**IJPSR (2026), Volume 17, Issue 11** **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 01 May 2025; received in revised form, 03 October 2025; accepted, 26 October 2025; published 01 November 2025

# ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF BUPIVACAINE AND MELOXICAM IN SYNTHETIC MIXTURE

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**Keywords:**

In-situ gel, Acyclovir, Anti-viral, HPMC E50 LV, Pluronic F-127

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**ABSTRACT:** Bupivacaine and Meloxicam are used in combination for treatment of Pain relief. Objective of present work was to develop simple, precise, accurate methods for simultaneous estimation of both drugs using HPTLC and RP-HPLC method. In HPTLC method silica gel G- F254 TLC plate as a stationary phase and a mobile phase of Toluene: Ethyl acetate: Methanol: Formic acid (6:2:1.5:0.5 v/v/v/v) used to resolve Bupivacaine and Meloxicam. Bupivacaine and Meloxicam were estimated at 270nm. The proposed method was validated according to ICH guideline Q2 (R1). For RP-HPLC method, chromatographic separation was achieved on Shim – Pack solar C18 (250 mm × 4.6mm, 5µm). Detection was carried out at 210nm using Acetonitrile:Methanol: Water pH-5, adjusted with 0.1% OPA (80: 10: 10 v/v/v). HPTLC method was found to be linear over the concentration range of 2000-6000ng/b and for BUP and 60-180 ng/band for MEL. HPLC method was found to be linear over the concentration range of 30-70µg/ml for BUP and 0.9-2.1µg/ml for MEL. All the methods were validated for linearity, precision, accuracy, LOD and LOQ according to ICH guideline Q2 (R1). All the method was accurate and precise.

**INTRODUCTION:** Pain is a generic phrase used to describe unpleasant bodily experiences. It results from the neurological system being activated. From bothersome to incapacitating, pain can vary widely. It could feel like a slow pain or a sudden stab. Other ways to describe it include scorching, stinging, pinching, throbbing, and sore [1]. Pain is the sensation that we connect to real or possible tissue injury. It is definitely a sensation in one or more body regions, but it is also usually unpleasant, making it an emotional experience as well [2]. According to a recent study on the worldwide burden of disease, the most common causes of impairment among individuals aged 10 to 49 are pain-related disorders.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.17(11).3066-80

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.17(11).3066-80

Effective pain management is still difficult despite a wealth of research on pain and analgesic techniques. Therefore, knowing the physiology of pain is crucial to knowing how to properly evaluate, diagnose, and treat it [3].

**Diagnosis of Pain:** Patients' accounts of their discomfort can reveal important details about the myriad of potential causes of the condition. To comprehend and treat pain, medical practitioners frequently use a mix of patient history, physical examinations, and diagnostic tests. Depending on the suspected cause, a variety of diagnostic tests can be used to look into the underlying causes of pain. These tests could include imaging tests like MRIs, CT scans, ultrasounds, or X-rays, as well as operations like biopsies or endoscopies. A thorough assessment of pain may also include evaluating nerve function and evaluating mental health.

**Treatment of Pain:** Depending on the underlying reasons of the patient's particular illness, clinicians frequently employ a range of treatments for treating

pain. Prescription of anti-inflammatory medications, such as corticosteroids or specific COX-2 inhibitors, which are advised for inflammatory conditions. Medications that assist control pain signals in the neurological system, such as antidepressants or neuropathic pain or functional pain syndrome, may be part of a therapy plan for chronic pain problems. While stress management helps lower overall pain levels, patients are urged to recognise and stay away from activities or triggers that make their pain worse. Combining medical treatments with lifestyle modifications can help patients manage their pain and improve their overall quality of life.

## MATERIALS AND METHODS:

**Chemical Reagents:** From Alcon Biosciences Pvt. Ltd. In Vapi, Bupivacaine was obtained and from Swati Spentose Pvt. Ltd. In Vapi, Meloxicam was obtained. Methanol and water used in the study were obtained from purification system of institute. Toluene, Ethyl acetate, Formic acid, Acetonitrile was used of analytical grade by Rankem.

### RP-HPLC Method:

**Equipment and Software:** For RP-HPLC instrument of SHIMADZU LC 2010HT, Auto sampler was utilized for the processing of chromatograms and data generation during research work.

**Chromatographic Parameters:** The separation of sample using Reversed phase high performance liquid chromatography (RP-HPLC) was conducted on the Shim-pack solar C18 column with dimensions of 250 × 4.6mm, 5µm. The mobile phase consisted of a mixture of acetonitrile, methanol and water by adjusting pH 5 with 0.1% OPA, with the ratio of 80:10:10. The equipment was used at a flow rate of 1 ml/min, and the entire duration of a single run was 20 minutes. The temperatures of column were kept constant at 27°C.

### Preparation of Sample:

**Standard Solutions:** Accurately weigh 10 mg of BUP (Bupivacaine) was accurately weighed and placed in a volumetric flask of 10 ml and dissolved in methanol make up the volume up to mark with methanol to get final concentration (1000µg/ml). A 10 mg of MEL (Meloxicam) was accurately weighed and placed in a volumetric flask of 10 ml and dissolved in methanol make up the volume up to mark with methanol to get final concentration (1000µg/ml).

**Working Solutions:** Accurately weigh 1 ml aliquot of the stock solution was pipette out and put into a 10 ml volumetric flask which was then filled with methanol. A 1ml aliquot of the stock solution was pipette out and put into a 10 ml volumetric flask which was then filled with methanol.

**Binary Mixture of BUP and MEL:** Accurately weigh about 3 ml of BUP (100 µg/ml) and 0.09 ml of MEL (100 µg/ml) working solutions were placed in a single volumetric flask and diluted with methanol to make final concentrations of BUP (30 µg/ml) and MEL (0.9 µg/ml).

**Method Validation:** The validated metrics for the developed method include specificity, linearity, precision, and accuracy, repeatability, robustness.

**System Suitability Studies:** To evaluate the system's suitability, six replicates of BUP and MEL at concentrations of 60 µg/ml of BUP and 1.8 µg/ml of MEL were analysed. Column efficiency, peak asymmetry, and resolution were computed for each replicate.

**Specificity:** Specificity is defined as the quantitative detection of an analyte in the presence of those components that are expected to be present in the sample matrix. The specificity of the new approach was demonstrated by spiking BUP and MEL in a hypothetical placebo (that could be assumed to be present) and demonstrating that the analytes peak was not affected by excipients.

**Linearity:** The linearity response was found by assessing 5 separate levels of concentration in the ranges of 30-70 µg/ml for BUP and 0.9-2.1 µg/ml for MEL. A calibration curve is set for this study, the BUP calibration curve comprised of five distinct concentrations of solution ranging from 30-70 µg/ml.

Peak area vs. Conc. calibration curve was plotted, and regression equation was derived. The MEL calibration curve comprised of five distinct concentrations of solution ranging from 0.9-2.1 µg/ml. Peak area vs. Conc. calibration curve was plotted, and regression equation was derived.

**Precision:** Different types of precision is studied like.

**Repeatability:** the repeatability of the devised method was evaluated by testing samples from the same batch six times with standard solutions containing concentrations of 60µg/ml for BUP and 1.8µg/ml for MEL and calculating the per cent R.S.D (Relative Standard Deviation).

**Intraday precision:** It was determined by evaluating samples from the same batch with three standard solutions with concentrations of 50, 60 and 70 µg/ml for BUP and 1.5, 1.8, 2.1 µg/ml for MEL. On the same day, solutions were analysed three times (n=3) in a short period of time, and the per cent R.S.D. was calculated.

**Interday Precision:** It was determined by testing samples from the same batch with three standard solutions containing concentrations of 50, 60 and 70 µg/ml of BUP and 1.5, 1.8, 2.1 µg/ml of MEL. The solutions were analysed three times (n=3) on three distinct days, and the per cent R.S.D. was calculated each time.

**Accuracy:** Preparation of sample solution for BUP, mixture solution BUP (30 µg/ml) + MEL (0.9 µg/ml), Solution Y:BUP contains (100 µg/ml) and Solution Z:MEL (100 µg/ml).

**LOD & LOQ:** The LOD (Limit of Detection) was calculated using a series of five calibration curves that were used to determine the method's linearity. The LOD was computed using the following formula:

$$LOD = 3.3 \times S.D./Slope.$$

Where, σ = standard deviation of the response

S = slope of the calibration curve.

The LOQ (Limit of Quantitation) was calculated using a series of five calibration curves that were used to determine the method's linearity. The LOQ was determined using the following formula:

$$LOQ = 10 \times S.D./Slope$$

Where, σ = standard deviation of the response

S = slope of the calibration curve.

**Robustness:** The technique's robustness was determined by subjecting it to minor changes in method conditions such as, mobile phase ratio and flow rate.

**Simultaneous Estimation of Bupivacaine and Meloxicam in Synthetic Mixture:** A 100 mg Bupivacaine and Meloxicam were weighed and obtained. Accurately weighed powder equivalent to about 58.5 mg of BUP and 1.76 mg MEL was transferred to 100 ml volumetric flask. About 10 ml methanol was added to flask and sonicated for 10-12 min. The final Volume was adjusted with Methanol (1000 µg/ml) of resulting solution Filtered through Whatman filter Paper No 41. This solution was used as Stock solution 1 ml of aliquot solution was pipette out and transferred to 10 ml volumetric flask and volume was made up mark with methanol (100 µg/ml).then 3 ml was withdrawn from above working solution and transferred into 10 ml volumetric flask and volume was made up mark with methanol to get final sample solution containing 30 µg/ml of BUP and 0.9 µg/ml of MEL respectively. The concentration of BUP and MEL was obtained by solving the regression equation.

**HPTLC Method:**

**Equipment and Software:** For HPTLC (High Performance Thin Layer Chromatography) instrument of Automatic TLC sampler (CAMAG LINOMET 5), UV cabinet (CAMAG), TLC Scanner 4 (CAMAG). The software was Win CATS was utilized for the processing of chromatograms and data generation during the research work.

**Chromatographic Parameters:** Using a TLC sampler applicator, a working standard solution or sample solution was spotted on a precoated TLC plate under nitrogen stream (CAMAG linomet 5). The plate was dried in the air before being developed in a twin trough chamber for 20 minutes with a mobile phase of toluene: ethyl acetate: methanol: formic acid 6:2:1.5:0.5. The plate was taken out of the chamber and dried after development. With the CAMAG TLC 4 scanner and win CATS software, photometric measurements were taken in absorbance mode. At 270 nm, BUP and MEL were scanned.

**Preparation of Sample:**

**Standard Solutions:** Accurately weighed BUP (50mg) then transferred into 50ml volumetric flask and dissolved in methanol make up the volume up to mark with methanol to get final concentration (1000 µg/ml).

Accurately weighed MEL (10mg) then transferred into 100ml volumetric flask and dissolved in methanol make up the volume up to mark with methanol to get final concentration (100 µg/ml).

**Working Solutions:** Accurately weigh 10 ml aliquot of the stock solution was pipette out and put into a 50 ml volumetric flask, which was then filled with methanol. (200 µg/ml). Then weigh 0.6 ml aliquot of the stock solution was pipette out and put into a 10 ml volumetric flask, which was then filled with methanol (6 µg/ml).

**Binary mixture of BUP and MEL:** Accurately weigh 20 ml of BUP (200 µg/ml) and 0.6 ml of MEL (6 µg/ml) standard solutions were combined in a volumetric flask of 50 ml of mobile phase of BUP and volumetric flask of 10 ml of mobile phase of MEL make up volume with diluted to make final concentrations of BUP (2000 µg/ml) and MEL (60 µg/ml).

**Method Validation:** The validated metrics for the developed method include specificity, linearity, precision, and accuracy, repeatability, robustness.

**Linearity:** The devised approach was used to spot and analyse a working standard solution (10, 15,20,25,30 µl). For BUP and MEL, the linearity response was evaluated by analysing five separate levels of calibration curves in the 2000-6000 ng/band and 60-180 ng/band, respectively. Peak area versus concentration calibration curves were plotted, and the correlation coefficient and regression line equation were calculated.

**Precision:**

**Intraday Precision:** On the same day, three different concentrations of working solution were tested three times, and the per cent RSD was computed.

**Interday Precision:** Three different concentrations of working solution were tested three times on different days to determine the per cent RSD.

**Accuracy:** The method accuracy was confirmed by a recovery analysis of a marketed formulation at three levels of standard addition. The per cent recovery of BUP and MEL was discovered. The method accuracy is justified by a recovery rate ranging from 98 to 102 per cent. Mixture solution X:BUP (2000µg/ml) + MEL (60µg/ml), Solution Y:BUP (1000 µg/ml), Solution Z:MEL (100 µg/ml).

**Robustness:** The method's resilience was demonstrated by introducing tiny changes in several parameters such as mobile phase composition and chamber saturation time. For the same purpose, several compositions of mobile phase were tested, and chromatograms were generated. The method's robustness was assessed by calculating per cent RSD values. The method's robustness was tested at concentration levels of BUP (4000ng/band) and MEL (120ng/band).

**Specificity:** Specificity is a strategy for detecting analytes quantitatively in the presence of components that are likely to be present in the sample matrix.

**Simultaneous Estimation of BUP and MEL in Synthetic Mixture:** From the mixture, 2 ml of solution was taken into 100 ml of volumetric flask and volume was made up to mark with methanol. Solution was filtered through Whatmann filter paper no. 42. Thus, resulting solution gave 585 µg/ml of BUP and 17.6 µg/ml of MEL. From the above solution, 1.70 ml was pipette out and transferred to 10 ml volumetric flask and volume was made up to mark with methanol in order to give a solution containing BUP (400 µg/ml) + MEL (12µg/ml). From the above solution, 20µl was spotted using CAMAG LINNOMET 5 applicator. Chromatogram was recorded and the concentration of BUP and MEL was obtained by solving the regression equation.

## RESULTS AND DISCUSSION:

**RP-HPLC Method Development:**

**Selection of Wavelength:** Bupivacaine and Meloxicam solutions were scanned at 200-400 nm using a UV-Visible Spectrophotometer. The wavelength was chosen from the overlay spectra of the above solutions. So, from the spectra, 210 nm was chosen for further determination in the case of HPLC.

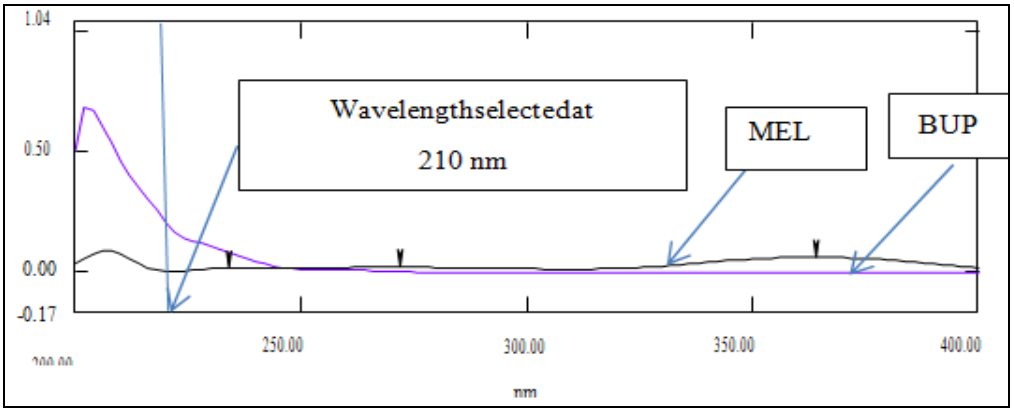


**FIG. 1: OVERLAY UV SPECTRUM OF BUP AND MEL SHOWING SELECTION OF WAVELENGTH**

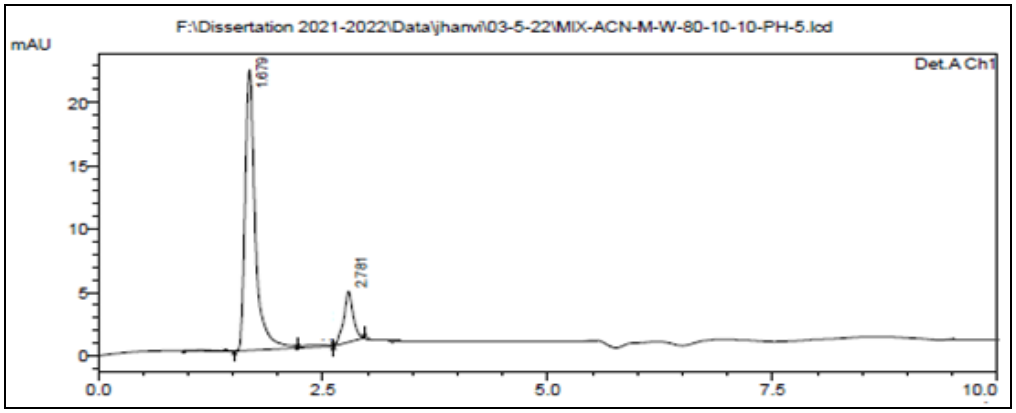


**FIG. 2: CHROMATOGRAM OF BUP AND MEL IN (ACN) ACETONITRILE: METHANOL: WATER PH-5, ADJUSTED WITH 0.1% OPA (80: 10: 10 V/V/V)**

**Development Method:** After optimization of mobile phase, final optimized chromatographic condition is as given below in the table.

**TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITION**

| Sr. no. | Parameters | Condition |
|---|---|---|
| 1 | Mobile phase | Acetonitrile : Methanol : Water pH-5, adjusted with 0.1% OPA (80 : 10 : 10 v/v/v ) |
| 2 | Flow rate | 1 ml/min |
| 3 | Run time | 20 min |
| 4 | Volume of injection | 10 µl |
| 5 | Detection wavelength | 210 nm |
| 6 | Retention time | BUP: 1.679, MEL: 2.781 |
| 7 | Theoretical plate | BUP: 7462.064, MEL: 14651.626 |
| 8 | Resolution | 2.103 |

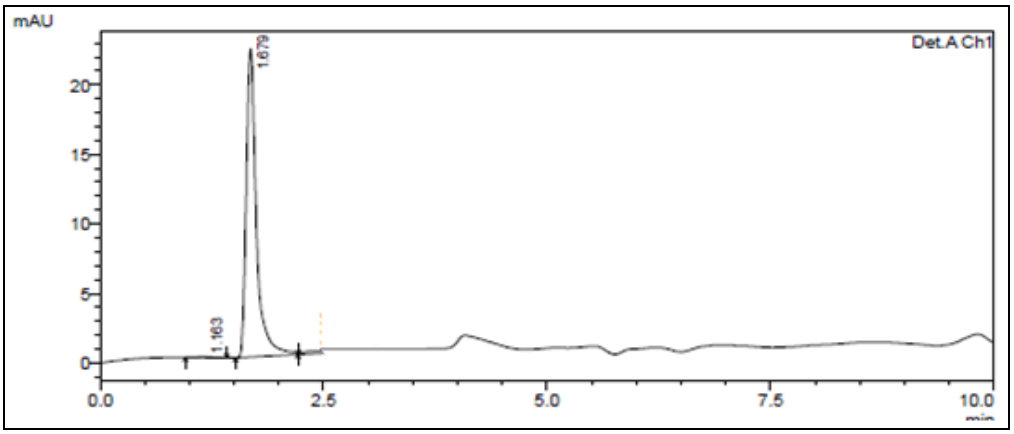


**FIG. 3: CHROMATOGRAM OF BUP (40 µG /ML) IN ACN: METHANOL: WATER PH-5, ADJUSTED WITH 0.1% OPA (80: 10: 10 V/V/V)**

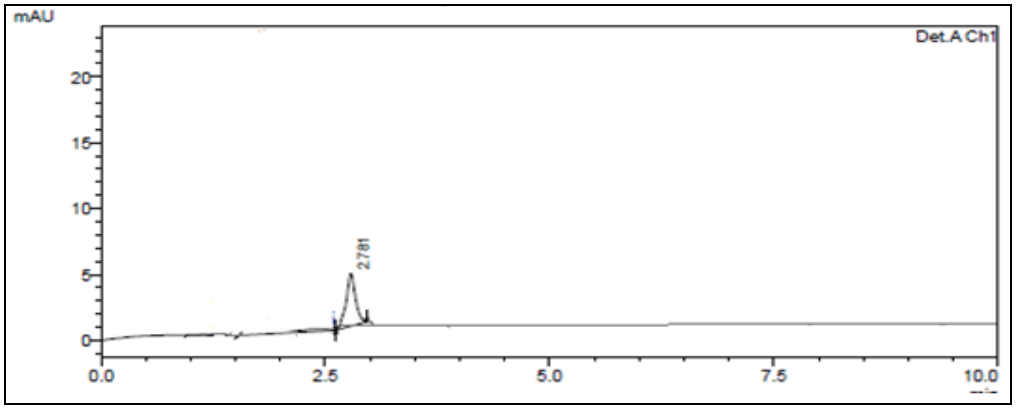


**FIG. 4: CHROMATOGRAM OF MEL (1.2 µG/ML) IN ACN: METHANOL: WATER PH-5, ADJUSTED WITH 0.1% OPA (80: 10: 10 V/V/V)**

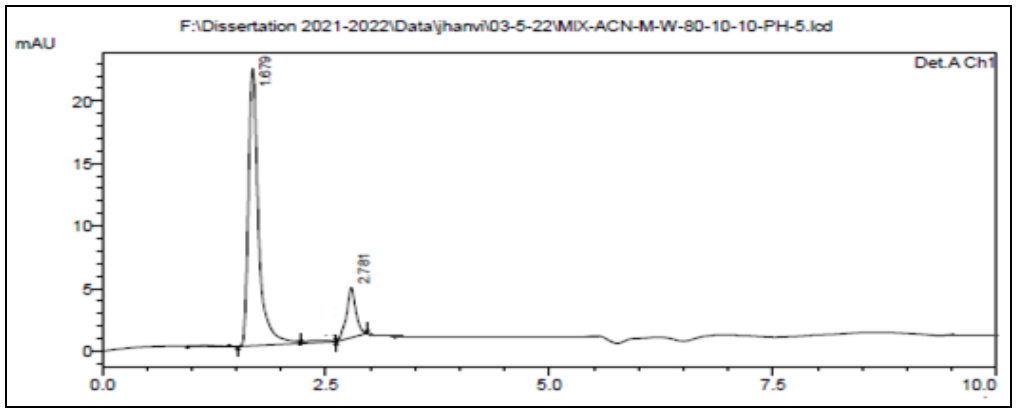


**FIG. 5: CHROMATOGRAM OF BUP (40 µG/ML) AND MEL (1.2 µG/ML) IN ACN: METHANOL: WATER PH-5, ADJUSTED WITH 0.1% OPA (80: 10: 10 V/V/V)**

**TABLE 2: SYSTEM SUITABILITY DATA FOR BUP AND MEL**

| Drugs | Parameters | Mean ± S.D. (n=6) | % R.S.D. |
|---|---|---|---|
| BUP | Retention time | 1.685±0.004764 | 0.282673 |
| | Theoretical plate | 7473.591±7.940593 | 0.106249 |
| | Tailing factor | 1.8175±0.004461 | 0.245444 |
| | Retention time | 2.8035±0.011811 | 0.421295 |
| MEL | Theoretical plate | 14702.48±29.06352 | 0.197678 |
| | Tailing factor | 1.040167±0.004665 | 0.448532 |

SD- Standard Deviation.

## Method Validation:

**Linearity:** The linearity study was carried out for both drugs at five different concentration levels. The linearity of BUP and MEL was in the range 30-70µg/ml and 0.9-2.1µg/ml and are depicted in table.

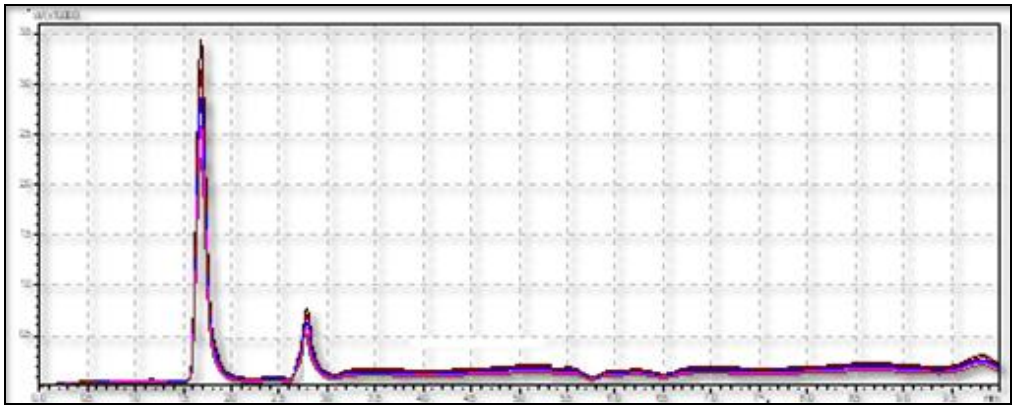

**FIG. 6: OVERLAIN CHROMATOGRAM OF BUP (30-70 MG/ML) AND MEL 0.9-2.1 MG/ML)**

**TABLE 3: LINEARITY DATA FOR BUP**

| Sr. no. | Conc. (µg/ml) | Mean Peak Area ± S.D. (n=5) | %R.S.D. |
|---|---|---|---|
| 1 | 30 | 294261.6±308.8468 | 0.104957 |
| 2 | 40 | 323553.7±372.022 | 0.114281 |
| 3 | 50 | 368498±516.6395 | 0.140201 |
| 4 | 60 | 409610±676.8663 | 0.165247 |
| 5 | 70 | 470065±1058.049 | 0.225086 |

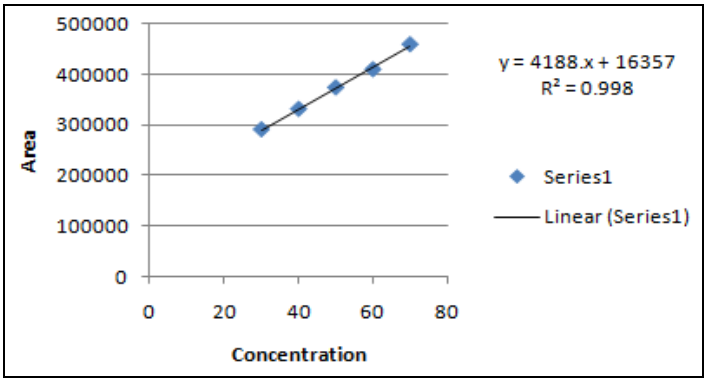


**FIG. 7: CALIBRATION CURVE OF BUP**

**TABLE 4: LINEARITY DATA FOR MEL**

| Sr. no. | Conc. (µg/ml) | Mean Peak Area ± S.D. (n=5) | %R.S.D. |
|---|---|---|---|
| 1 | 0.9 | 175213±399.4425 | 0.227975 |
| 2 | 1.2 | 225523±374.9413 | 0.166254 |
| 3 | 1.5 | 257314.4±362.9509 | 0.141053 |
| 4 | 1.8 | 294228±387.2945 | 0.131631 |
| 5 | 2.1 | 330419±510.09510 | 0.154378 |

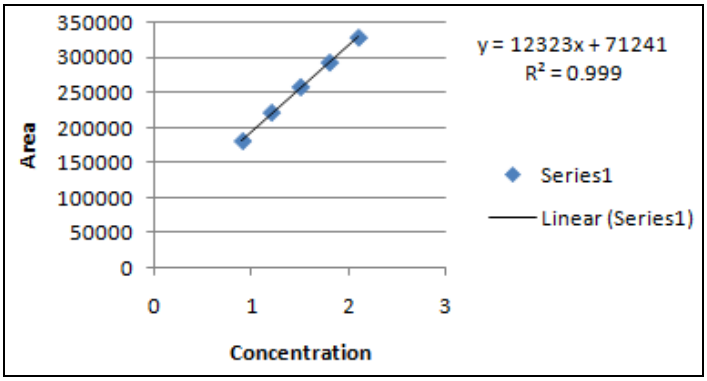


**FIG. 8: CALIBRATION CURVE OF MEL**

## Specificity:

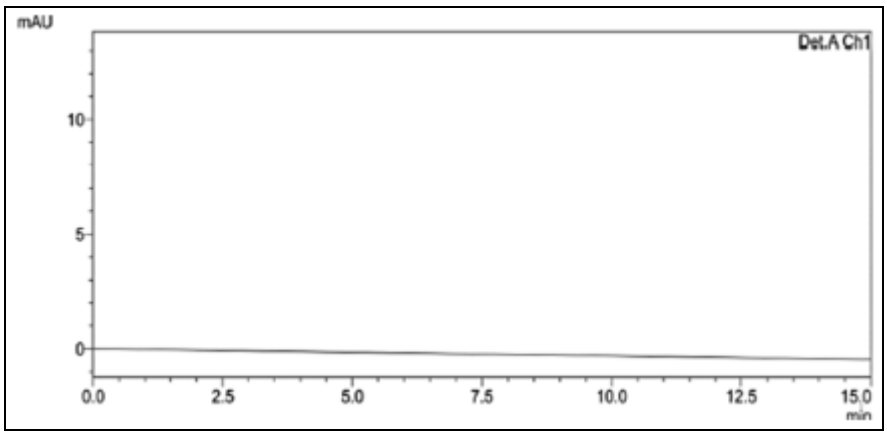


**FIG. 9: CHROMATOGRAM OF MOBILE PHASE**

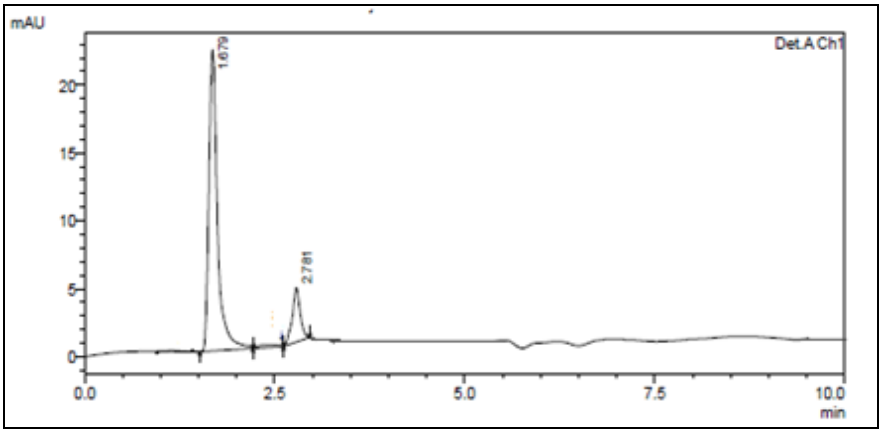


**FIG. 10: CHROMATOGRAM OF BUP AND MEL**

**Precision:**

**Repeatability:** The data of repeatability for BUP and MEL are depicted in **Table 5.**

**TABLE 5: REPEATABILITY DATA FOR BUP AND MEL**

| Drugs | Conc. (µg/ml) | Mean Peak Area ± S.D. (n=6) | %R.S.D. |
|---|---|---|---|
| BUP | 60 | 367962±2823.929 | 0.224287 |
| MEL | 1.8 | 257026.5±576.4782 | 0.767451 |

**Intraday Precision:** The data for intraday precision for BUP and MEL are depicted in the **Table 6.**

**TABLE 6: INTRADAY DATA OF BUP AND MEL**

| Drugs | Conc. (µg/ml) | Mean Peak Area ± S.D. (n=3) | %R.S.D. |
|---|---|---|---|
| BUP | 50 | 324778±515.5065 | 0.158726 |
| | 60 | 366905.3±616.277 | 0.167966 |
| | 70 | 409937±894.5708 | 0.218222 |
| MEL | 1.5 | 224596±344.1816 | 0.153245 |
| | 1.8 | 256339.3±322.8395 | 0.125942 |
| | 2.1 | 293344.3±353.4817 | 0.120501 |

**Interday Precision:** The data for intraday precision for BUP and MEL are depicted in the **Table 7.**

**TABLE 7: INTERDAY DATA OF BUP AND MEL**

| Drugs | Conc. (µg/ml) | Mean Peak Area ± S.D. (n=3) | %R.S.D. |
|---|---|---|---|
| BUP | 50 | 325179±545.2146 | 0.167666 |
| | 60 | 367614.7±684.0975 | 0.186091 |
| | 70 | 4092607±915.4054 | 0.223671 |
| MEL | 1.5 | 225131±496.4806 | 0.22053 |
| | 1.8 | 256796.7±405.8255 | 0.15803 |
| | 2.1 | 293364±382.0903 | 0.130244 |

**Accuracy:** The amounts of BUP and MEL were estimated by using the regression equation of the calibration curve. The low value of standard deviation indicates that the proposed method is accurate. Results of recovery studies are shown in **Table 8.**

**TABLE 8: ACCURACY DATA OF BUP AND MEL**

| Drugs | Level | Amt. of sample (µg/ml) | Amt. of drug spiked (µg/ml) | Total conc. Found (µg/ml) | Mean Peak Area ± S.D. (n=3) | Amt. of sample found (µg/ml) | % Recovery |
|---|---|---|---|---|---|---|---|
| BUP | 0 | 30 | 0 | 30 | 293698±215.4228 | 29.5 | 98.33 |
| | 80 | 30 | 24 | 54 | 304556.7±388.0288 | 53.58 | 99.22 |
| | 100 | 30 | 30 | 60 | 334958.7±457.7874 | 59.87 | 99.78 |
| | 120 | 30 | 36 | 66 | 403006.7±607.7749 | 65.98 | 99.96 |
| | 0 | 0.9 | 0 | 0.9 | 174741.7±274.3453 | 0.89 | 98.88 |
| | 80 | 0.9 | 0.72 | 1.62 | 195824±363 | 1.61 | 99.38 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| MEL | 100 | 0.9 | 0.9 | 1.8 | 213879.7±201.7829 | 1.79 | 99.44 |
| | 120 | 0.9 | 1.08 | 1.98 | 240228±3306.877 | 1.97 | 99.49 |

**LOD & LOQ:** The LOD for BUP and MEL was found to be 0.0461 µg/ml and 0.1399 µg/ml respectively.

The LOQ for BUP and MEL was found to be 0.1720 µg/ml and 0.5224 µg/ml respectively.

**Robustness:** The robustness of the method was established by making deliberate minor variations in the following method parameters.

- Flow rate:
- Mobile phase:

**TABLE 9: ROBUSTNESS DATA OF BUP**

| Parameters | Level | Peak area ± S.D. (n=3) | % R.S.D. | Rt ± S.D. (n=3) | % R.S.D. |
|---|---|---|---|---|---|
| Mobile phase | 78:11:11 | 95505±561.076 | 0.3451 | 1.17±0.0540 | 0.3215 |
| (80:10:10v/v) | 82:9:9 | 98509±446.556 | 0.4632 | 1.18±0.0572 | 0.2536 |
| Flow rate (1ml/min) | 0.8ml/min | 96503±546.099 | 0.2378 | 1.21±0.0504 | 0.3572 |
| | 1.2ml/min | 97814±383.221 | 0.1342 | 1.24±0.0452 | 0.2241 |

**TABLE 10: ROBUSTNESS DATA OF MEL**

| Parameters | Level | Peak area ± S.D. (n=3) | %R.S.D. | Rt ± S.D. (n=3) | %R.S.D. |
|---|---|---|---|---|---|
| Mobile phase | 78:11:11 | 314180±1526.6 | 0.4859 | 2.781.± 0103 | 0.4721 |
| (80:10:10v/v) | 82:9:9 | 308852±897.672 | 0.2906 | 2.764±0.0055 | 0.3586 |
| Flow rate (1ml/min) | 0.8ml/min | 305576±626.476 | 0.2050 | 2.716±0.1102 | 0.3274 |
| | 1.2ml/min | 328722±1042.57 | 0.3171 | 2.705±0.0069 | 0.2654 |

## Assay:

**TABLE 11: ASSAY**

| Amount taken (µg/ml) | | Amount obtained (µg/ml) (n=3) | | %BUP ± S.D. (n=3) | %MEL ± S.D. (n=3) |
|---|---|---|---|---|---|
| BUP | MEL | BUP | MEL | | |
| 40 | 1.2 | 38.64 | 1.19 | 99.25 ± 0.9457 | 99.83 ± 0.2502 |

**TABLE 12: SUMMARY OF RP-HPLC METHOD DEVELOPMENT**

| Parameters | BUP | MEL |
|---|---|---|
| Linearity (n=5) | 30-70 | 0.9-2.1 |
| Regression equation | Y=4188.1x + 163576 | Y=123237x + 71241 |
| Correlation coefficient ($R^2$) | 0.998 | 0.999 |
| Repeatability (n=6) | 0.224287 | 0.767451 |
| Intraday precision (n=3) | 0.158726-0.218222 | 0.153245-0.120501 |
| Interday precision (n=3) | 0.167666-0.223671 | 0.22053-0.130244 |
| LOD (n=5) | 0.0461 | 0.1399 |
| LOQ (n=5) | 0.1720 | 0.5224 |
| %Recovery (n=3) | 98.33-99.96 | 98.88-99.49 |
| %Assay ± S.D. (n=3) | 99.25 ± 0.9457 | 99.83 ± 0.2502 |

## HPTLC Method:

**Mobile Phase Optimization:** Different solvent systems have been tried for separation of BUP and MEL but good separation was found in Toluene: Ethyl acetate: Methanol: Formic acid (6 : 2 : 1.5 : 0.5 v/v/v/v). It gave good resolution in BUP and MEL with Rf value 0.73 and 0.25 respectively.

**TABLE 13: TRIALS FOR SELECTION OF MOBILE PHASE**

| Sr. no. | Trails | Observation | Remark |
|---|---|---|---|
| 1. | Hexane: Ethyl acetate: Glacial acetic acid (6.5:3:0.5 v/v/v) | No separation observed | Not satisfied |
| 2. | Toluene: Ethyl acetate: Methanol: Formic acid (8:1:0.5:0.5 v/v/v/v) | Separation with tailing | Not satisfied |
| 3. | Toluene: Ethyl acetate: Methanol: Formic acid (6:2:1.5:0.5 v/v/v/v) | Separation but bupivacaine goes into solvent front | Not satisfied |
| 4. | Toluene: Ethyl acetate: Methanol: Formic acid (6:2:1.5:0.5 v/v/v/v) | Good separation | Satisfactory Rf value: Bupivacaine: 0.73 Meloxicam: 0.25 |

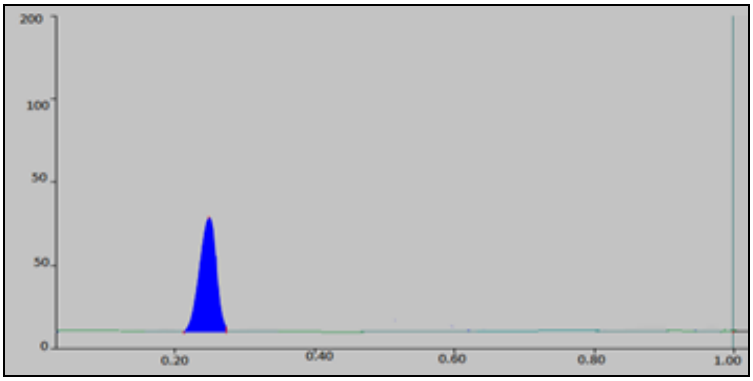


**FIG. 11: CHROMATOGRAM OF MEL (120 NG/BAND)**

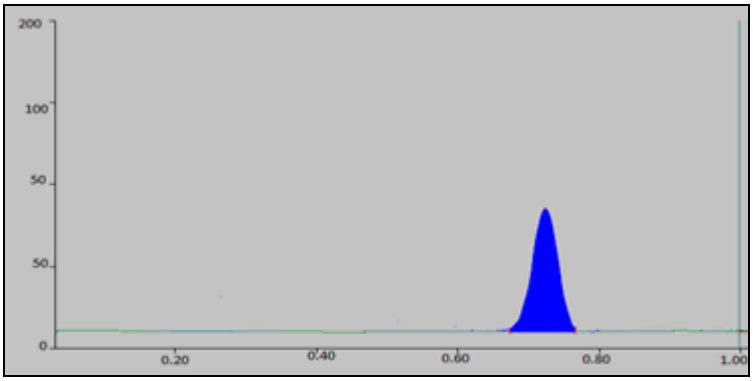


**FIG. 12: CHROMATOGRAM OF BUP (4000NG/BAND)**

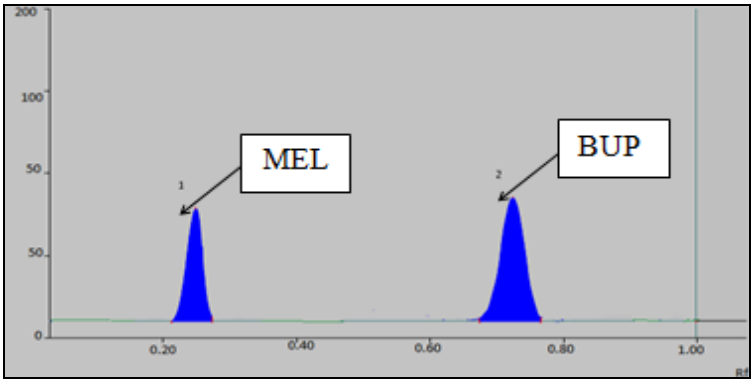


**FIG. 13: CHROMATOGRAM OF MIXTURE MEL (120NG/BAND) AND BUP (4000NG/BAND)**

**Selection of Wavelength:** Overlain spectra of BUP and MEL were taken from TLC scanner 4. The λmax for BUP and MEL were found to be 270nm on both. 270nm was selected as detection wavelength because both BUP and MEL shows good absorptivity at this wavelength.

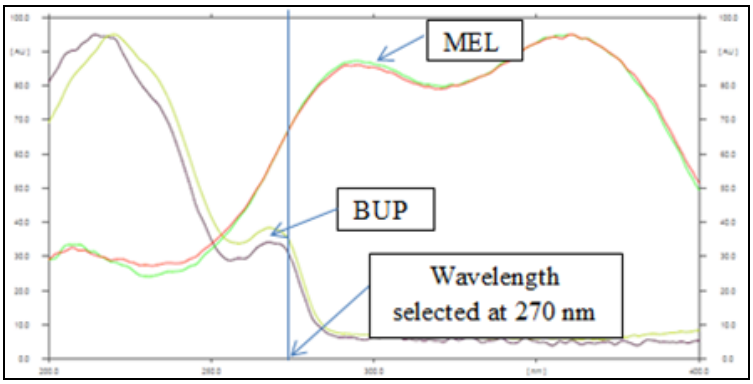


**FIG. 14: OVERLAY SPECTRUM OF BUP AND MEL TAKEN FROM TLC SCANNER 4 SHOWING SELECTION OF WAVELENGTH FOR DETECTION**

**Method Validation:**

**Linearity and Range:** The table shows linearity data for MEL and BUP at 60-180 ng/band and 2000-6000 ng/band, respectively. The image depicts a chromatogram demonstrating the linearity of BUP and MEL. The Rf values for MEL and BUP were found to be 0.25 and 0.73, respectively.

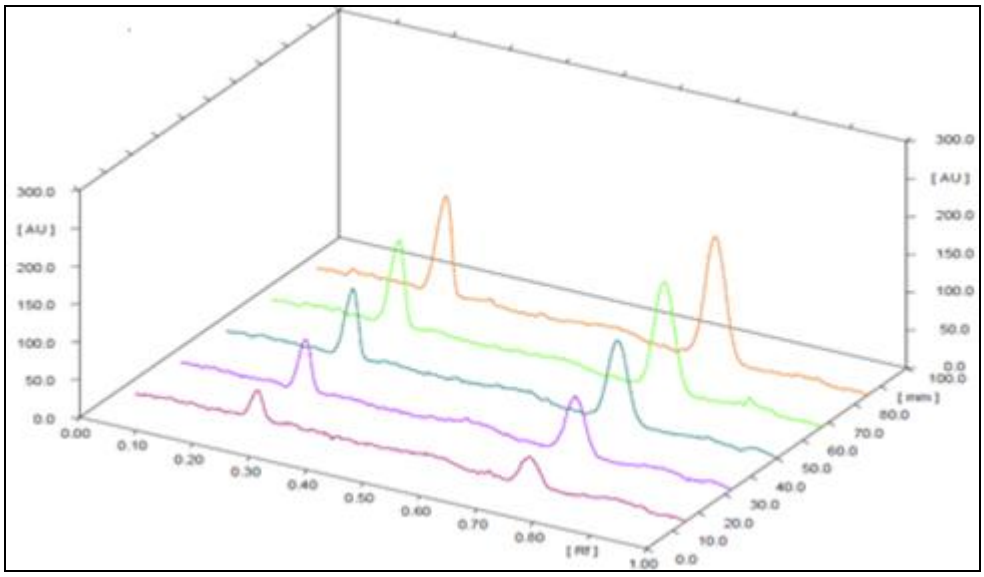


**FIG. 15: OVERLAIN CHROMATOGRAM OF MEL (60-180 NG/BAND) AND BUP (2000-6000NG/BAND)**

## Linearity Data for BUP:

**TABLE 14: LINEARITY FOR BUP**

| Concentration (ng/band) | Area mean ± S.D. (n=5) | %R.S.D. |
|---|---|---|
| 2000 | 975.3±6.234822 | 0.639233 |
| 3000 | 2107.4±9.528903 | 0.452164 |
| 4000 | 3066.4±5.07937 | 0.165646 |
| 5000 | 4156.6±4.926662 | 0.118524 |
| 6000 | 4799.74±5.63143 | 0.117328 |

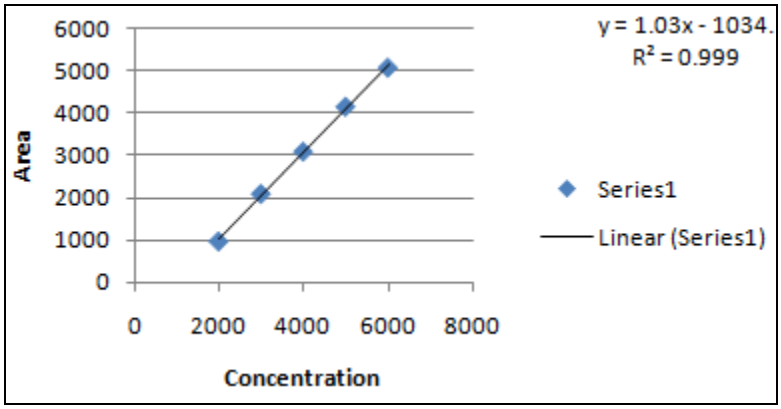


**FIG. 16: CALIBRATION CURVE OF BUPIVACAINE**

Linearity data for MEL:

**TABLE 15: LINEARITY FOR MEL**

| Concentration (ng/band) | Area mean ± S.D. (n=5) | %R.S.D. |
|---|---|---|
| 60 | 832.2±5.403703 | 0.649327 |
| 90 | 1241.46±6.393982 | 0.515037 |
| 120 | 1839.54±4.902346 | 0.266498 |
| 150 | 2496.18±5.737334 | 0.229845 |
| 180 | 2965.7±6.174545 | 0.208199 |

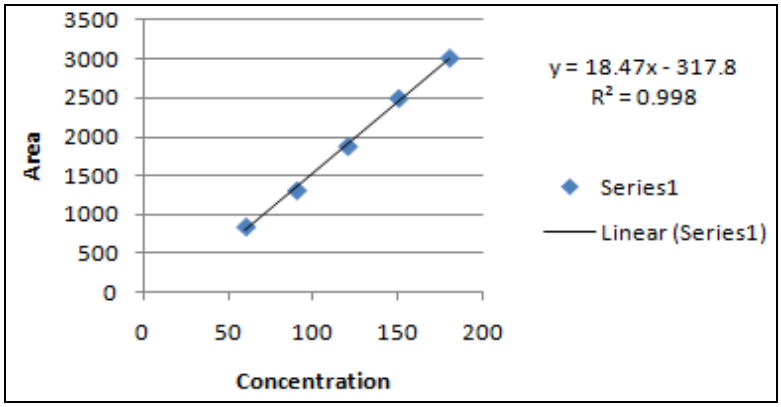


**FIG. 17: CALIBRATION CURVE OF MELOXICAM**

## Precision:

## Repeatability:

**TABLE 16: REPEATABILITY**

| Drugs | Conc. (ng/band) | Area mean ± S.D.(n=6) | %R.S.D. |
|---|---|---|---|
| BUP | 4000 | 3070.8±7.756718 | 0.252593 |
| MEL | 120 | 1890.5±5.176872 | 0.273836 |

## Intraday Precision:

**TABLE 17: INTRADAY PRECISION**

| Drugs | Conc. (ng/ml) | Area mean ± S.D. (n=3) | %R.S.D. |
|---|---|---|---|
| BUP | 3000 | 2097.66±5.033233 | 0.369644 |
| | 4000 | 3062±4 | 0.130634 |
| | 5000 | 4151.667±4.50925 | 0.108613 |
| MEL | 90 | 1234.1±4.172529 | 0.338103 |
| | 120 | 1886.06±3.906832 | 0.207142 |
| | 150 | 2483.33±4.760602 | 0.191702 |

## Interday Precision:

**TABLE 18: INTERDAY PRECISION**

| Drugs | Conc. (ng/ml) | Area mean ± S.D. (n=3) | %R.S.D. |
|---|---|---|---|
| BUP | 3000 | 2101.33±7.767453 | 0.38706 |
| | 4000 | 3063.33±4.50923 | 0.147201 |
| | 5000 | 4155.33±5.507571 | 0.132542 |
| MEL | 90 | 1245.167±4.76801 | 0.382664 |
| | 120 | 1884.9±4.7571 | 0.252379 |
| | 150 | 2485.7±6.062178 | 0.192782 |

## Accuracy:

**TABLE 19: ACCURACY**

| Drug | Level | Amt. of sample taken (ng/band) | Amount of standard spiked (ng/band) | Total conc. (ng/band) | Mean peak area ± SD (n=3) | Total concentration found Mean ± SD (ng/band) (n=3) | %Mean recovery± SD (n=3) |
|---|---|---|---|---|---|---|---|
| BUP | 0 | 2000 | 0 | 2000 | 957.4667±4.11015 | 1977 | 98.85 |
| | 80 | 2000 | 1600 | 3600 | 1017.067±4.701418 | 3589 | 99.69 |
| | 100 | 2000 | 2000 | 4000 | 1138.333±4.50995 | 3989 | 99.72 |
| | 120 | 2000 | 2400 | 4400 | 1246.667±4.63211 | 4399 | 99.97 |
| MEL | 0 | 60 | 0 | 60 | 833.3333±6.027714 | 58.89 | 98.15 |
| | 80 | 60 | 48 | 108 | 848±3 | 107 | 99.07 |
| | 100 | 60 | 60 | 120 | 936.6667±4.50925 | 118.96 | 99.13 |
| | 120 | 60 | 72 | 132 | 991.6667±4.50367 | 131.89 | 99.91 |

## Robustness:

**TABLE 20: ROBUSTNESS**

| Method parameter | Delibrate changes | %RSD of peak area (n=3) | | % RSD of Rf (n=3) | |
|---|---|---|---|---|---|
| | | BUP | MEL | BUP | MEL |
| Chamber saturation time (20 min±5 min) | 15 min | 0.2461 | 0.1352 | 0.3472 | 0.8245 |
| | 25 min | 0.2782 | 0.1672 | 0.3871 | 0.2638 |
| Mobile phase (6:2:1.7:0.3 ± 0.2) | 6:2:1.7:0.3 | 0.3272 | 0.2571 | 0.4357 | 0.2976 |
| | 6:2.2:1.5:0.3 | 0.3567 | 0.2863 | 0.4893 | 0.3256 |

**Specificity:** The specificity of the method was ascertained by analysing standard drugs and sample of Bupivacaine and Meloxicam. The results suggested that proposed method is specific, the excipients present in the formulation does not affect the result.

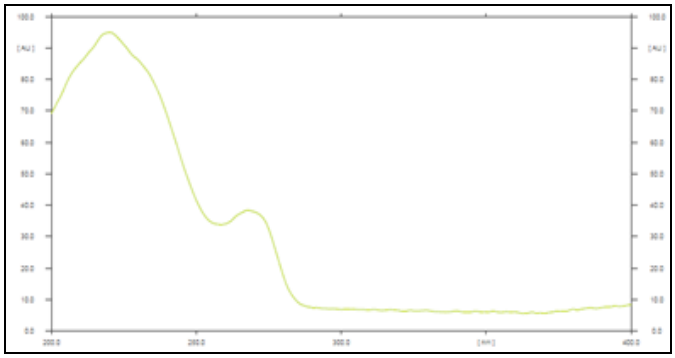

**FIG. 18: BUP STANDARD SPECTRA**

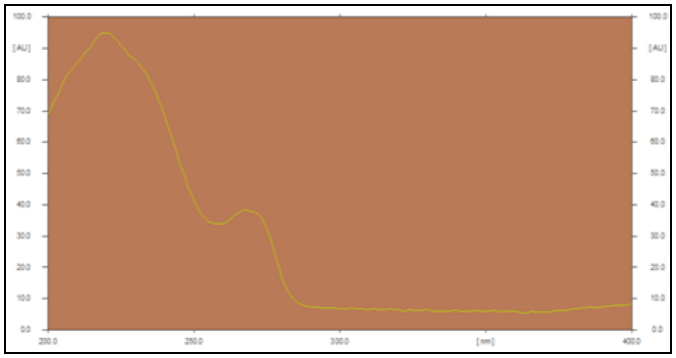

**FIG. 19: BUP SAMPLE SPECTRA**

**TABLE 21: SPECIFICITY OF BUP**

| | R(s,m) | R(m,e) |
|---|---|---|
| Standard | 0.999 | 0.999 |
| Sample | 0.999 | 0.999 |

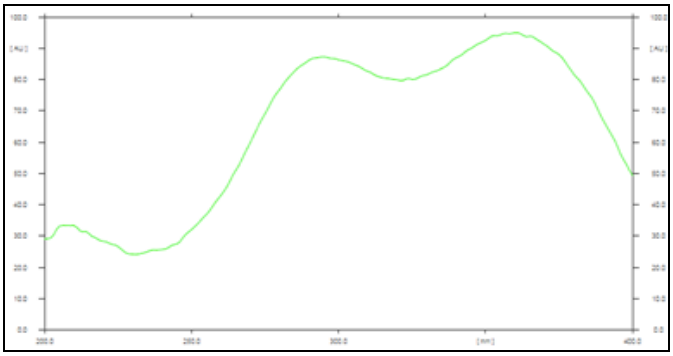

**FIG. 20: MEL STANDARD SPECTRA**

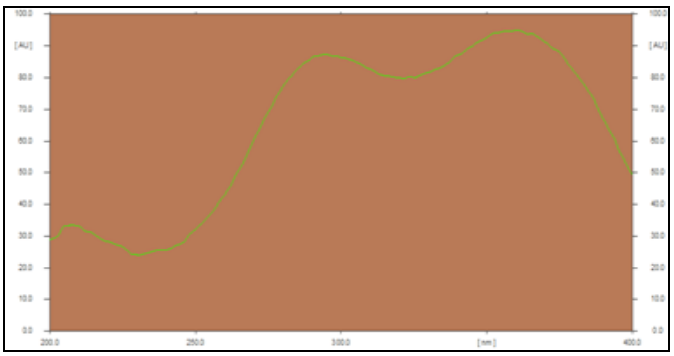

**FIG. 21: MEL SAMPLE SPECTRA**

**TABLE 22: SPECIFICITY OF MEL**

| | R(s,m) | R(r,e) |
|---|---|---|
| Standard | 0.999 | 0.999 |
| Sample | 0.999 | 0.999 |

## Assay of Synthetic Mixture:

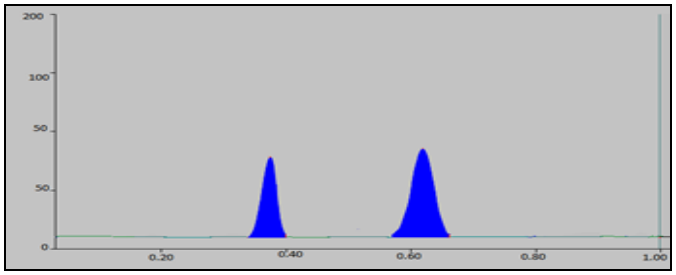


**FIG. 22: CHROMATOGRAM OF MIXTURE (BUP 4000NG/BAND AND MEL 120NG/BAND)**

**TABLE 23: ASSAY**

| Synthetic Mixture | Actual Concentration (µg/ml) | | Amount obtained (µg/ml) | | %BUP ± S.D. (n=3) | %MEL ± S.D. (n=3) |
|---|---|---|---|---|---|---|
| | BUP | MEL | BUP | MEL | | |
| | 4000 | 120 | 3976.11 | 119.86 | 99.54±0.4674 | 99.39±0.366 |

**TABLE 24: SUMMARY OF VALIDATION PARAMETER OF HPTLC METHOD**

| Result | BUP | MEL |
|---|---|---|
| Wavelength for measurement | 270nm | 270nm |
| Concentration range | 2000-6000ng/band | 60-180ng/band |
| Regression equation (Y) | Y=1.03x - 1034.8 | Y=18.477x - 317.82 |
| Slope (b) | 1.03 | 18.477 |
| Intercept (a) | 1034.8 | 317.82 |
| Correlation coefficient ($R^2$) | 0.999 | 0.998 |
| % Recovery | 99.53-100.06 | 98.83-100.56 |
| Repeatability (n=6) | 0.252593 | 0.273836 |
| Intraday precision (n=3) | 0.36944-0.108613 | 0.338103-0.191702 |
| Interday precision (n=3) | 0.38706-0.132542 | 0.382664-0.192782 |
| Limit of Detection (LOD) | 0.34691ng/band | 0.23617ng/band |
| Limit of Quantitation (LOQ) | 1.0407ng/band | 0.7085ng/band |
| %Assay | 99.54±0.4674 | 99.39±0.3661 |

## CONCLUSION:

**HPTLC Method:** HPTLC method developed and validated for simultaneous estimation of BUP and MEL. In which mobile phase is toluene: ethyl acetate: methanol: formic acid (6:2:1.5:0.5 v/v/v/v) saturation time was kept for 20 min. drugs were separated at Rf value of 0.73 and 0.25 and linearity range showed was 2000-6000 ng/band and 60-180 ng/band with correlation coefficient 0.9991 and 0.998 for BUP and MEL respectively. The method was found to be simple, precise and accurate. Limit of detection for BUP and MEL were found to be 0.34691 ng/band and 0.23617 ng/band and Limit of Quantitation for BUP and MEL were found to be 1.0407ng/band and 0.7085 ng/band respectively. The % assay was found to be 99.54% and 99.39 % for BUP and MEL respectively. Further %RSD was found to be less than 2% for precision, intraday and interday study.

The overall result obtained for both drug suggested that all proposed method are specific for estimation Bupivacaine and Meloxicam. The result of recovery study indicate the all method were accurate. The result of intraday and interday variation with low value of %RSD showed that develop method were precise.

**RP-HPLC Method:** For development of RP-HPLC method, Shim-pack solar C18 (250 x 4.6mm, 5µm) column was used as stationary phase and ACN: Methanol: Water pH-5, adjusted with 0.1% OPA (80:10:10 v/v/v) as mobile phase. The flow rate was 1 ml/min and both drugs were quantified at 210 nm. The retention time for BUP and MEL was found to be 1.689 min and 2.781 min respectively. The linearity range obtained for RP-HPLC method was 30-70µg/ml and 0.9-2.1 µg/ml for BUP and MEL respectively.

Limit of detection for BUP and MEL 0.0461µg/ml were found to be 0.1399 µg/ml and Limit of Quantitation for BUP and MEL were found to be 0.1720 µg/ml and 0.5224 µg/ml respectively. The % assay was found to be and for BUP and MEL respectively. Further %RSD was found to be less than 2% for precision, intraday and interday study.

The overall result obtained for both drug suggested that all proposed method are specific for estimation of Bupivacaine and Meloxicam. The result of recovery study indicate the all method were accurate. The result of intraday and interday variation with low value of %RSD showed that develop method were precise.

**ACKNOWLEDGEMENTS:** We would like to thank our Principle of Rofel Shri G. M. Bilakhia College of Pharmacy, Vapi, Gujarat, India and Alcon Biosciences Pvt. Ltd., Gujarat, India and Swati Spentose Pvt. Ltd., Gujarat, India for providing all the facilities to carry out this research work.

**CONFLICT OF INTEREST:** We declare that we have no conflict of interest.

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**How to cite this article:**
Jhanvi HB and Shuchi MD: Analytical method development and validation for simultaneous estimation of bupivacaine and meloxicam in synthetic mixture. Int J Pharm Sci & Res 2026; 17(11): 3066-80. doi: 10.13040/IJPSR.0975-8232.17(11).3066-80.