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STABILITY INDICATING METHOD DEVELOPMENT OF RP-UPLC, VALIDATION OF SIMULTANEOUS QUANTITATION OF BUPIVACAINE and MELOXICAM IN PURE AND FORMULATION

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ABSTRACT
An easy, accurate, and precise RP-UPLC was established and also validated for simultaneous quantitation of Bupivacaine and Meloxicam in the formulation as well as in pure form. An acceptable separation of Bupivacaine and Meloxicam was shown by the developed technique Bupivacaine and Meloxicam retention times were 1.359 min. and 3.687 min. correspondingly. From regression equations, LOQ and LOD were obtained and observed to be 0.2ppm and 0.6ppm - Bupivacaine, 0.06 and 0.0 I 8ppm - Meloxicam, respectively. Stability studies of the injectable dosage form under forced degradation conditions of acid, alkali, peroxide, thermal, hydrolysis, and ultraviolet rays showed that the technique was particular without interferences from any type of possible contaminations that might form over the shelf life. The newly established RP-UPLC technique was stability indicating, accurate, rapid, and sensitive, for active pharmaceutical ingredients and pharmaceutical dosage forms.

Keywords: Bupivacaine, Meloxicam, Accurate, Simultaneous, RP – UPLC.
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INTRODUCTION

Bupivacaine (Fig.-I)(% purity - 99.99) chemically known as "(RS) - I -butyl-N -(2,6- dimethyl phenyl) piperidine-2-carboxamide", isa potent long-lasting local anesthetic belongs to amide group. lt works by blocking the transmission of nerve impulses from motor nerve endings (higher doses) and sensory nerve endings.[1,2]Bupivacaine HCL is a white crystal-clear powder slightly soluble in chloroform or acetone or ether, soluble in water, and freely soluble in ninety-five percent (95 %) ethanol.[3-6] Bupivacaine (BP) is used for post-operative analgesia, intraoperative local anesthesia, and in the therapy of obstetrics and chronicpain.[7]

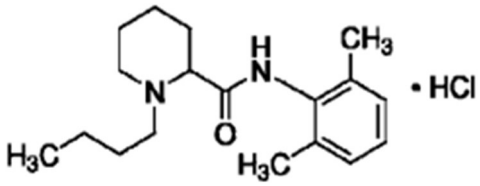

Fig.-1: Chemical structure of Bupivacaine Hydrochloride

Meloxicam (Fig.-2) (% purity - 99.98) is chemically designated as 4- hydroxy-2-methyl-N-(5-methyl-1,3-thiazol-2-yl)-2H-1,2-benzothiazine-3-carboxamide-1, 1-dioxide). It's empirical formula is $C_{14}H_{13}N_3O_4S_2$. The molecular weight is 351.4 g/mol.[8] Meloxicam is an enol carboxamide NSAID similar to piroxicam. It is a solid, pastel-colored crystalline powder. It's higher solubility is observed in strong acids and bases, very slightly soluble in methanol, and practically insoluble in water.[9]

Meloxicam is widely used in the treatment of spondylitis, osteoarthritis, rheumatoid arthritis, colorectal polyps, and cancer, in addition to its antipyretic and analgesic effects.[10,11]In order to separate the combined

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formulation Meloxicam and Pentaprazole, Raneem Ahmad used a mobile phase - phosphate buffer/acetate in the ratio of 30:70 v/v and adjusted 1.0 mL/min flow rate (25 •C). The wavelength detection was at 310 nm. Meloxicam and Pantoprazole retention times were 6 and 9 min. The linearity of drugs ranges from 0.1 to 200 mg/L.[12]Nalini Kanta Sahoo achieved results on the "Develosil ODS HG -5 RPC l8" analytical column (150 mm x 4.6 mm internal diameter, 5 µm) with mobile-phase, Acelonilrile: Phosphate buffer in the ratio of 60:40 and a flow rate was adjusted to l mL/min.

Fig.-2: Chemical structure of Meloxicam

The wavelength detection was done at 268 nanometers. Linearity was established in the range of 20 — 120 µg/ml.[13] Madhu Sudhan Reddy validated the results on "Xterra symmetry C 18 column" (l0cm x4.6 mm, 5 µm) with a mobile phase composed of phosphate buffer: methanol (50: 50 v/v), and the flow rate was arranged at 0.8 L/min and eluents detected at 244nm.[14] Muhammad Zaman proposed the HPLC process with a "Kromasil C18 column" with dimensions of (25cm long x 4.6) mm id,5µm particle size), Methanol: Water (80: 20) v/v) was used as a mobile phase. Mobile phase pH was altered to 3.0 by using OPA and combination drugs were monitored at 228 nm. The flow rate was adjusted to 0.8 mL/min. The retention time of Tinidazole HCL was 2.613 min and of Meloxicam was 6.992 min with a resolution of 3.23 and the linearity in the range of 5-50 µg/mL[15]. PCHGS Prathyusha, employed HT C18 Column (50mm x 4.6mm, 1.8µm) using 5:40:650 v/v/v (Phosphate buffer, ACN, and Milli Q water) as a mobile phase, a flow rate was altered to 1.0 ml/min. The detected wavelength was 234 nm. at 45°C column temperature[16]. Konda Sidda Reddy presented a simultaneous estimation on UPLC-MS with an "Acquity HSS T3 column" (2.1 x 50 mm, 1.8 µm) and also mobile phase A (10 mM Ammonium Formate) and Mobile phase B – Acetonitrile: water: Formic acid (96:5:0.2, v/v/v).[17]The aim of the Particular research work is to develop, and validate an easy, accurate, quick, sensitive reverse phase UPLC, stability-indicating method for the simultaneous quantitation of Bupivacaine and Meloxicam in commercial products as well as in active pharmaceutical ingredients. Additionally, it aims to conduct degradation studies of pharmaceutical dosage form under forced degradation conditions of acid, alkali, peroxide, dry heat, UV light, and neutral to show that the method is distinct without any interference. The goal of this study is to create and validate a quick, sensitive, accurate, stability-indicating, and simple RP-UPLC method for the simultaneous estimation of Bupivacaine and Meloxicam in Pharmaceutical formulation as well as the active pharmaceutical ingredients. Additionally, it aims to conduct degradation studies of pharmaceutical dosage form under forced stress conditions of oxidation, acid, base, temperature, water, and UV light to show that the method is specific without any interference.

EXPERIMENTAL

Material and Methods

Bupivacaine and Meloxicam pure bulk drugs (API), Bupivacaine and Meloxicam combined fixed dosage form injection (Zynrelef*)*, distilled water, acetonitrile, Tri ethanolamine, Ortho phosphoric acid buffer. All the chemicals and reagents listed above were obtained from Rankem.

Instrumentation and Equipment

The chemicals were weighed on electronics balance-Denver TL-64. Eutech pH 700 - pH meter was used to measure the pH of the buffer. The solutions were sonicated or degassed using an ultra sonicator - Eutech Enterprises. The chromatographic analytical method was carried out with Agilent 1290 Infinity II model LC system and the signal obtained was detected using PDA detector with empower - 2 software. The absorbances of drugs were measured using UV-VIS spectrophotometer PG instruments T60 with UV win 6 software programs.

Preparation of Buffer

1ml Tri ethanol amine (TEA) mixed in 1 liter of water and adjust its pH to 2.5 with Phosphoric acid (H_3PO_4).

Method of Preparation of Mobile Phase
Based on drugs solubility diluent was selected. Acetonitrile and buffer were mixed in the proportion of 50:50 and passed over a 0.45µ pore size filter.

Method of Preparation of Standard Solution
Weigh exactly 200mg of Bupivacaine and 6mg of Meloxicam working standards, transfer into100ml volumetric flask then add diluent (70ml), sonicate for 10min to get solubilize the contents and finally add the diluent up to the mark.

Method of Preparation of Standard Working Solution
5 ml of stock solution (sample) was taken in a 50ml measuring container and diluent was added up to the mark. (200ppm Bupivacain and 6ppm Meloxicam).

Method of Preparation of Sample Solution
7 mL of sample was taken (equivalent to 200 mg of Bupivacaine and 6 mg of Meloxicam), into a 100 ml VF, then 70 mL of diluent was added, sonicated for a period of 30 minutes to dissolve the contents, added sufficient quantity of diluent to make up the mark

Chromatographic Conditions
A Waters Acquity UPLC quarternary pump with a Photo diode array detector and a Phenyl analytical column (100x2.1mm, 1.7µm) was used in the UPLC method development and validation. Acetonitrile: Tri ethanol amine in the proportion of 50:50 v/v, was used as a mobile phase, and the flow rate was adjusted to 0.5 ml/minute. The normal column temperature was maintained. The injection volume of the sample –is 10µl. The eluted compounds were monitored at 240nm.

Method Development
The mobile phase was pumped for around thirty minutes to saturate the column properly and correct the baseline. Various mobile phase ratios, buffers, and other parameters were changed to create the method.

Validation Parameters
Assay of Bupivacaine and Meloxicam
According to the RP-UPLC assay method, the commercial formulation of Bupivacaine (200ppm) and Meloxicam (6ppm) injection was assayed. Six sample sets were prepared and analyzed.

Linearity and Range
Six reference solutions with concentrations of approximately 50–300 ppm of Bupivacaine and 1.5–9 ppm of Meloxicam—corresponding to 25, 50, 75, 100, 125, and 150%—were created to show the linearity, range of the reverse phase UPLC assay technique. The prepared solutions were injected into the chromatographic system to determine the correlation coefficient and graphs were plotted between response area and concentration to find out the 'correlation coefficient' (R^2)

Accuracy
By spiking known amounts of the drug analyte and calculating percent recovery, the method's accuracy was measured at 3 distinct concentration levels - 50 percent, 100 percent, and 150 percent.

Precision - Method Precision To determine the method precision six working standard solutions were used. All the injections 'peak areas were measured and calculated the SD and % RSD.

Intermediate Precision
Six working standard solutions were injected on separate days by different analysts or with different instruments to ascertain the intermediate precision. Then calculated the SD and % RSD.

LOD and LOQ
The lower quantification limit and the detection limit were determined by means of the following equations depending on the linear graph slope as well as the responses of standard deviation.
"LOD = 3.3 x Standard deviation/slope"; "LOQ = 10 × Standard deviation/slope"

System Suitability Parameters
Six (6) duplicates of working standard samples of Bupivacaine and Meloxicam were administered to assess system suitability, and characteristics such as plate number, tailing factor, resolution, and peak asymmetry of solutions were examined.

Robustness
Small adjustments in chromatographic settings like 'flow rate'(0.9 ml/minute and 1.1 ml/minute), and 'mobile phase' (55:45, 45:55) were used to test the method's robustness.

Specificity and Selectivity
The absence of adjuvant interference during the application of the planned approach to the study of pharmaceutical formulations demonstrated its selectivity. The method's specificity was assessed in terms of interference caused by the occurrence of any additional placebos. Two dissimilar samples were administered and compared to their placebo counterparts.

Forced Degradation Studies
Acid Degradation
1 ml Bupivacaine andMeloxicam (stock) solution was added to 1 ml of 2N HCl, then the obtained mixture was allowed to reflux for 30 min. at sixty degrees centigrade. The resulting composition was diluted to get 200ppm and 6ppm solutions, respectively, and solutions of ten micro liters were administered in the chromatographic system, to evaluate sample stability.

Base Degradation
A stock solution of 1 milliliter of Bupivacaine and Meloxicam. was mixed with 2NNaOH (1 ml) and refluxed at 60°C for 30 minutes. The obtained solution was diluted to get solutions of 200ppm and 6ppm and solutions of ten micro liters were administered in the chromatographic system, to determines ample stability.

Peroxide Degradation
Twenty percent of H_2O_2 (1ml) was mixed separately with one ml of stock solutionof Bupivacaine and Meloxicam. The solutions were maintained at 60°C for thirty minutes. The subsequent composition was diluted to get 200 ppm and 6 ppm solutions, and solutions of ten micro liters were administered in the chromatographic system, to evaluate the sample's stability.

Thermal Degradation
To evaluate heat degradation, the standard solution of the drug was heated in an oven at one hundred and five degrees Celsius (105°C) for a time duration of 1 hour. The resulting solution was diluted to 200ppm and 6ppm solution for the UPLC analysis, and solutions of ten micro liters were administered in the chromatographic system, to evaluate the stability of the sample.

Photo (UV light) Stability
The drug photo (UV light) stability was examined by subjecting 2000ppm and 60ppm solutions to ultraviolet luminescence for one day in a UV chamber or 200-Wh/m^2 in a light stability chamber. The obtained mixture was diluted to get 200ppm and 6ppm solutions for the UPLC investigation, and solutions of ten microliters were administered in the chromatographic system, to analyze the sample's stability.
Hydrolytic Degradation
The medication was refluxed in water about for 1 hour at a temperature of 60°C for stress testing under neutral conditions. For the UPLC investigation, the resulting composition was diluted to 200 ppm and 6 ppm, and 10 µl was administered into the chromatographic system, to determine the sample's stability.

RESULTS AND DISCUSSION

Assay
The research demonstrated that the developed UPLC technique was reliable and user-friendly enough to be utilized regularly. Bupivacaine and Meloxicam were determined to have % contents of 99.3 and 99.9, respectively. Table-1 presented the outcomes.

Table-1: Results of Marketed Formulation Analysis

Compound Name	Brand Name	Label Claim	% Content
Bupivacaine	Zynrelef	400mg	99.3
Meloxicam	Zynrelef	12ml	99.9

Linearity

Six linear concentrations of Bupivacaine (50-300 ppm) and Meloxicam (1.5-9 ppm) were administered in a twofold manner. The linearity equation for Bupivacaine was y = 11,370.02x + 18535.5 and for Meloxicam was y = 9794.64x + 729.54, and the peak areas were previously mentioned. The outcomes were displayed in Tables-2 and Table-3 and obtained graphs were displayed in Fig.-3, and Fig.-4.

Table-2: Bupivacaine and Meloxicam Linearity Studies

S.No.	Concentration (ppm)	Area Count	Concentration (ppm)	Area Count
1	50	611295	1.50	15864
2	100	1142112	3.00	30058
3	150	1756325	4.50	45230
4	200	2265157	6.00	60301
5	250	2864676	7.50	73695
6	300	3429409	9.00	88490

Table-3: Optical characteristics of Bupivacaine and Meloxicam

Parameters	Bupivacaine	Meloxicam
Linearity (ppm)	50-300 ppm	1.5-9 ppm
Regression equation	y = 11,370.02x+18535.5	y = 9794.64x + 729.54
Slope	11,370.02	9794.64
Intercept	18535.5	729.54
Correlation coefficient (R^2)	0.9998	0.9998

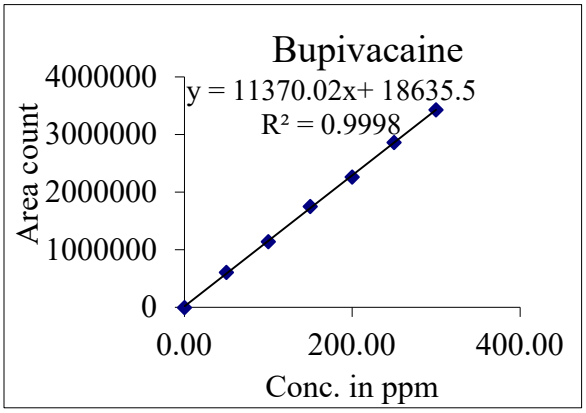


Fig.3: Linearity of Bupivacaine

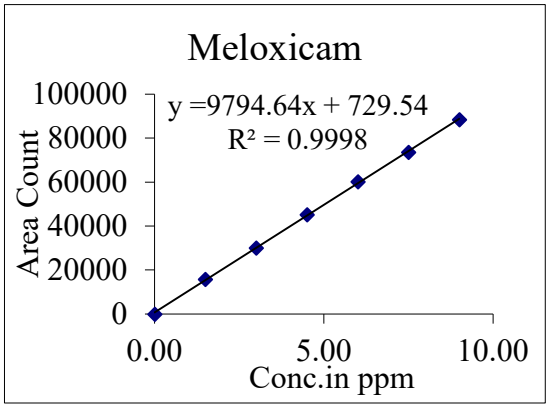


Fig.- 4: Linearity of Meloxicam

Accuracy

The method's accuracy was demonstrated using standard addition and recovery studies. The range of recovery was 98-102%. The results were shown in Tables-4and5. The mean percent recovery for Bupivacaine and Meloxicam was 100.3 percent and 100.2 percent, respectively.

Table-4: Recovery Studies of Bupivacaine

Accuracy- Bupivacaine				
% Level	Amount Added (mg)	Amount Recovered (mg)	% Recovery	Mean % Recovery
50	100	99.91	99.9	100.3
	100	101.66	101.7	
	100	99.54	99.5	
100	200	199.15	99.6	
	200	202.24	101.1	
	200	200.19	100.1	
150	300	296.03	98.7	
	300	300.99	100.3	
	300	304.83	101.6	

Table-5: Recovery studies of Meloxicam

Accuracy- Meloxicam				
% Level	Amount Added (mg)	Amount Recovered (mg)	% Recovery	Average% Recovery
50	3	3	100.0	100.2
	3	3.05	101.7	
	3	3.02	100.7	
100	6	6.02	100.3	
	6	6.04	100.7	
	6	5.98	99.7	
150	9	9.01	100.1	
	9	8.94	99.3	
	9	8.98	99.8	

Precision

For two drugs, the average area, SD, and percent RSD were calculated. % RSD yielded 1.01 percent and 0.46 percent for Bupivacaine and Meloxicam, respectively.

Table-6: Method Precision of Bupivacaine and Meloxicam

Method Precision		
S.No.	Area Count of Bupivacaine	Area Count of Meloxicam
1	2234872	60452
2	2224569	60979
3	2272318	60598
4	2254803	60218
5	2256562	60327
6	2286321	60734
Avg	2254907	60551
Std.dev	22867.12	279.194
%RSD	1.01	0.46

The average area, SD, and percent RSD for two medications were calculated. % RSD was 1.15 percent and 0.73 percent for Bupivacaine and Meloxicam, respectively. The method precision and intermediate precision results were shown in Table-6 and Table-7.LOD andLOQ data in Table-8 and system suitability data were shown in Table -9.

Table-7: Intermediate Precision of Bupivacaine and Meloxicam

Intermediate precision		
S.No.	Area Count of Bupivacaine	Area Count of Meloxicam
1	2267912	60618
2	2218345	60779
3	2253701	61392
4	2247263	60945
5	2294197	60397
6	2238549	60114
Avg	2253327.83	60707.5
Std.dev	25931.14	444.23
%RSD	1.15	0.73

Table-8: LOD and LOQ of Bupivacaine and Meloxicam

Drug	LOD (ppm)	LOQ (ppm)
Bupivacaine	0.6	0.2
Meloxicam	0.018	0.06

Robustness

In all of the robustness scenarios, there was a very minimal variance in the resolution and tailing factor values, demonstrating the method's robustness. The outcomes were displayed in Table-10.

Table-9: System Suitability Parameters Results

S. No.	Bupivacaine			Meloxicam		
Injection	Retention Time (Minute)	Plate Count of USP	Tailing	Retention Time (Minute)	Plate Count of USP	Tailing
1	1.359	3072	1.12	3.587	10999	1.04
2	1.352	3069	1.15	3.684	10987	1.06
3	1.358	3073	1.11	3.679	10976	1.05
4	1.354	3077	1.13	3.678	10991	1.04
5	1.351	3067	1.10	3.680	10998	1.08
6	1.356	3078	1.14	3.682	10984	1.02

Table-10: Robustness of Bupivacaine and Meloxicam

Parameter	% RSD of Bupivacaine	% RSD of Meloxicam
Flow(1.1ml/min)	0.75	0.35
Flow (0.9 ml/min)	0.77	0.77
Mobile phase ((55:45)	0.95	0.4
Mobile Phase (45:55)	0.68	0.36

Specificity and Selectivity

The UPLC chromatograms for the drug matrix (a mixture of the medication and the placebo) showed almost no interference peaks in the retention time ranges. As a result, the UPLC technique suggested in this study was specific. By examining the chromatograms of the placebo and blank samples for interference peaks, the method's specificity and selectivity were evaluated. The excipients prevented any interference peaks from occurring. The process was therefore selective and specific. Blank chromatogram as shown in Fig.-5 and placebo solution chromatogram as shown in Fig.-6. Optimized chromatogram shown in Fig.-7.

Forced Degradation Studies

Degradation experiments carried out in compliance with the ICH recommendations proved the method's capacity to indicate method stability. Bupivacaine and Meloxicam were shown to degrade to the greatest degrees (17.8% and 13.9%, respectively) when exposed to peroxide conditions. Bupivacaine and Meloxicam both underwent minimal degradation when exposed to water, with Bupivacaine experiencing a degradation of 2.9% and Meloxicam experiencing a degradation of 3.3%. There were no interferences in the retention times of bupivacaine and meloxicam under any of the conditions of degradation. Table-11 and Fig.-8 provides specifics on Bupivacaine and Meloxicam's degradation under various stress circumstances.

Table-11: Degradation data of Bupivacaine and Meloxicam

S. No.	Degradation Condition	Degradation of Bupivacaine		Degradation of Meloxicam	
		% Drug undegraded	% Drug degraded	% Drug undegraded	% Drug degraded
1	Acid	87.5	12.5	86.9	13.1
2	Alkali	86.7	13.3	87.7	12.3
3	Peroxide	82.2	17.8	86.1	13.9
4	Reduction	88.3	11.7	90.7	9.3
5	Thermal	89.8	10.2	93.3	6.7
6	UV	96.2	3.8	96.7	3.3
7	Water	97.1	2.9	95.2	4.8

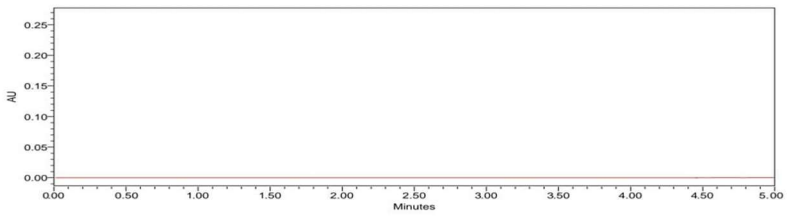


Fig.-5: Blank Chromatogram of Bupivacaine and Meloxicam

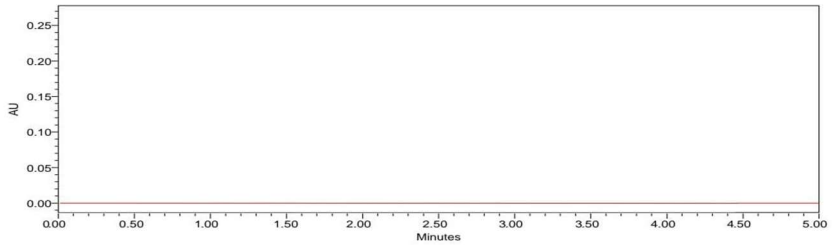


Fig.-6: Placebo Chromatogram of Bupivacaine and Meloxicam

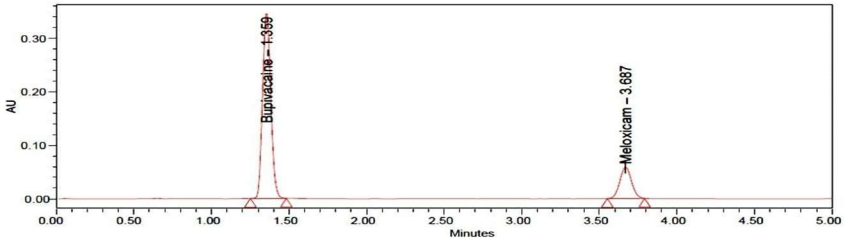
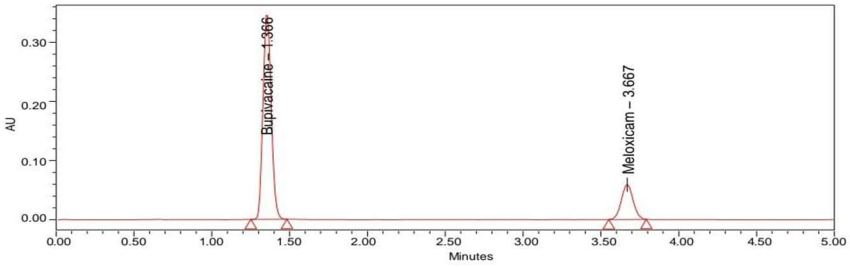
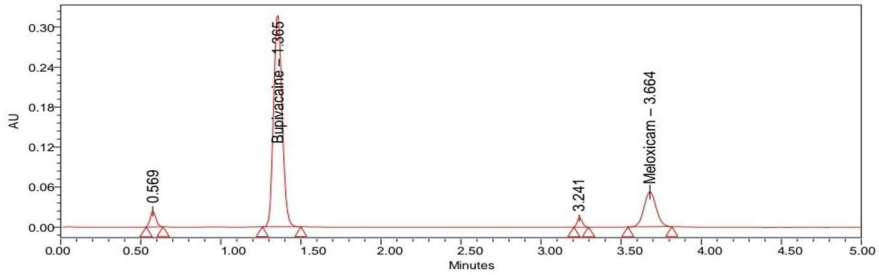


Fig.-7: Optimized Chromatogram of Bupivacaine and Meloxicam



(a)



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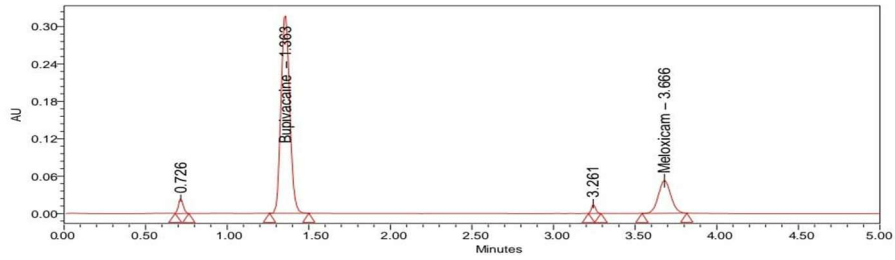

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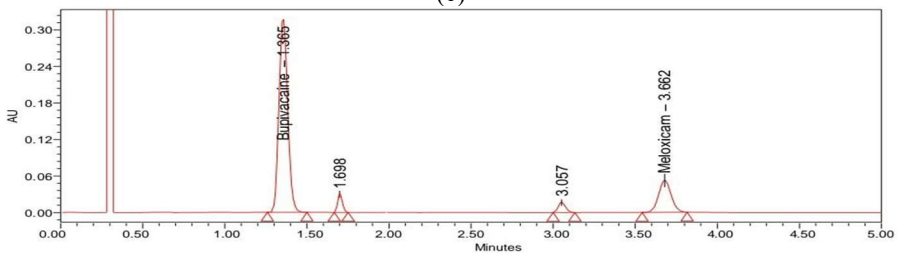

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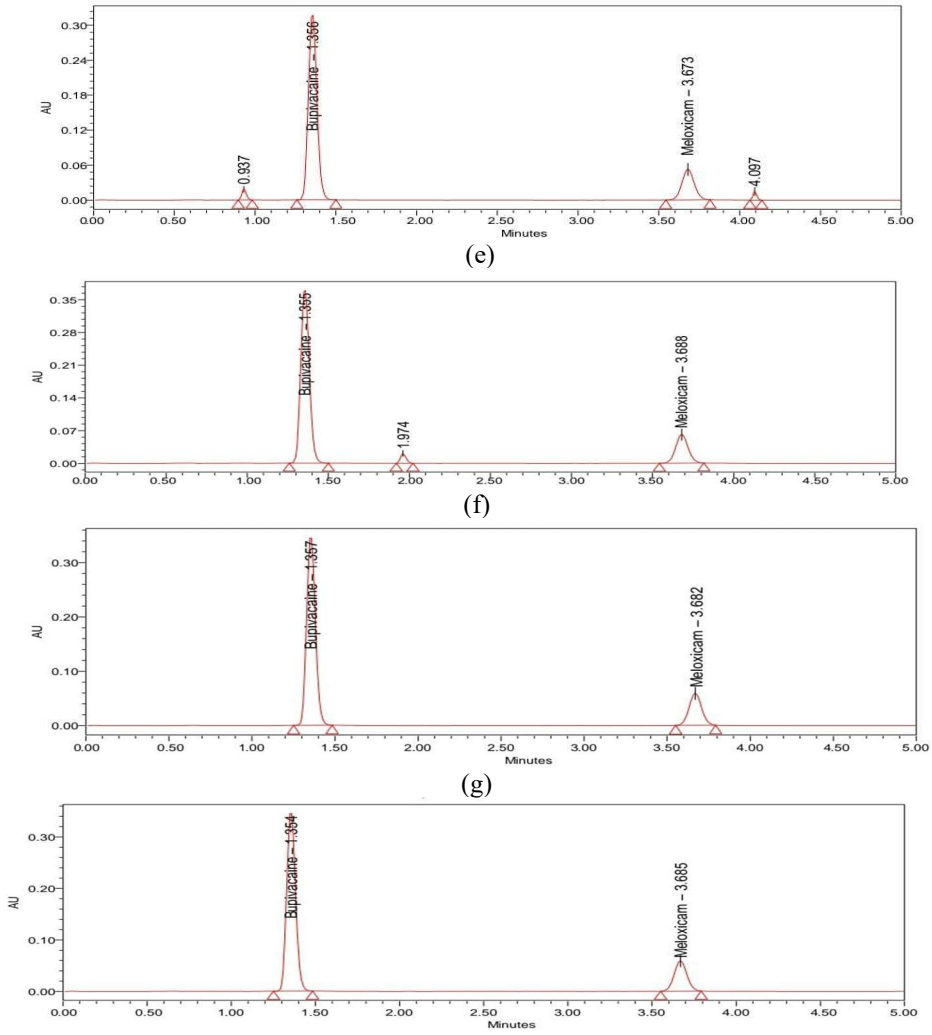

Fig.-8: Degradation studies of (a) Control (b) Acid (c) Alkali (d) Peroxide (e) Redox (f) Thermal (g) UV (h) Water

CONCLUSION

For the simultaneous quantification of Bupivacaine and Meloxicam in injectable formulation, a simple, accurate and specific approach was established. Bupivacaine and Meloxicam had retention times of 1.359 and 3.687 minutes, respectively. The percent RSD of Bupivacaine and Meloxicam, respectively, was found to be 1.01 and 0.46 a percentage. For Bupivacaine and Meloxicam, percent recoveries were 100.3 percent and 100.2 percent, respectively. The limit of detection and limit of quantitation for Bupivacaine andMeloxicam determined from the 'regression equation' were 0.6 and 0.018 ppm,0.2 and 0.06 ppm, consecutively. Regression equation of Bupivacaine y = 11370.02x + 18635.5, and Meloxicam y = 979464x + 729.54. The findings of the forced degradation test showed that the peaks were clean under all stressful situations. The developed approach might be applied in regular quality control tests in industries since retention times and run times were decreased.

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CONFLICT OF INTERESTS

The authors state that the submission of this work has no conflicts of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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