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# SIMULTANEOUS ESTIMATION OF MEFENAMIC ACID AND HYOSCINE-*N*-BUTYL BROMIDE BY NOVEL RP-HPLC METHOD IN BULK AND PHARMACEUTICAL DOSAGE FORM

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**Abstract:**

*A novel, precise, accurate, rapid and cost effective isocratic reverse-phase high performance liquid chromatographic (RP-HPLC) method was developed, optimized and subsequently validated for the estimation of Mefenamic acid and Hyoscine-N-Butyl bromide in the bulk and pharmaceutical dosage form. Separation was achieved on Inertsil ODS-3 column (250 × 4.6 mm, 5 µm) on Agilent HPLC 1260 system using potassium phosphate buffer (5 mM, pH 3.0): acetonitrile (25:75 v/v) as mobile phase and flow rate of 1 mL/min at 210.0 nm. The method was validated according to USP category I requirements. The system suitability shows the response with retention time, theoretical plate, tailing factor and peak area for both the drugs. Mefenamic acid and Hyoscine-N-butyl bromide marketed drug products were obtained from a drug distributor and assayed for potency using the validated method. Validation acceptance criteria were met in all cases. The linearity ranges were obtained 10-30 µg/mL for MFA and 20-60 µg/mL for HBB with retention times ($R_t$) 9.3 and 5.27 min respectively, and the linearity was $r^2$ > 0.999. The method was determined to be specific and robust. Both accuracy (98.95-99.12%) and precision (0.808-1.549%) were established across the analytical range for low, intermediate and high QC concentrations. Method applicability was demonstrated by analyzing marketed product (Hyocimax MF) of MFA and HBB, in which results showed potency >98%. The developed method was successfully used for the quality assessment of marketed drug products and assesses the product quality of novel dosage forms.*

**Key Words:** *Mefenamic acid (MFA), Hyoscine-N-butyl bromide (HBB), RP-HPLC, Method Validation*

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## INTRODUCTION:

Analysis includes a wide range of simple and instrumental analytical methods, but the most widely used analytical methods for quality assurance are spectroscopy and chromatography based. Most quantitative analysis requires measuring specified components in the presence of sample matrix and related substances, therefore isolation or separation of the components are required preceding quantitative analysis. In such cases chromatographic techniques are used for quantitative analysis. Analytical methods are a measure of quality of the drugs play a very comprehensive role in drug development and follow up activities, to assure that a drug product meets the established standard is stable and will continue to meet purported quality throughout its shelf life[1]. Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients. The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable [2]. Reverse phase is the choice for the majority of samples, but if acidic or basic analytes are present then reverse phase ion suppression or reverse phase ion pairing (for strong acids or bases) should be used. The stationary phase should be $C_{18}$ bonded. For low/medium polarity analytes, normal phase HPLC is a potential candidate, particularly if the separation of isomers is required [3]. Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B) [4]. In the development of method, an important issue is for the drug in the form of a formulation due to the interference caused by the formulation excipients in a short time period and minimum trials. Mefenamic acid (MFA), an anthranilic acid derivative, is a member of the fenamate group of nonsteroidal anti-inflammatory drugs. Mefenamic acid is analgesic, anti-inflammatory and antipyretic which inhibit the activity of the enzymes cyclooxygenase I and II, resulting in a decreased formation of precursors of prostaglandins and thromboxanes [5]. Hyoscine N-butyl bromide (HBB), also known as scopolamine, is a type of antimuscarinic agent and has central and peripheral actions (figure 1).

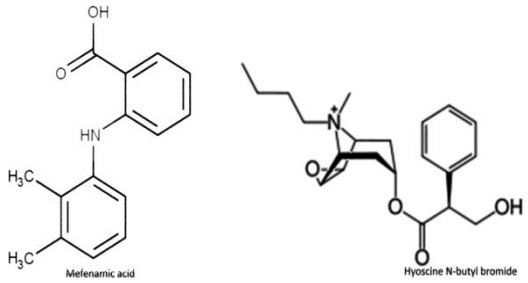


**Fig 1: Structure of Mefenamic acid and Hyoscine N-butyl bromide**

Attachment of the butyl-bromide moiety prevents the movement of this drug across the blood-brain barrier, thereby minimizing its potential neurologic side effects. It is often used as an antispasmodic treatment for the pain and discomfort induced by abdominal cramps or menstrual cramps [3]. Method developed can be conveniently used for quality control and routine determination of drug in pharmaceutical preparation in pharmaceutical industry. A highly sensitive and reproducible, simple and accurate analytical technique can be validated for the estimation combined dosage form. The present study is to develop and validate analytical method for mefenamic acid and hyoscine *N*-butyl bromide in bulk and pharmaceutical dosage form. Fixed dose combination containing MFA and HBB is a combination of anti-spasmodic drugs. Few spectrophotometric and chromatographic methods have been reported for estimation of HBB in bulk, pharmaceutical formulations. Several spectrophotometric, chromatographic methods are also reported for determination of MFA alone and in combination with other drugs from pharmaceutical formulations and biological fluids. In order to robust performance and ease of system setup, it was thought to develop a simple, fast, accurate reproducible and economical method for the simultaneous estimation of MFA in combination with HBB in solid dosage form.

## MATERIALS AND METHODS:

### Materials and reagents

Mefenamic acid and hyoscine n-butylbromide were obtained as gift sample from Ajanta Pharma Ltd. Mumbai, India. Methanol, water, acetonitrile, triethylamine, ortho phosphoric acid was procured from Merck, India. All the chemicals and reagents were used of analytical (HPLC) grade.

**Method development of mefenamic acid and hyoscine-*N*-butyl bromide**

**Instrumentation**

The analysis was carried out on an Agilent Technologies 1260 infinity (Wil-mington, DE) HPLC system using quaternary pump, online degasser, column heater, auto sampler and VWD/PDA detector. Column used was Inertsil ODS-3 column with dimensions 250 × 4.6 mm i. d. and particle size 5µm in series with stationary phase C18. The software used for data integration was Autochro 3000.

**Chromatographic conditions**

The elution isocratic with mobile phase consisting of 5 mM $KH_2PO_4$ (pH 3.0) buffer-acetonitrile in a ratio of 25:75 (v/v) was used at a flow rate of 1mL/min. Analysis was performed with UV-visible detector at 210 nm on ambient temperature. The mobile phase was used as diluents for preparation of standard and sample preparations [6].

**Preparation of mobile phase**

Mobile phase was prepared by mixing 250 mL of buffer with 750 mL of acetonitrile in to 1000mL measuring cylinder and transferred in to reagent bottle. The mobile phase was sonicated for 15 min and filtered through 0.45µ membrane filter paper.

**Preparation of standard stock solution**

Accurately weighed MFA and HBB (25mg each) and transferred in to 100mL volumetric flasks separately and dissolved in the mobile phase. The volume was adjusted with mobile phase to give composite solution of 100µg/mL each of MFA and HBB.

**Preparation of sample solution from the marketed product**

Twenty tablets (Hyocimax MF tablet) were weighed and grind to finely powdered. Tablet powder equivalent to 250 mg of MFA and 10 mg of HBB was transferred to a 250 mL volumetric flask and dissolved in 200 mL of mobile phase. The volume was made up to the mark with diluents and mixed well. The solution was sonicated for 15 min and filtered through 0.45µ membrane filter. The solutions were further diluted with mobile phase. The final concentration of solution was 20 g/mL of MFA and 40 µg/mL of HBB. The amount of drugs in samples was calculated from the peak area of MFA and HBB [6]. The amount of drug in a tablet was calculated using following formula;

$$mg/tablet = \left\{\frac{AT1 \times WS1 \times Ds \times P1}{AS1 \times WT \times Dt}\right\} \times Avg.wt.$$

…… (1)

Where,

$AT_1$ = Average area of MFA/HBB peaks in Test chromatogram

$AS_1$ = Average area of MFA/HBB peaks in Standard chromatogram

$D_s$ = Dilution factor for standard

$D_t$ = Dilution factor for test

$P_1$ = Potency of working standards of MFA/HBB of % w/w basis

Avg. wt = Average weight of 20 Tablets

Further calculate the amount of MFA/HBB present in % of label claim using following formula;

$$\% \text{ label claim} = \frac{Assay\ (\frac{mg}{tabet}) \times 100}{label\ claim\ of\ MFA/HBB}$$

…… (2)

**Method validation**

The optimized chromatographic conditions were validated by evaluating specificity, range, linearity, accuracy, precision, robustness, limit of detection, limit of quantitation and system suitability parameters in accordance with the ICH guidelines [7, 8].

**Linearity and range**

Linearity study was performed by preparing standard solutions at five different concentrations (10, 15, 20, 25 and 30µg/mL for MFA and 20, 30, 40, 50 and 60µg/mL for HBB) over the concentration range of 10-30µg/mL and 20-60 µg/mL for MFA and HBB respectively. The response was measured as peak area and the calibration curves were obtained by plotting peak area against concentration. Linear least square regression analysis was then employed to correlate peak areas and drug concentrations.

**Specificity**

The specificity study was performed by injecting each drug individually and in drug mixture. Method robustness was evaluated by analyzing the system suitability standard and evaluating system suitability parameters after varying the HPLC pump flow rate (± 20%), auto-sampler injector volume (± 10%), column compartment temperature (± 2°C) and mobile phase pH (± 0.1), respectively. Full spectrum (200-560 nm) chromatograms were acquired and Agilent Chem Station peak purity algorithms were also used to evaluate specificity. The specificity studies proved the absence of any undesired interference.

**Precision**

Precision was considered at repeatability and intermediate precision in accordance with ICH recommendations. From the standard stock

solutions, mixed standards containing MFA and HBB were prepared. Standard solutions (n=6) were injected using a universal rheodyne injector with injection volume of 10µL. The intraday and inter-day precisions were determined. The resultant standard curves were evaluated for intra and inter-day reproducibility. Precision was expressed by the relative standard deviation (RSD %) of the analyte peak areas.

**Accuracy**

Accuracy was expressed as percent recovery (%) by comparing the amount determined from the quality control standards against the respective nominal values. Accuracy of the method was also tested on drug products at three concentrations with 3 replicates at each level, using the ICH Q2R1 approach of standard addition [4]. The accuracy of an analytical method was determined by the standard addition method. A known amount of standard MFA and HBB corresponding to 80%, 100%, and 120% of the label claim was added to pre-analyze sample of tablet dosage form separately. The recovery studies were carried out three times at each level of recovery.

**Limit of detection (LOD) and limit of quantitation (LOQ)**

The LOD and LOQ were separately determined on the basis of residual standard deviation of the regression line or the standard deviation of y-intercepts of regression lines. Following formulae were used;

$$LOD = 3.3 \times \sigma/S \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

$$LOQ = 10 \times \sigma/S \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Where, 3.3 for LOD and 10 for LOQ are signal-to-noise ratio; S-standard deviation of the response of the minimum detectable drug concentration; σ is the slope of the calibration curve [6].

## RESULTS AND DISCUSSIONS:

An HPLC method void of problems is necessary to best assess the product quality of combined drugs. The aim was to develop a simple and robust HPLC method, ideally possessing buffering capacity and using UV detection at the absorbance maxima. The reverse phase HPLC was selected for separation because it was convenient and rugged than other forms of the liquid chromatography and was more likely to give good resolved peaks at a reasonable retention time at a specific pH.

**Selection of analytical wavelength ($\lambda_{max}$) for isobestic point**

An isobestic point is the point at which both the drugs in a particular combination will have same absorbance at a single wavelength. From the overlay spectra two wavelengths 223.0 nm ($\lambda_{max}$ of MFA) and 205.0 nm ($\lambda_{max}$ of HBB) were selected for estimation of drugs using simultaneous equation method (SEM). The isobestic point of Mefenamic acid and Hyoscine *N*-butyl bromide is 210 nm (figure 2).

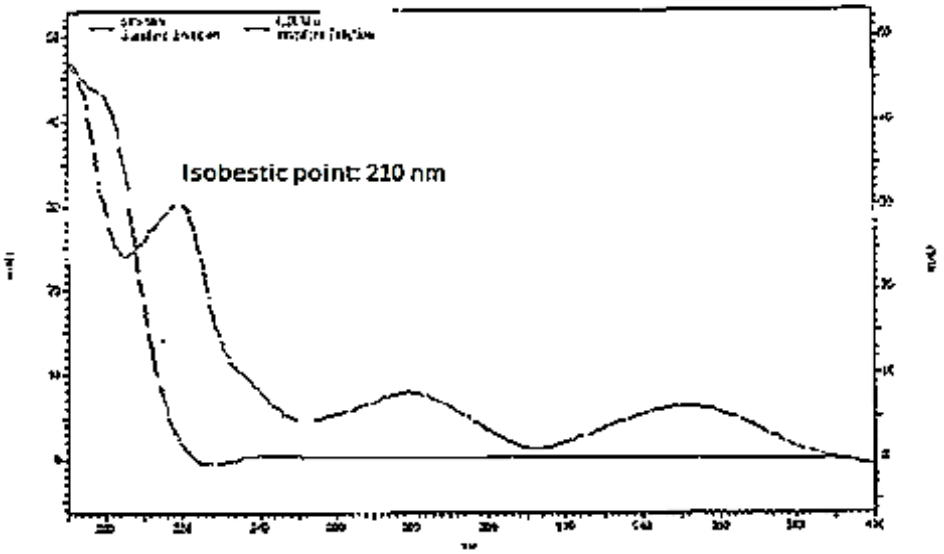


**Fig 2: Overlain Spectra of MFA and HBB**

**Optimization of chromatographic conditions**

Based on drugs solubility and various mobile phase compositions were evaluated to achieve acceptable separation using selected chromatographic conditions. For optimization of chromatographic conditions mainly focus was given in mobile phase, composition of mobile phase as well wavelength and flow rate. For mobile phase optimization two organic solvents (water and acetonitrile) was used while for selection of stationary phase. In the first trial water and acetonitrile (60:40) was used as diluent with flow rate 1.5mL/min (figure 3A). The blank baseline was not proper and the peak was not eluted. Hence this method was not suitable for drug estimation. In second condition, buffer and acetonitrile was chosen as mobile phase in the ratio of 20:80 with the same diluent. But more response obtained from the standard. Hence this method was not suitable and inject less concentrated standard solution and less injection volume (Figure 3B).

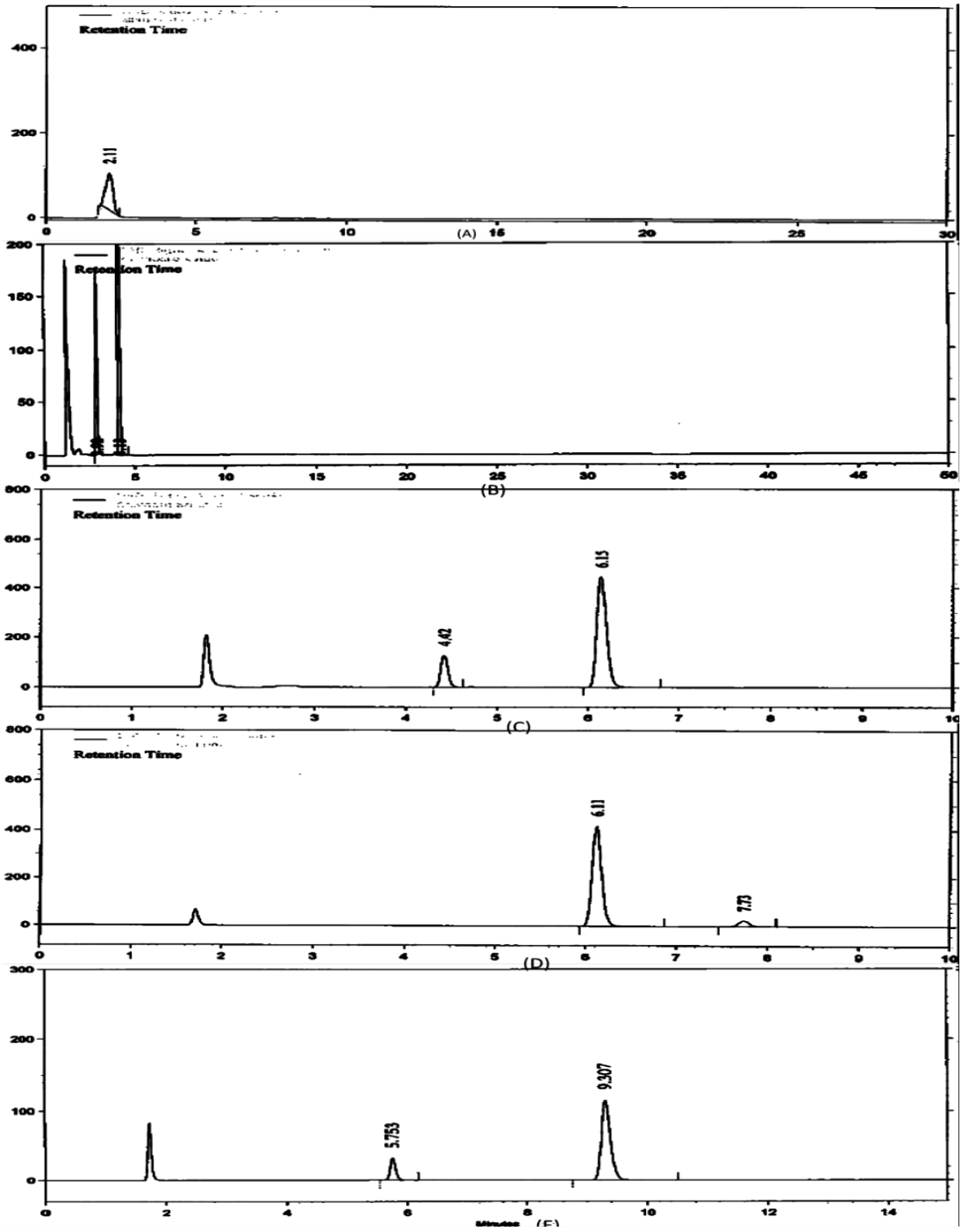


**Fig 3: Chromatogram of MFA and HBB for water: ACN (60:40%), B) $KH_2PO_4$ buffer: ACN (20:80%), C) $KH_2PO_4$ buffer: ACN (20:80%), D) $KH_2PO_4$ (pH 3.0) buffer: ACN (20:80%) and E) $KH_2PO_4$ (pH 3.0) buffer: ACN (25:75%)**

In third trial, injection volume was reduced from 100 µL to 10 µL, flow rate 1.0 mL/min and working standard concentration was decreased to 50 µg/mL of MFA and 30 µg/mL of HBB. But still more response obtained from the standard. Hence this method was not suitable for determination of drugs in combined form. After that tried for fourth trial with the same mobile phase but in this trial mobile phase itself had been used as diluent. Injection volume was reduced to 10 µL. But due to change in diluents, the $R_t$ was also changes (Figure 3D). Then fifth trial was taken mobile phase, by using above method, MFA and HBB were separated with buffer and acetonitrile in ratio 25:75 with pH adjusted to 3.0. The concentration of standard MFA and HBB was taken 20µg/mL and 40µg/mL respectively. The changes in pH resulted in better resolution (figure 3E). Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that more or less symmetrical peaks on the chromatogram after the detection of all the compounds. By slight change of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram was the one in which all the peaks are symmetrical and well separated in less run time. The mobile phase was selected on the basis of best separation, peak purity index, peak symmetry, theoretical plate etc. Finally the optimum mobile phase containing buffer: acetonitrile 25:75 (v/v) was selected because of ability to resolve the peaks of MFA (RT = 9.307± 0.05) and HBB (RT = 5.753± 0.09) with a resolution factor of 5.52. The optimization of the stationary phase and HPLC column selection were performed. As observed, MFA and HBB bound to C18 stationary phase tightly so that TEA was necessary to form an ion pair to achieve efficient elution. A major factor contributing to this strong interaction is the fourteen carbon chain of palmitate. In order to reduce the affinity of both drugs to the column, a cyano stationary phase with lower hydrophobicity was selected since it is more suitable for efficient elution of basic amines. Due to basic nature of analyte**,** Inertsil ODS-3, 5 micron C-18 column 250mm x 4.6mm column was used as a stationary phase because properties of such columns are reversed-phase with hybrid-based for good peak shape performance for highly basic compounds. The best resolution and peak shape, without excessive tailing and ensure the consistency; Inertsil ODS-3 (250x4.6mm) 5 micron C18 column was selected. Quantification was achieved with UV detection at 210nm [6]. As per European Pharmacopoeia any peak due to the bromide ion which appears close to the solvent peak is disregarded (figure 3E). The best resolution with reasonable retention time was obtained with mobile phase containing buffer and acetonitrile in gradient elution mode with 1.0 mL/min flow rate in gradient mode at 210 nm and run time 30 min. Stationary phase change allowed for significant modifications to the mobile phase composition. Given the low UV-cutoff at 200 nm, potassium phosphate buffer (5 mM, pH 3.0) was employed and results showed that this mobile phase could provide better peak symmetry and less baseline noise. Under the current chromatographic condition, MFA and HBB had a retention time of 9.307 and 5.753 min respectively when isocratically eluted with a flow rate of 1 mL/min at 25°C. In summary, a novel *RP*-HPLC analysis method for MFA and HBB is established on Inertsil ODS-3 C18 column (250×4.6 mm, 5 µm, 100 Å) performed at 25°C. After minor adjustment, the mobile phase was composed of phosphate buffer (5 mM, pH 3.0)-acetonitrile (25:75 v/v). MFA and HBB was eluted isocratically at a flow rate of 1 mL/min with a run time of 10 min; its retention time was around 5.753 and 9.307 min, detected at 210 nm. This method was a significant improvement over the previous trial for better resolution and enhanced selectivity over MFA and HBB and is a potential analytical tool to evaluate product stability.

**Validation of the proposed RP-HPLC method**

When method development and optimization are complete, it is necessary to accomplish method validation. For validation of analytical method, the guidelines of the International Conference on the Harmonization of technical requirements for the registration of pharmaceuticals for human use has recommended validation characteristics including system suitability, accuracy (% recovery), linearity, precision (% RSD) were investigated. System suitability is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be carried out. It is performed to ensure that the system is operating properly and read to deliver results with acceptable accuracy and precision.

**System suitability parameters**

The system suitability test is designed to ensure the validity of the analytical procedure as well as the conformation of the resolution between different peaks of interest. All critical parameters tested met the acceptance criterion on all days, as listed in Table 1. Adequate resolution >1.5 between MFA and HBB demonstrated the method specificity in the presence of its synthetic precursor, as shown in figure 3E.

**Table 1: System suitability data**

| USP Criteria | Specification | MFA | HBB | Pass/Fail |
|---|---|---|---|---|
| Retention time | RSD ≤ 2.0% | 0.04% | 0.12% | Pass |
| Capacity factor k' | > 1.0 | 2.724 | 2.774 | Pass |
| Symmetry | > 0.5 | 0.749 | 0.807 | Pass |
| Area | RSD ≤ 2.0% | 1.62% | 1.34% | Pass |
| Theoretical plates/meter | > 13,333 (2000/column) | 20,412 | 20,464 | Pass |
| USP tailing | < 2.0 | 1.38 | 1.12 | Pass |
| Resolution | > 1.5 | 2.19 | 2.24 | Pass |

MFA and HBB another possible impurity, was also well separated from drugs. The acceptance criteria were: relative standard deviation (RSD) for peak area less than 2.0%, a peak width at half height of less than 0.25 minute, a resolution for MFA and HBB greater than 1.5, a USP tailing factor less than 2.0.

**Linearity and calibration standards**

Linear regression data showed a good relationship over a concentration range of 10-30 µg/mL for MFA and 20-60 µg/mL for HBB. The linear regression equations for MFA and HBB were found to be y = 84.01x-1.71 and y = 7.3269x+1.3264, respectively. The regression coefficient values ($r^2$) were found to be 0.9998 and 0.9987 for MFA and HBB respectively indicating a high degree of linearity. The results demonstrated an excellent correlation between analyte peak area and concentration over the analytical range with $r^2 \geq 0.999$ using a least squares linear regression functions [9].

**Specificity and stability studies**

The specificity studies revealed the absence of any interference by excipients since none of the peaks appeared at the same retention time of MFA and HBB. The interaction study in standard solution was also carried out by comparing peak of each drug individually and in drug mixture. Results indicated that the analyte did not interact with each other. As per European Pharmacopoeia any peak due to the bromide ion which appears close to the solvent peak is disregarded. The stability of the analyte solutions was determined by comparing the analyte solutions at 3rd day and 7th day with that of the freshly prepared solution at 1st day. The differences determined on 3rd day were ±0.8 and ±1.15 for MFA and HBB respectively. The differences determined up to 7th day were ±1.72 and ±1.32 for MFA and HBB respectively. Together with all the aforementioned observations, we determined that this method is specific for MFA and HBB analysis.

**LOD and LOQ**

LOD and LOQ of MFA and HBB were calculated using the equations according to ICH guidelines. The limit of detection and limit of quantification were found to be 0.33 and 1.0 µg/mL for MFA and 0.802 and 2.43µg/mL for HBB respectively. The values indicate that the method is sensitive.

**Accuracy and precision**

Accuracy and precision was established across the analytical range for MFA and HBB. The intra and inter day accuracy and precision were calculated from the QC samples for drugs. In order to meet the experimental requirements, the acceptance criterion for low concentrations was set to be ± 10%, and ± 5% for medium and high concentrations. For evaluation of accuracy of the developed method, recovery studies were carried out using standard addition method at three different levels. The average % recoveries for MFA and HBB in marketed formulation were found to be 99.12 ±0.85 and 98.95±1.54 % respectively**.** The results revealed that there was no interference of excipients. The intra-day and inter-day precisions were assessed by analyzing standard solutions. A low % RSD (Relative Standard Deviation) value of 0.8081 and 1.5492 for MFA and HBB respectively indicates that the method is precise [6, 9]. The intra-day and inter-day results were calculated statistically are given in Table 2.

**Table 2: Intra-day and inter-day precision of HPLC study**

| Drug | Amount Taken [µg/ml] | Intra-day [n=3] | | Inter-day [n=3] | | Mean % Assay | SD | RSD |
|---|---|---|---|---|---|---|---|---|
| | | Area of sample 105 | % Assay | Area of sample 105 | % Assay | | | |
| MFA | 20.38 | 161.69 | 98.5 | 167.31 | 99.5 | 96.25 | 0.778 | 0.8081 |
| | 20.38 | 159.12 | 96.8 | 156.62 | 95.4 | | | |
| | 20.38 | 157.17 | 95.7 | 156.08 | 95 | | | |
| HBB | 40.28 | 27.7 | 98.3 | 28.31 | 98.1 | 100.06 | 1.55 | 1.5492 |
| | 40.28 | 28.56 | 101.2 | 28.62 | 101.5 | | | |
| | 40.28 | 28.39 | 100.7 | 28.55 | 100.2 | | | |

Data of intra-day and inter-day data did not differ significantly in terms of precision. Moreover, we were aware that even small increase of the lower limit of quantitation would significantly impact the accuracy in a positive manner. The percent recovery of the added standard to the assay samples was calculated and tabulated in Table 3. The average percent recoveries obtained as 98.95% for hyoscine-*N*-butyl bromide and 99.12% for mefenamic acid with % RSD less than 2 which fully agree with system suitability. This showed that, the proposed HPLC method for the determination of MFA and HBB in a tablet was found to be sufficiently accurate.

**Robustness**

It is important to demonstrate robustness of the method to ensure the HPLC method insensitive to minor changes in the experimental conditions. As pointed out by Dejaegher *et al*., the responses were evaluated: MFA and HBB retention time, peak area, capacity factor, USP tailing, resolution between MFA and HBB, and theoretical plate numbers [10]. Results are summarized in Table 4 and none of the alterations caused a significant change in these responses and all responses met the acceptance criteria for system suitability. Therefore the method is robust and reliable to quantitation MFA and HBB. Average retention times ± SD for MFA and HBB were 5.23±1.56, 9.26±1.56 min respectively for six replicate analyses.

**Table 3: Percent recovery data of Mefenamic Acid and Hyoscine-*N*-Butyl bromide**

| Drug | Level of % Accuracy | Trials | Amount added (mg) | Amount recovered (mg) | % Recovery | Mean % Recovery | SD | % RSD |
|---|---|---|---|---|---|---|---|---|
| MFA | 80 | I | 195.12 | 196.3 | 100.6 | 100.52 | 0.2 | 0.07 |
| | | II | 195.32 | 196.3 | 100.49 | | | |
| | | III | 195.39 | 196.3 | 100.46 | | | |
| | 100 | I | 244.06 | 240.66 | 98.61 | 98.49 | 0.2 | 0.1 |
| | | II | 244.44 | 240.66 | 98.45 | | | |
| | | III | 244.52 | 244.66 | 98.42 | | | |
| | 120 | I | 292.69 | 288.37 | 98.34 | 98.35 | 0.4 | 0.1 |
| | | II | 292.36 | 288.37 | 98.46 | | | |
| | | III | 292.9 | 288.37 | 98.26 | | | |
| HBB | 80 | I | 8.08 | 7.93 | 98.24 | 98.45 | 0.18 | 0.19 |
| | | II | 7.95 | 7.93 | 98.49 | | | |
| | | III | 7.96 | 7.93 | 98.61 | | | |
| | 100 | I | 10.01 | 9.95 | 99.4 | 99.17 | 0.24 | 0.25 |
| | | II | 10.06 | 9.95 | 98.91 | | | |
| | | III | 10.03 | 9.95 | 99.2 | | | |
| | 120 | I | 12.56 | 12.52 | 99.68 | 99.15 | 0.41 | 0.41 |
| | | II | 12.63 | 12.52 | 99.13 | | | |
| | | III | 12.66 | 12.52 | 98.89 | | | |

**Analysis of the marketed products**

Analysis of marketed tablets (Hyocimax MF) was performed on developed method. Assay of marketed tablet containing 250 mg MFA and 10 mg HBB was performed by preparing the sample solutions as described in the previous section. A typical chromatogram of MFA and HBB is shown in figure 4.

**Table 4: Robustness test results for the HPLC method for MFA and HBB analysis**

| Parameters | Drug | Nominal condition | Flow Rate (mL/min) | | Column temperature (°C) | | pH | |
|---|---|---|---|---|---|---|---|---|
| Change | - | - | 0.9 (Low) | 1.1 (High) | 23 (Low) | 27 (High) | 2.8 (Low) | 3.2 (High) |
| Retention time, min (RSD %) | MFA | 9.240 (0.062) | 9.585 (0.065) | 9.584 (0.050) | 9.579 (0.080) | 9.586 (0.057) | 9.588 (0.064) | 9.598 (0.124) |
| | HBB | 5.233 (0.062) | 5.285 (0.065) | 5.284 (0.050) | 5.379 (0.080) | 5.386 (0.057) | 5.288 (0.064) | 5.298 (0.124) |
| Peak area (RSD %) | MFA | 168.35 (1.654) | 165.9 (1.713) | 163.5 (0.856) | 167.4 (1.586) | 164.9 (1.739) | 165.6 (1.588) | 163.7 (1.710) |
| | HBB | 45.63 (1.654) | 49.9 (1.713) | 43.5 (0.856) | 52.4 (1.586) | 54.9 (1.739) | 51.6 (1.588) | 53.7 (1.710) |
| Resolution (RSD %) | MFA | 2.19 (1.507) | 2.333 (1.155) | 2.312 (0.550) | 2.421 (1.527) | 2.339 (0.559) | 2.445 (2.080) | 2.606 (0.807) |
| | HBB | 2.308 (1.507) | 2.313 (1.155) | 2.321 (0.550) | 2.401 (1.527) | 2.329 (0.559) | 2.415 (2.080) | 2.106 (0.807) |

The % drug content found to be between 98.49% for MFA and 99.17% for HBB with not more than 2 % RSD. Data indicated that the estimation of dosage forms were accurate. The results are given in the Table 5. This method demonstrated strong applicability for product potency analysis where product potency was found >98%. In addition, this method has been proved applicable for potency analysis of novel MFA and HBB formulations prepared in house and also demonstrated an applicability to quantitation MFA and HBB release from these novel formulations during dissolution testing.

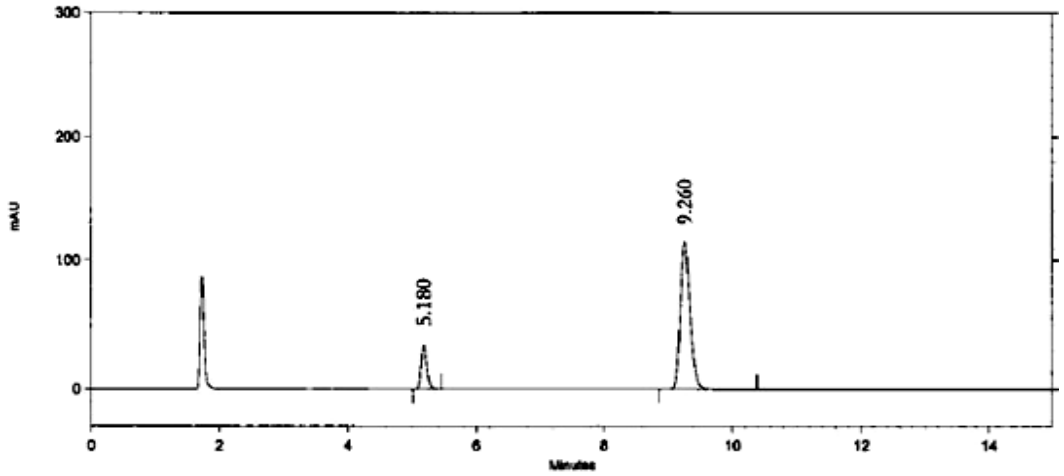


**Fig 4: Typical chromatogram of MFA and HBB in tablets sample**

**Table 5: Potency of MFA and HBB in marketed formulation by RP-HPLC (n = 3)**

| Product | Conc. (µg/ml) | Area | Amount found | Drug potency | Mean | SD | % RSD |
|---|---|---|---|---|---|---|---|
| MFA | 20 | 166 | 20.82 | 102.01 | 100.68 | 1.148 | 1.141 |
| | 20 | 194 | 20.38 | 100.02 | | | |
| | 20 | 194 | 20.37 | 100.02 | | | |
| HBB | 40 | 27.7 | 40.28 | 98.9 | 98.98 | 0.141 | 0.142 |
| | 40 | 32.3 | 40.28 | 99.15 | | | |
| | 40 | 27.8 | 40.29 | 98.91 | | | |

Hence it can be concluded that the developed RP- HPLC method is an accurate, precise and robust method and can be employed successfully for the estimation of MFA and BHH in bulk and formulation.

## CONCLUSION:

A simple, efficient, robust and less time consuming RP-HPLC method for simultaneous estimation of MFA and HBB was developed and validated. The significant advantages of this method were the simplicity of mobile phase preparation, buffering capacity, reduced consumption of organic solvents and avoidance of ion pairing agents. The proposed reversed phase high-performance liquid chromatographic method has been evaluated over the linearity, precision, accuracy, specificity and proved to be convenient and effective for the quality control of MFA and HBB in given application. The method has been successfully tested for the analysis of marketed tablets and can be adopted for the routine analysis of formulations containing any one of the above drugs or their combinations without any alteration in the chromatographic conditions. Thus, the proposed methodology is rapid, selective, requires a simple sample preparation procedure and represents a good procedure of MFA and HBB determination in pharmaceutical dosage forms.

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