chromatography-MS/MS method has been established and validated for concurrent quantification of Aceclofenac and Thiocolchicoside in human plasma and it was enriched with the help of Protein Precipitation technique. Hibar® C18 (5µm, 50 x 4.6 mm i.d.) column used as adsorbent and the following eluant is used to separate the aceclofenac and thiocolchicoside by using Cyanomethane and 10mM Ammonium acetate buffer (pH 4.0) with 80:20 v/v ratio and the elution rate was fixed 0.5ml/min and the detection was performed by Triple quadrupole LC-MS/MS using electron spray ionization as positive and negative mode. The calibration curve was consistently accurate for the Aceclofenac and Thiocolchicoside between the range of 785 to 15700ng/ml and 6 to 120ng/ml using Etodolac as the internal standard. Aceclofenac detected (LOD) and quantified (LOQ) 1.5ng/ml and 4.5ng/ml and similarly, thiocolchicoside was detected 1ng/ml and quantified 3ng/ml. The precision, accuracy and the correlation coefficient (R^2) of Aceclofenac and Diclofenac are within the limits as per USFDA guidelines, so this can be used for the quantification of Aceclofenac and Thiocolchicoside in Human plasma for the study of biomedical and biopharmaceutical sciences.

Keywords: LC-MS/MS, Bio-analytical, Aceclofenac, Thiocolchicoside, Human Plasma.

INTRODUCTION

Nowadays there are many people are severely suffering from joint pain or rheumatic pain traumatological pain or orthopedic pain with inflammations. To treat there are various drugs and drug combinations are available in the market. There are few combinations are available in the market which are approved by the CDSCO/DCGI in India. Aceclofenac and Thiocolchicoside is one of the combinations among them that is currently available in the market to treat the pain.

Aceclofenac potent non-steroidal anti-inflammatory agent that inhibits COX-2 and prostaglandin E_2 (PGE_2) synthesis in blood mononuclear and polymorphonuclear cells due to this easily penetrates inflammatory tissues of joints and thus exhibits an excellent action of inhibiting Prostaglandins production as compared to other anti-inflammatory analgesics drugs. *Thiocolchicoside* is an Angiotensin Receptor Antagonists (ARBs) that inhibits the binding of angiotensin II to Angiotensin1 (AT1), which is found in many tissues such as vascular smooth muscle and the adrenal glands. It has selective affinity for γ-aminobutyric acid (GABA) receptors and acts on the muscular contraction by activating the GABA inhibitory pathways, thereby acting as a potent muscular relaxant.

Anti Inflammatory and analgesics are often used in combination with muscle relaxants for symptomatic relief of low back pain. This combination is used in rheumatologic or orthopedic or traumatological pain. Our aim is to develop single method for the multiple drug or drug in fixed dose combination. Keeping this in our mind we want to establish the method for the combination of drugs which is available in the market. In this connection we have chosen the anti-inflammatory drugs which are available in the combination many moleucles are available in the combination but we have chosen the new fixed dose combination. We selected 3 drugs with

combination like Thiocolchicoside, Aceclofenac, and Etodolac. Bio-analysis helps for the quantitative measurement of the combination in the biological system like plasma, blood, saliva, urine, etc. The methods developed with this combination can be used for bioavailability and bioequivalence studies, therapeutic drug monitoring and new drug combination studies. The method developed using an internal standard Etodolac which is a non-steroidal anti-inflammatory drug and a potent analgesic. The structures of three drugs are given in figures1, 2, and 3.

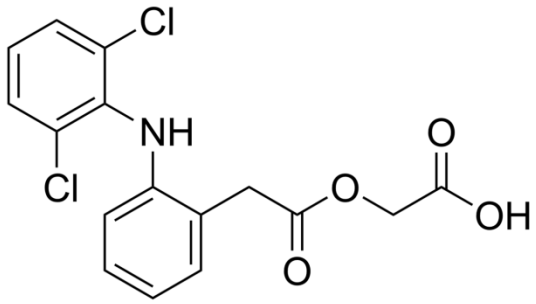

Fig. 1 -ACECLOFENAC

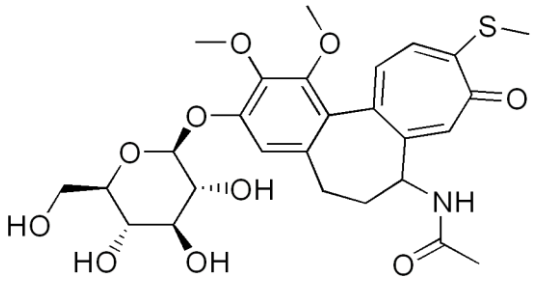

Fig. 2 THIOCOLCHICOSIDE

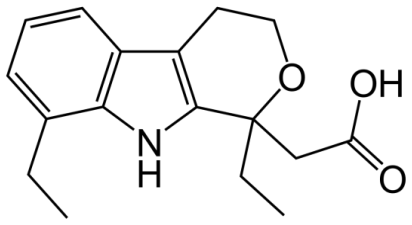

Fig. 3- ETODOLAC

The objective of the study is aimed at developing and validating the New Bio-analytical method which is to be suspected as accurate, precise, linear, selective and stable as per USFDA guidelines for the concurrent estimation of Aceclofenac and Thiocolchicoside in human plasma using LC-MS/MS method.

EXPERIMENTAL METHODS

Chemicals and Eluent Used

Cyanomethane of LC grade and Ammonium Acetate of Analytical grade was procured from S.D. Fine Chemicals Ltd and LC grade aqua from the Milli-Q system were used. Working Standard of Aceclofenac, Thiocolchicoside, and internal standard Etodolac obtained from SARV BIO LABS-Himachal Pradesh.

Instruments used

Weighing done by Sartorius digital balance (R200D & 1702) and pH detected by Systronics - pH meter. Shimadzu gradient HPLC system used with LC-10 AT-VP as a pump, SPD M-10AVP as Photo Diode Array detector and Class-VP for obtaining the data after the analysis. UV detection is done by Shimadzu (UV-1700) spectrophotometer. Shimadzu LC-MS/MS 8030 for mass analysis with Lab solution data station, Triple Quadrupole analyzer and ESI interface. Ultra Sonicator, Centrifuge, and Vortexer also used during the process. Analytical columns used were Hibar® C18 (5µ, 50 x 4.6 mm i.d.) and Zorbax C18 (5µ, 50 x 4.6 mm i.d.).

Separation conditions:

LC/MS Conditions

Stationary Phase was Hibar® C18 (5µ, 50 x 4.6 mm i.d.) and eluant was Ammonium acetate: Cyanomethane (20:80, V/V) pH 4.0 at a Flow rate of 0.5 ml/min. The injection volume was 10 µl with ambient temperature with a mass detector and run time 3.0 minutes.

Electrospray Ionization with Negative and Positive Mode used as an ion source that provides negative polarity for Aceclofenac and Thiocolchicoside and Etodolac as the positive mode. The probe temperature was ambient and 250 ºC as Desolvation Line (DL) temperature. The retention time of Aceclofenac, Thiocolchicoside, and Etodolac was 1.135 min, 1.171 min, and 1.321 min respectively. Scan type was MRM (Multiple Reactions Monitoring).

Eluent preparation for the separation:

Cyanomethane : 10mM Ammonium Acetate (pH4.0), ration 80:20 V/V

Composition of Aceclofenac, Thiocolchicoside, and Etodoloc concentrated stock solution

10.0 mg of Aceclofenac was weighed and shited into a 10.0 ml standard flask. Dissolved the Aceclofenac with cyanomethane and made the volume with the same solvent and homogenized well by shaking the volumetric flask. Similarly, Thiocolchicoside and Etodolac stock solutions were prepared respectively to 1.0mg/ml by using cyanomethane. Labeled both the container and stored at 2 to 8 °C.

Composition of Aceclofenac and Thiocolchicoside working standard solution:

Prepared 10.0 ml each of 785.0, 4710.0 and 15700ng/ml of Aceclofenac standard solutions and 6.0, 36.0, and 120.0ng/ml of Thiocolchicoside solution using the respective stock solutions. The solutions were made the volume with mobile phase and were stored at –20 ± 2°C.

Composition of Linearity and range samples (CC):

Prepared, 10.0 ml each of 785, 1570, 2355, 3140, 4710, 6280, 9420, 12560, and 15700ng/ml of Aceclofenac calibration curve samples using the Aceclofenac standard stock solution and 10.0 ml each of 6, 12, 18, 24, 36, 48, 72, 96, and 120ng/ml of Thiocolchicoside calibration curve samples using Thiocolchicoside standard stock solution diluted with eluent and stored at –20 ± 2˚C until analysis.

Composition of Quality control (QC) Samples:

Prepared 10.0 ml each of 785.0, 4710.0 and 15700.0 ng/ml of Aceclofenac and 6, 36, 120ng/ml of Thiocolchicoside Quality control samples using the standard stock solution and by using eluent and stored at – 70 ± 2°C.

Validation Methodology

Validation of the method was performed as per USFDA guidelines.

Protocol for sample preparation:

Prepared calibration curve sample and quality control sample solution. Transferred 0.1 ml of both samples separately into a 2.0ml centrifuge tube and added 0.1ml of 100ng/ml of IS, 0.5ml blank plasma, 0.3ml of precipitating agent. Vortexed the resulting solution for 30 seconds and

centrifuged at 3500 r/min for 10 minutes, separated the supernatant layer and preserved for the analysis.

RESULTS AND DISCUSSIONS

Validation of the method:

The main motto of the selected study is to establish a bio-analytical method for the concurrent estimation of Aceclofenac and Thiocolchicoside using LC-MS/MS.

Accuracy of the established was determined by relative and absolute recovery experiments. The recovery values for Aceclofenac in plasma were ranged from 90.4 to 92.6% and similarly for Thiocolchicoside in plasma solution were ranged from 94.6 to 95.7%. It was, therefore, derived that the established method was accurate and reliable (Table 1)

Table 1- RECOVERY STUDIES OF ACECLOFENAC AND THIOCOLCHICOSIDE

Concentration (ng/ml)	**Aceclofenac**		
	Mean (ng/ml) ± SD	**CV (%)**	**Accuracy (%)**
LQC(785)	710±2.83	0.39	90.43
MQC(4710)	4352.354±4.6	0.10	92.4
HQC(15700)	14549±43.2	0.30	92.66
Concentration (ng/ml)	**Thiocolchicoside**		
	Mean (ng/ml) ± SD	**CV (%)**	**Accuracy (%)**
LQC(6)	5.67±0.02	0.4	94.6
MQC(36)	34.1±0.16	0.48	94.9
HQC(120)	114.8±0.3	0.32	94.7

The estimation of the drugs was found to be precise with the methods optimized. This was evident from the coefficient of variation values, which were in limits for all concentration (Table 2)

Table 2-PRECISION STUDIES OF ACECLOFENAC AND THIOCOLCHICOSIDE

Analyte	QCs (ng/ml)	Mean concentration found (ng/ml) ± SD	Intra-day	
			Accuracy (%)	Precision (% CV)
Aceclofenac	LQC(785)	716.85±5.9	91.32	0.8
	MQC(4710)	4337.73±14.03	92.09	0.32
	HQC(15700)	14584.33±13.27	92.89	0.09
Thiocolchicoside	LQC(6)	5.57±0.06	92.83	1.22
	MQC(36)	35.13±0.58	97.58	1.66
	HQC(120)	114.51±0.77	95.4	0.6
Analyte	QCs (ng/ml)	Mean concentration found (ng/ml) ± SD	Intra-day	
			Accuracy (%)	Precision (% CV)
Aceclofenac	LQC(785)	728.05±5.17	92.74	0.7
	MQC(4710)	4354.04±19.30	92.44	0.44
	HQC(15700)	14593.78±6.36	92.94	0.04
Thiocolchicoside	LQC(6)	5.67±0.06	94.50	1.18
	MQC(36)	34.77±0.33	96.58	0.95
	HQC(120)	113.82±1.24	94.85	1.09

Six samples were analyzed and recorded the chromatograms. No endogenous interferences detected at the selected drugs and internal standard during the retention time. No observation of additional peaks in the sample chromatograms.

The Retention time was 1.135 min, 1.171 min and 1.321 respectively for Aceclofenac and Thiocolchicoside and etodolac.

The linearity and range for Aceclofenac between 785 – 15700 ng/ml and similarly for Thiocolchicoside the range between 6 - 120 ng/ml. The R2 value was found to be 0.9956 and 0.9907 respectively (Table 3) and it shows linear.

Table 3- LINEARITY & RANGE

S. No.	Aceclofenac (ng/ml)	Thiocolchicoside (ng/ml)	Internal Standard (ng/ml)	Response Factor (Aceclofenac)	Response Factor (Thiocolchicoside)
1	785	6	10	0.86	0.18
2	1570	12	10	1.31	0.43
3	2355	18	10	1.87	0.54
4	3140	24	10	2.35	0.52
5	4710	36	10	3.12	0.87
6	6280	48	10	4.48	1.13
7	9420	72	10	6.67	1.44
8	12560	96	10	9.68	1.94
9	15700	120	10	11.78	2.56

The stability of the drug spiked human plasma samples at three levels was studied for three freeze-thaw cycles and similarly for short term (6 hours) and benchtop (8 h) stability studies. The results showed that the selected drugs were stable in plasma when stored at frozen state (Table 4a, 4b, 4c).

Table 4a- STABILITY OF ACECLOFENAC AND THIOCOLCHICOSIDE IN PLASMA DURINGSTORAGE AND SAMPLE HANDLING

Freeze and Thaw Stability data

Aceclofenac				Thiocolchicoside			
Sl.No	**LQC (785 ng/ml)**	**MQC (4710 ng/ml)**	**HQC (15700 ng/ml)**	**Sl. No**	**LQC (6ng/ml)**	**MQC (36 ng/ml)**	**HQC (120 ng/ml)**
1	717.02	4355.84	14615.25	**1**	5.758	34.163	115.84
2	725.56	4368.92	14625.32	**2**	5.982	35.254	115.78
3	735.84	4372.25	14645.19	**3**	5.613	35.596	116.25
4	748.14	4379.1	14662.45	**4**	5.611	35.11	116.35
5	754.02	4385.23	14684.72	**5**	5.823	34.685	116.46
6	768.50	4389.05	14692.36	**6**	5.912	34.998	116.16
Mean	741.51	4375.06	14654.15	**Mean**	5.78	34.96	116.17
S.D (+/-)	16.1	10.21	26.35	**S.D (+/-)**	0.12	0.41	0.23
C.V	2.1	0.23	0.17	**C.V**	2.23	1.19	0.21

Table 4b- STABILITY OF ACECLOFENAC AND THIOCOLCHICOSIDE IN PLASMA DURING STORAGE AND SAMPLE HANDLING

Short Term Stability data (at ambient temperature)

Aceclofenac				Thiocolchicoside			
Sl.No	**LQC (785 ng/ml)**	**MQC (471 ng/ml)**	**HQC (15700 ng/ml)**	**Sl.No**	**LQC (6 ng/ml)**	**MQC (36 ng/ml)**	**HQC (120ng/ml)**
1	718.25	4367.45	14698.12	**1**	4.96	34.258	114.99
2	729.48	4375.29	14725.51	**2**	5.23	35.095	115.021
3	732.80	4381.5	14738.91	**3**	5.38	35.335	115.225
Mean	726.84	4374.74	14720.84	**Mean**	5.19	34.89	115.08
S.D (+/-)	0.8	4.97	14.7	**S.D (+/-)**	0.15	0.39	0.08
C.V	6.7	0.11	0.09	**C.V**	2.8	1.14	0.07

Table 4c- STABILITY OF ACECLOFENAC AND THIOCOLCHICOSIDEIN PLASMA DURINGSTORAGE AND SAMPLE HANDLING

Bench Top Stability data

Aceclofenac				Thiocolchicoside			
Sl.No	**LQC (785 ng/ml)**	**MQC (4710 ng/ml)**	**HQC (15700 ng/ml)**	**Sl.No**	**LQC (6 ng/ml)**	**MQC (36 ng/ml)**	**HQC (120 ng/ml)**
1	746.13	4354.84	14745.54	**1**	4.98	34.26	114.58
2	758.48	4367.3	14752.85	**2**	5.27	34.84	115.11
3	765.02	4379.52	14766.90	**3**	5.41	35.11	115.25
Mean	756.54	4367.22	14755.09	**Mean**	5.22	34.74	114.98
S.D (+/-)	6.78	8.7	7.67	**S.D (+/-)**	0.15	0.3	0.25
C.V	0.8	0.19	0.05	**C.V**	2.97	0.8	0.21

The limit of detection (LOD) values for Aceclofenac and Thiocolchicoside were found to be 1.5ng/ml and 1ng/ml respectively and their limit of quantification (LOQ) value was 4.5ng/ml and 3ng/ml respectively. (Table 5)

By changing the experimental conditions like pH, column and the analyst, we have studied the robustness and ruggedness. No specific changes in the separation parameters were observed

when changing the experimental conditions and optimized conditions. The System suitability parameters such as column efficiency (theoretical plates), resolution factor of the optimized methods were found satisfactory (Table 5)

Table 5- SYSTEM SUITABILITY PARAMETERS

Parameters	Aceclofenac	Thiocolchicoside
Linearity and Range	785 - 15700 ng/ml	6 - 120 ng/ml
Correlation Factor	0.9956	0.9907
Theoretical Plates	6956	3694
Asymmetric factor	1.04	1
Limit of Detection (LOD)	1.5ng/ml	1ng/ml
Limit of Quantification (LOQ)	4.5ng/ml	3ng/ml

Estimation of Aceclofenac and Thiocolchicoside by LC-MS/S method:

The standards were injected and chromatograms, spectra were recorded. The typical spectra and chromatograms of the Aceclofenac, Thiocolchicoside, and Etodolac (IS) are given in Fig. 4-11.

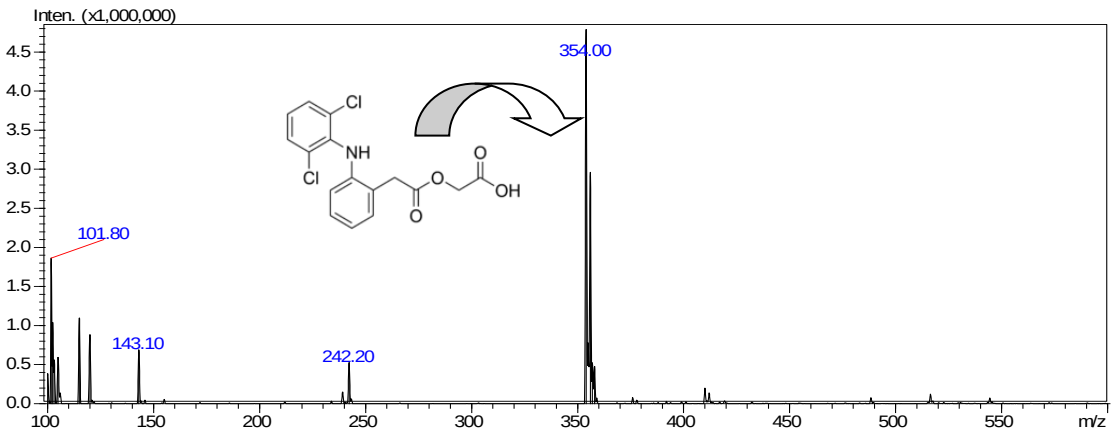

Fig.4: Typical standard ms -spectrum of Aceclofenac

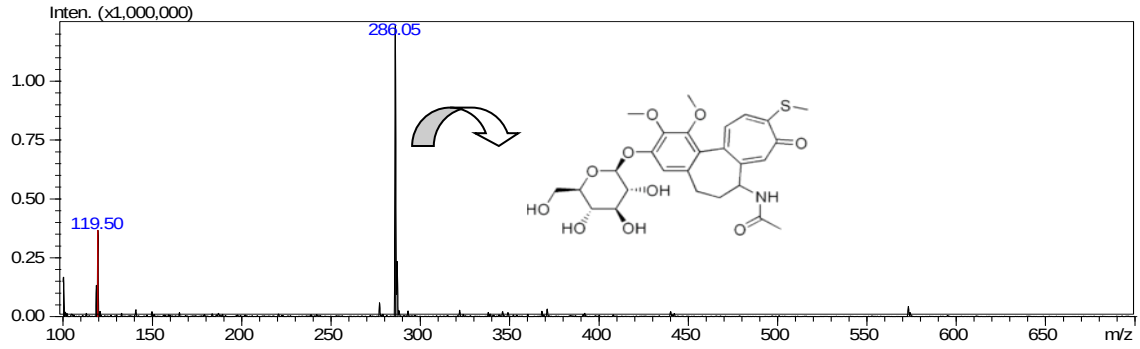

Fig.5: Typical standard ms -spectrum of Thiocolchicoside

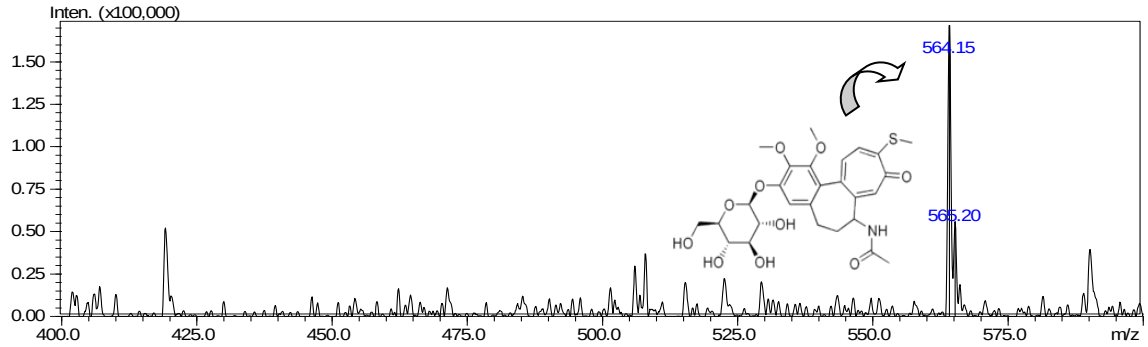

Fig.6: Typical standard ms -spectrum of Etedolac

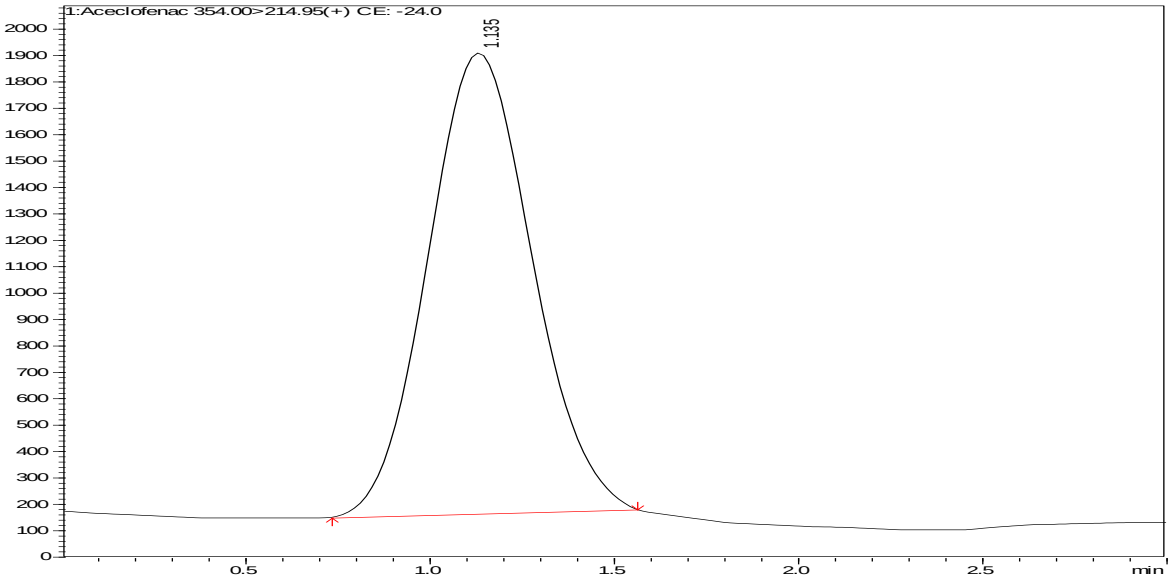

Fig.7: Typical standard chromatogram of Aceclofenac in Plasma

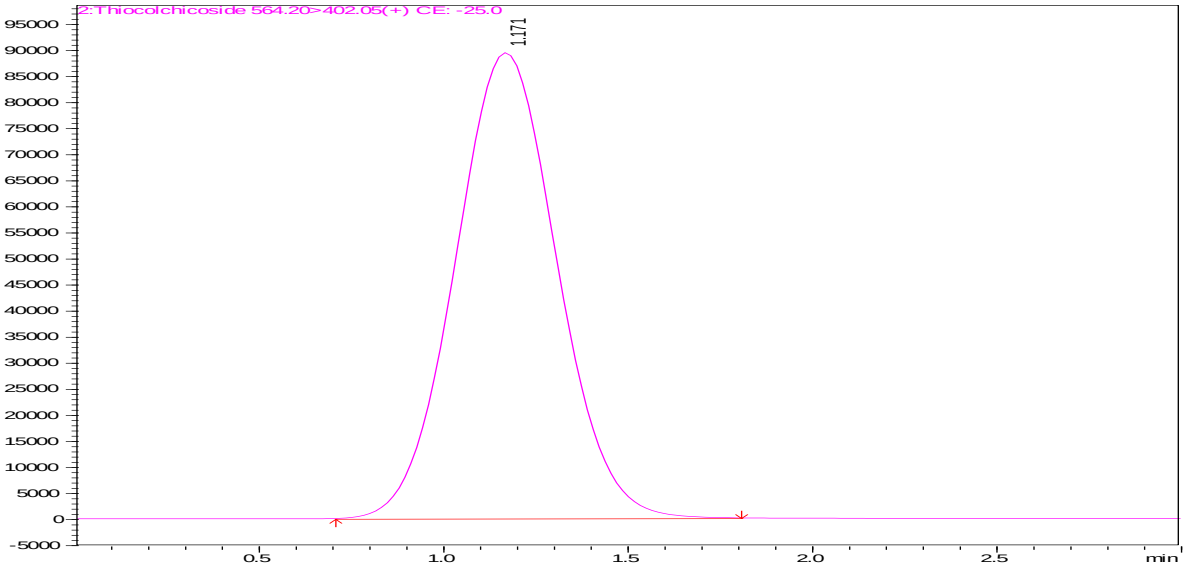

Fig.8: Typical standard chromatogram of Thiocolchicoside in Plasma

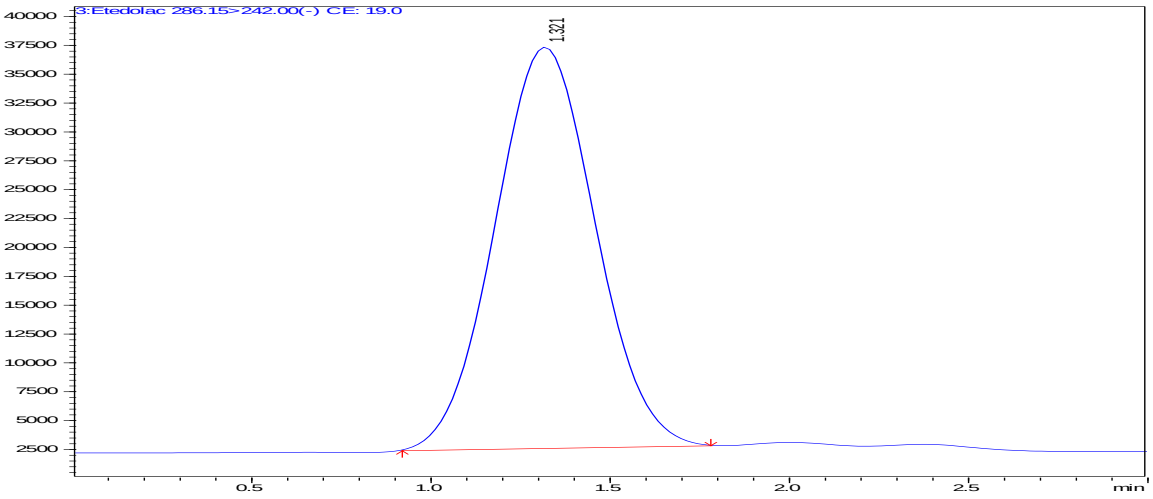

Fig.9: Typical standard chromatogram of Etedolac in Plasama

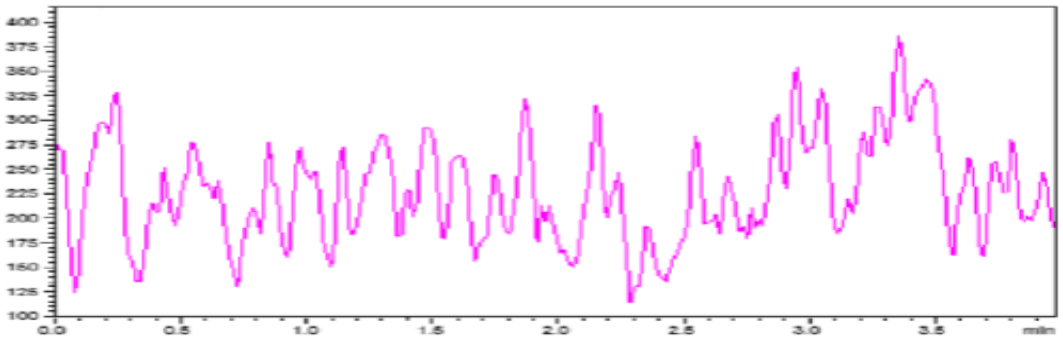

Fig.10: Typical chromatogram of blank plasma

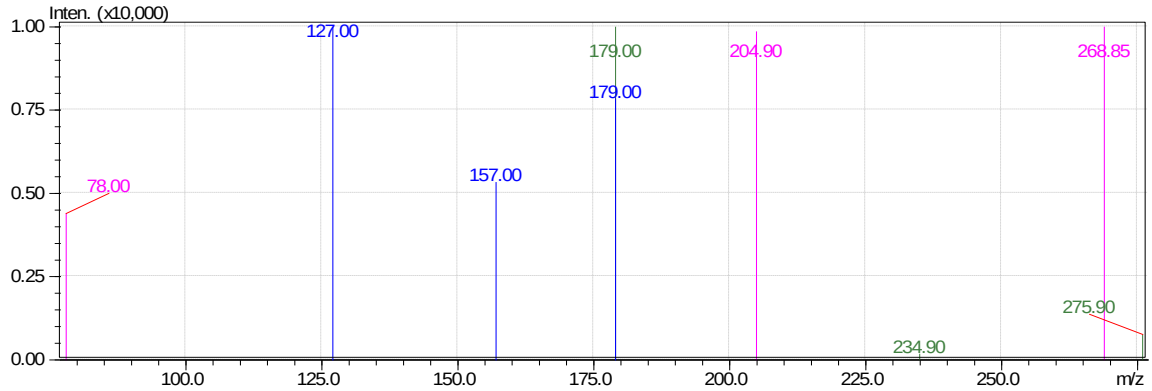

Fig11 : Product ion scan of Acecloenac, Thiocolchicoside and Etedolac(IS)

The linearity curves (fig. 12, 13) were constructed regularly for spiked plasma containing Aceclofenac and Thiocolchicoside and internal standard during the process of pre-study validation and in-study validation.

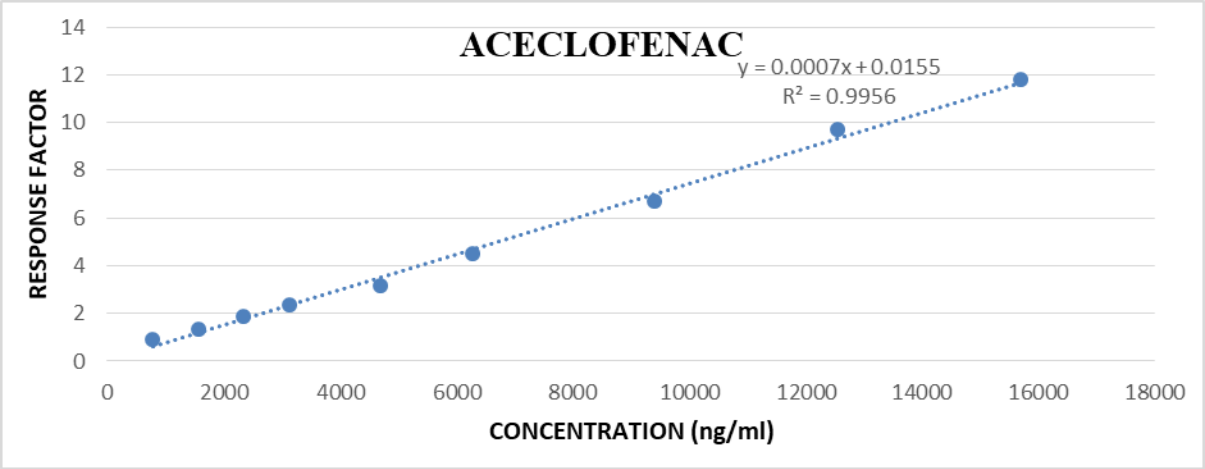

Fig.12: Calibration curve of Aceclofenac

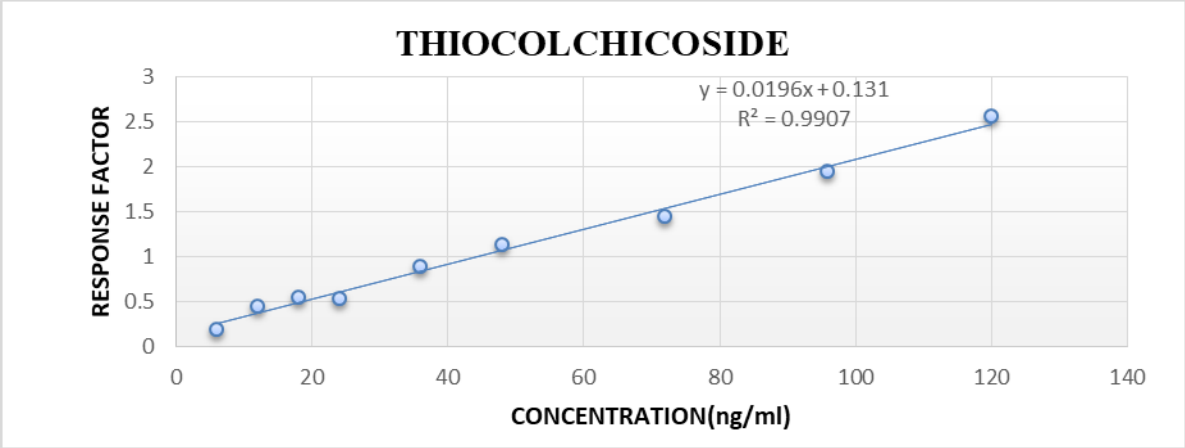

Fig.13: Calibration curve of Thiocolchicoside

The eluent that used for the establishment of the method provided a well-defined separation between the Aceclofenac, Thiocolchicoside and Etodolac, and endogenous components. No interference has been observed at the retention time of Aceclofenac, Thiocolchicoside, and Etodolac.

The response factor of Aceclofenac and Thiocolchicoside was calculated. The concentration of Aceclofenac and Thiocolchicoside present in plasma samples were calculated and presented.

CONCLUSION

Based on the above experiments performed, it can be concluded that the intended method was validated for Aceclofenac and Thiocolchicoside estimation. The validated methods are developed as per USFDA guidelines. The validated methods such as ruggedness, robustness, stability studies, accuracy (90.43 to 92.66% for Aceclofenac and 94.6 to 94.90% for Thiocholchicoside), and precision (% for Aceclofenac and % for Thiocholchicoside were within the acceptable limits. Limit of Detection and Quantification for the these drugs are 1.5 ng/ml and 4.5ng/ml for Aceclofenac and 1ng/ml and 3ng/ml for Thiocolchicoside. So, the article can be concluded that the method can be used for sample subject analysis. The method was found to be established, and it can be used for the individual drug analysis of the selected drugs or in the combination form like Aceclofenac and Thiocolchicoside or Thiocolchicoside and Etodolac or Aceclofenac and Etodolac quantification in human plasma, especially for Bioequivalence studies.