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# METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC ANALYSIS OF BROMFENAC IN RABBIT PLASMA

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**ABSTRACT**

A simple, precise, rapid, and accurate reverse phase high performance liquid chromatography method was developed for the estimation of Bromfenac in Rabbit plasma by using Paracetamol as IS. The protein precipitation technique was used for the extraction of analyte from rabbit plasma. The chromatographic separation was performed on Agilent, Zorbas SB C18 (4.6mm×250mm×5µ) column with PDA detection at 267nm. The mobile phase comprising of mixture of methanol and buffer 0.1% ortho phosphoric acid (pH 2.6) in the ratio of 30:70v/v, at the flow rate 0.8ml/min. The analytical method described here is valid for the estimation of Bromfenac in Rabbit plasma over a range of 50µg/ml-150µg/ml. The retention times of Bromfenac and Paracetamol were found to be 3.781 and 2.470mins respectively. The correlation coefficient for Paracetamol and Bromfenac were 0.998 and 0.999 respectively. The LOD and LOQ values for Bromfenac were found to be 0.13µg/ml and 0.40µg/ml respectively. %RSD of Bromfenac and Paracetamol was obtained to be 0.41and 0.28 respectively. This method is time efficient and samples are easy to monitoring Bromfenac in plasma.

**Keywords**: HPLC-PDA method, Rabbit Plasma, Bromfenac, Protein precipitation.

## INTRODUCTION

The method measuring drugs in biological media are becoming increasingly important for the study of bioavailability and bioequivalence studies, quantitative evaluation of drugs and their metabolites, clinical pharmacokinetics, research in basic biomedical and pharmaceutical sciences and therapeutic drug monitoring.

Ocular drug delivery is one of the most fascinating and challenging tasks facing the pharmaceutical researchers. Enhancement of ocular penetration of eye drops remains one of the most challenging tasks in ophthalmology [1-4]. Topically applied non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in the management and prevention of ocular inflammation and cystoids macular edema related to cataract surgery [5, 6].

Brofenac is a Non-steroidal anti-inflammatory drug (NSAID) and is chemically-2-[2-amino-3-(4-bromobenzoyl) phenyl] acetic acid. Bromfenac ophthalmic solution is indicated for the treatment of postoperative inflammation in patients who have undergone cataract extraction. Bromfenac inhibit the COX-1 and COX-2 activity [7]. Paracetamol is an Analgesic and antipyretic and chemically N-(4-hydroxyphenyl) acetamide. It inhibits the synthesis of PGs by competing with arachidonic acid for the active site on the COX enzyme [8]. It is used as an internal standard.

It is a specific, linear, precise, accurate and rapid method. In this study, we have developed a HPLC method with a protein precipitation extraction for the determination of Bromfenac in rabbit plasma and the developed method is validated as per regulatory requirements [9, 10]. The structure of Bromfenac is shown in figure 1.

## MATERIALS AND METHODS

### Chemicals

Standard samples of Bromfenac, Paracetamol and Rabbit plasma were taken from rainbow pharma training lab. HPLC Grade solvent Methanol was obtained from Merk and milli-Q water. AR Grade ortho phosphoric acid was used.

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**Equipment**

The chromatographic equipment used was HPLC-Waters e 2695 was equipped with 2998 PDA, 2690 Pump and autosampler. Chromatographic separation were performed using the Agilent, Zorbax SB C18 (4.6mm×250mm×5µ) column and analyzed by Empower-2 software.

**Preparation of Standard**

Bromfenac and Paracetamol stock solutions were prepared at a concentration of 1mg/ml by dissolving in water and the stock solutions were stored in the refrigerator. Transferred 10 ml of standard stock solution into a 100ml volumetric flask and dilute to volume with HPLC grade water (100µg/ml). The bulk spiked calibration standards and quality control samples were stored in the freezer.

**Plasma preparation**

Blood samples were collected and taken to the lab where they were centrifuged at 3000rpm for 20min at room temperature. The resulting plasma samples were stored at -30$^0$C until analysis.

**Sample preparation**

Bromfenac sample was extracted by protein precipitation method. 100µl of rabbit plasma spiked with 200µl of Bromfenac drug taken in a 2ml centrifuge tube and mixed for 20 sec. To this 200µl of Paracetamol (IS), solution added and mixed for 20 sec. The drug was extracted by centrifuging with 100µl of methanol at 10,000rpm for 5min. The supernatant was finally collected and directly injected into the HPLC.

**HPLC METHOD**

The mobile phase used was mixture of methanol and buffer 0.1% of ortho phosphoric acid (pH 2.6) in the ratio of 30:70v/v, at the flow rate 0.8ml/min. Before analysis, the mobile phase was filtered through 0.45µm filter and then degassed ultrasonically for 5min. The eluent was monitored at a wavelength of 267nm. The injection volume was 10µl.

**METHOD VALIDATION**

The method was validated for accuracy, precision, linearity, specificity, limit of detection and limit of quantitation, robustness etc.

**Accuracy and precision**

The absolute recovery of Bromfenac was determined by comparison of the peak areas corresponding to 50, 100, 150µg/ml respectively with three replicates each. The precision was calculated as the relative standard deviation of the mean (RSD) with RSD (%) = (standard deviation of the mean/mean) ×100.

**Linearity**

Standard plasma samples with concentration range of 50-150 µg/mL were prepared by spiking different concentration of Bromfenac and Paracetamol working standard solutions into blank plasma. Calibration curve was constructed by plotting the peak height of Bromfenac and Paracetamol against the corresponding concentrations of standard plasma samples. Linear regression equation was applied and correlation coefficient was determined. The linearity was determined from five standard calibration curves over a concentration range of 50 – 150 µg/mL (R2, y = mx + c).

**Specificity**

The specificity of the method was determined by comparing the chromatograms obtained from the plasma samples containing Bromfenac and Paracetamol with those obtained from blank plasma samples and analyzing them for peaks interfering with the detection of Bromfenac or the internal standard. Subsequent peaks were tested to determine the level of purity compared to the stored spectra in order to confirm specificity.

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

The LOD and LOQ of the developed method were estimated based on standard deviation and slope of the calibration curve as 3.3 σ/S and 10 σ/S respectively. Here, σ was the regression standard deviation of intercept and S was the slope of calibration curves.

**Robustness**

The robustness of developed method was studied by evaluating the effect of small but deliberate variations in chromatographic conditions. The parameters studied were flow rate and column temperature.

**Table 1. Recovery studies for Bromfenac and Paracetamol**

| S. No. | Drug | Spiked level (%) | Amount taken (µg/ml) | Amount founded (µg/ml) | Percent recovery n=3 | Mean recovery |
|---|---|---|---|---|---|---|
| 1 | Bromfenac | 50 | 49.850 | 49.87 | 100.05 | 100.08 |
| | | 100 | 99.700 | 99.84 | 100.14 | |
| | | 150 | 149.550 | 149.66 | 100.07 | |
| 2 | Paracetamol | 50 | 49.550 | 49.60 | 100.10 | 100.02 |
| | | 100 | 99.100 | 99.04 | 99.94 | |
| | | 150 | 148.650 | 148.68 | 100.02 | |

n- Number of replicate injections

**Table 2. Observation of Precision**

| Injection | Paracetamol Area | Bromfenac Area |
|---|---|---|
| Injection 1 | 466290 | 1741964 |
| Injection 2 | 468429 | 1742418 |
| Injection 3 | 467042 | 1727458 |
| Injection 4 | 465150 | 1730352 |
| Injection 5 | 468040 | 1729610 |
| Average | 466990 | 1734360 |
| SD | 1327.73 | 7228.69 |
| % RSD | 0.28 | 0.41 |

**Table 3. Robustness study of Bromfenac**

| S. No. | Parameter | $R_t$ | Tailing | Plate Count | Resolution |
|---|---|---|---|---|---|
| 1 | Temperature minus | 3.770 | 1.43 | 5687 | 7.22 |
| 2 | Temperature plus | 3.727 | 1.44 | 6195 | 7.65 |
| 3 | Flow minus | 4.943 | 1.36 | 6345 | 7.67 |
| 4 | Flow plus | 2.992 | 1.39 | 5478 | 7.26 |

**Table 4. Validation parameters of Bromfenac by HPLC**

| S. No | Parameters | Results |
|---|---|---|
| 1 | System suitability | Pass |
| 2 | Accuracy | Pass |
| 3 | Precision | Pass |
| 4 | Linearity | $R^2$=0.999 |
| 5 | LOD | 0.13 |
| 6 | LOQ | 0.40 |
| 7 | Robustness | Pass |
| 8 | Retention time ($R_t$) (min) | 3.781 |

**Figure 1. Chemical structure of Bromfenac**

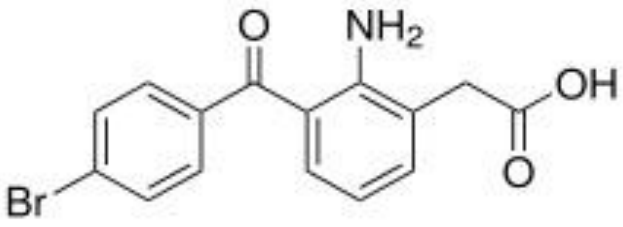



**Figure 2. Chromatogram of blank plasma sample**

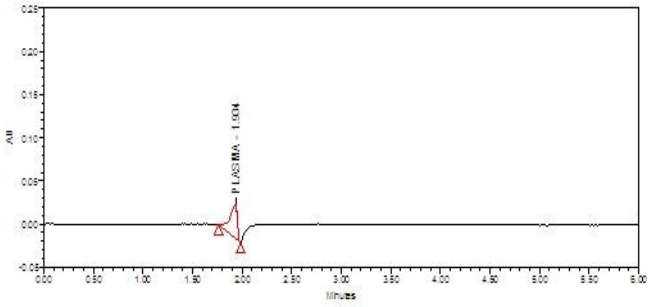



**Figure 3. Chromatogram of blank plasma spiked with Paracetamol and Bromfenac**

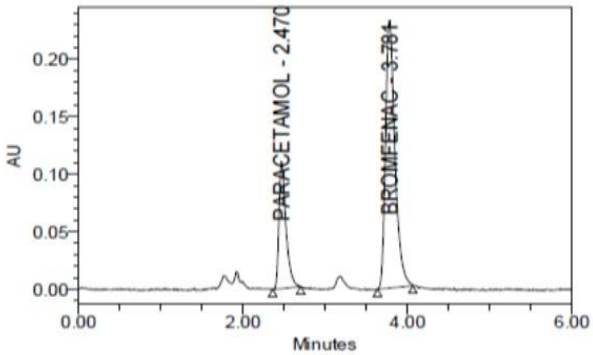
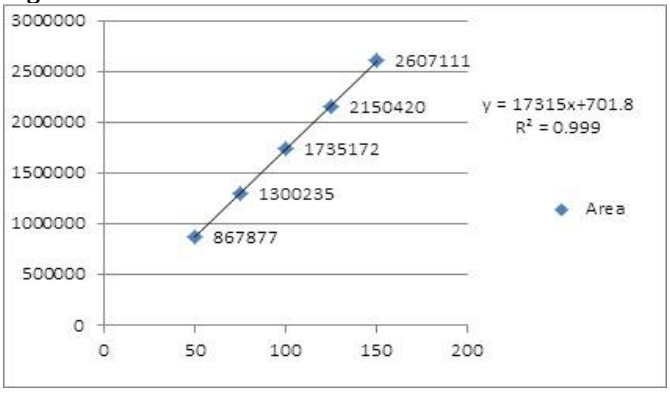



**Figure 4. Calibration curve for Bromfenac**



## RESULTS AND DISCUSSION

**Accuracy and Precision:** Accuracy and precision was evaluated by analyzing three batches. Each batch consists of three replicates. The mean accuracy for Bromfenac was 100.08% and for Paracetamol was 100.02% and the mean precision for Bromfenac was 0.41% and for Paracetamol was 0.28%.

**Linearity:** The calibration curve from 50.0µg/ml to 150.0µg/ml was generated using five calibrations. The figure 3 shows the calibration curve of Bromfenac. The slope, intercept and correlation coefficient were found to be 17315, 701.8 and 0.999 respectively.

**LOD and LOQ:** The LOD and LOQ were found to be 0.13µg/ml and 0.40µg/ml respectively. These low values were indicative of high sensitivity.

**Robustness:** The developed method robustness results were given in table 3. The result obtained implies method was robust for routine quantitative analysis.

## CONCLUSION

The bio-analytical method development and validation process for the analysis of Bromfenac by HPLC has been investigated in the study. The mobile phase is simple to prepare and it's economical. The optimized method showed good resolution with appropriate retention time for the respective peaks and good system suitability. The parameters which are validated for the developed method offered satisfactory results within acceptable limits, which reveal that developed method is validatable, transferable, robust, reliable, accurate and precise and sensitive. It can be concluded that the method is suitable for the routine quantification of Bromfenac, bioavailability and pharmacokinetic studies using HPLC.

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