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**Research**

# Optimization and Verification of A RP - HPLC Method for Analysis of Assay of Bumetanide API.

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## Abstract

The purity and quality of drug substances are paramount in pharmaceutical research, influencing their safety and efficacy. This study aimed to develop and validate a reliable reverse-phase ultra-performance liquid chromatography (RP-HPLC) method for the analysis of bumetanide, a potent loop diuretic associated with performance enhancement in athletics. Utilizing the Waters (Empower 2.0) equipped with a photodiode array detector, we established a method that provides enhanced resolution, speed, and sensitivity compared to traditional HPLC techniques. Materials, including HPLC-grade chemicals, were employed to formulate a mobile phase ensuring optimal performance during analysis. A 0.1% orthophosphoric acid solution was prepared for buffer use, and bumetanide was accurately quantified through a meticulously prepared standard solution. This method not only facilitates the detection of bumetanide in pharmaceutical formulations but also contributes to understanding its degradation profile. The findings affirm the effectiveness of RP-UPLC as a robust analytical tool in pharmaceutical quality control, addressing the pressing need for precise monitoring of compromised drugs.

**Keywords:** Bumetanide, HPLC, Acetonitrile.

## INTRODUCTION

The most effective way to demonstrate the quality of a drug substance is by confirming its purity. The fundamental objective of pharmaceutical research is to ensure the quality, safety, and efficacy of medications [1]. Drug quality includes factors such as potency, consistency, purity, pharmacological activity, and stability. Therefore, it is crucial for manufacturers to uphold these quality standards and create effective, safe, and non-

toxic drug formulations by developing advanced analytical techniques. Analytical methods for identifying and quantifying drug substances and products are categorized into physical, chemical, physicochemical, and biological methods. Physical analysis methods involve the reactions between the substance being analyzed and the reagents. Physicochemical techniques investigate the physical phenomena resulting from chemical reactions [2]. These methods typically focus on studying optical (such as emission techniques like fluorimetry), absorption (including UV, visible, and IR spectrophotometry), electrochemical (like potentiometry, amperometry, and polarography) characteristics, and chromatographic approaches (such as HPLC, GLC, and HPTLC), which encompass both separation and quantification (e.g., using photodiode array detectors). Chromatography is a highly utilized analytic technique with various forms, including paper chromatography, gas chromatography, liquid chromatography, thin layer chromatography (TLC), ion exchange chromatography, high-performance liquid chromatography (HPLC), and ultra-performance liquid chromatography (UPLC) [3]. HPLC, also known as high-pressure liquid chromatography, is a well-established analytical method for separating, identifying, and quantifying the components within a mixture. It is an advanced form of column liquid chromatography where the solvent flows through the column not solely by gravity but is forced through under pressures reaching up to 400 atmospheres. This pressure allows for the separation of sample components based on their varying affinities. In HPLC, liquid solvent is pressurized and combined with the sample mixture that then enters a column containing a solid adsorbent. The distinct interactions among each component lead to different flow rates, resulting in their separation within the column [4]. Bumetanide, a loop diuretic prescribed for heart failure, is recognized by the World Anti-Doping Agency (WADA) and the National Football League (NFL) as a prohibited substance for athletes. Its suspected use involves masking steroids by promoting urine excretion. Bumetanide [3-(Aminosulfonyl)-5-(butylamino)-4-phenoxy-benzoic] acid is a powerful high-ceiling or loop diuretic, demonstrating potency 40 to 60 times greater than furosemide [8]. The chemical structure and molecular weight of bumetanide are represented by the formula C17H20N2O5S, with a molecular weight of 364.416. While this compound falls within the sulfonamide classification, its structure varies significantly from that of furosemide and other similar drugs. The primary aim of this study was to develop and validate a method and conduct degradation studies of bumetanide in pharmaceutical dosage forms using RP-HPLC.

## MATERIALS AND METHODS

**Instrumentation**

| Name of the Instrument | Make/Model | Instrument ID |
|---|---|---|
| HPLC System | Waters (Empower 2.0) | GIL/HPLC/007 |
| Analytical Balance | Sartorius | GIL/ABAL/002 |
| pH meter | PICO+ | GIL/pHM/002 |
| Column | Inertsil ODS 3V 3.9mm x 150mm x 5µm | GIL/HPLC/COL-41 |

**Chemicals**

All chemicals, including HPLC-grade acetonitrile and orthophosphoric acid, were obtained from Rankem Chemical Division in Hyderabad. HPLC-grade water was consistently used throughout the experiment.

***Preparation of Orthophosphoric Acid (0.1%)***

To prepare a 0.1% orthophosphoric acid solution, 0.1 grams of orthophosphoric acid was accurately measured and placed into a 1000 ml volumetric flask. Water was added to reach a total volume of 1000 ml, and the solution was degassed to eliminate any air bubbles.

***Preparation of Mobile Phase***

The mobile phase was prepared by mixing a buffer solution with HPLC-grade acetonitrile in a 30:70 ratio in a volumetric flask. Prior to use, the mobile phase was degassed in an ultrasonic water bath for 5 minutes and then filtered through a 4.5 µm filter paper under vacuum filtration.

***Standard Solution Preparation***

To prepare a standard solution, 5 mg of bumetanide was accurately weighed and transferred into a clean, dry 10 ml volumetric flask, followed by the addition of 7 ml of diluent. The mixture was sonicated for 30 minutes and then brought to the final volume with more diluent. From this solution, 1 ml was taken and diluted in a 10 ml volumetric flask up to the final volume with diluent.

***Sample Preparation***

For sample preparation, twenty tablets were weighed to determine the average tablet weight. The equivalent weight of one tablet was then transferred to a 100 ml volumetric flask. After adding 80 ml of diluent, the mixture was sonicated for 25 minutes. The volume was adjusted with diluent, and the solution was filtered. Finally, 1 ml of the filtered solution was moved to a 10 ml volumetric flask and diluted to the final volume with diluent.

**Method Development**

Initially, attempts were made to develop a reverse phase liquid chromatography separation using various ratios of water, methanol, and acetonitrile as mobile phases. However, the drug did not respond well, and the resolution remained poor. The organic content of the mobile phase was then examined to enhance drug separation. Subsequently, a mixture of orthophosphoric acid and acetonitrile in a 30:70% v/v ratio was utilized at a flow rate of 0.4 ml/min. An Acquity SB C18 column (2 x 100 mm, 1.8 µm, 5µ particle size) was chosen as the stationary phase to improve resolution and significantly reduce peak tailing, bringing it close to 1.5. Various wavelengths from 210 nm to 280 nm were tested for drug analysis, and the wavelength showing maximum absorption for bumetanide at 254 nm was selected for the PDA detector. The retention time was approximately 0.852 minutes, as illustrated in Fig. 5.

**METHOD VALIDATION**

**System Suitability**

Sample solutions and six replicate injections were analyzed using freshly prepared standard solutions to assess each solute's peak area, theoretical plates (N), resolution (R), and tailing factors.

**Linearity**

Five solutions with concentrations ranging from 12.5 to 75 µg/ml were prepared. Each experiment was conducted in triplicate following the optimized chromatographic conditions. The calibration curve was generated by plotting the peak area of the chromatograms against the concentration of bumetanide.

**Precision**

Precision was evaluated through repeatability and intermediate precision, in line with ICH guidelines, by analyzing samples on the same day and on subsequent days. Each precision stage involved three sequential replicate injections at concentrations of 50, 100, and 150 µg/ml. Precision was expressed as the relative standard deviation (RSD).

**Accuracy**

The study of recovery of bumetanide was evaluated in triplicate at three concentration levels, i.e. 50%, 100% and 150% of working concentration of the sample [12]. The percentage of recoveries were calculated

## RESULTS

**Chromatograms depicting the method development of bumetanide**

Different chromatographic conditions were experimented to achieve the better efficacy of the chromatographic system. Parameters such as mobile phase composition, the wavelength of detection, column, column temperature, pH of mobile phase and diluents were optimized. Several proportions of buffer and solvents were evaluated in order to obtain an appropriate composition of the mobile phase. Choice of retention time, tailing, theoretical plates and runtime were major tasks while developing the method. A perfect peak was eluted at 30:70 (buffer: solvent) in an isocratic mobile phase flow rate. All the trails and the typical chromatogram obtained for bumetanide are shown in Fig 1.

**System suitability**

System suitability parameters such as Tailing factor for Bumetanide peak should be not more than 2.0 (1.12), Theoretical plates for Bumetanide peak in standard Solution should be more than 2000 (8366), RSD for Bumetanide peak in standard solution should not be more than 1.0% (0.03%) were evaluated for six replicate injections of the drug. The results were given in table 1.

**Table 1: System suitability data of bumetanide**

| S.No | Acceptance Criteria | Result |
|---|---|---|
| 1 | Tailing factor for Bumetanide peak should be not more than 2.0 | 1.12 |
| 2 | Theoretical plates for Bumetanide peak in standard Solution should be more than 2000 | 8366 |
| 3 | RSD for Bumetanide peak in standard solution should not be more than 1.0% | 0.03% |

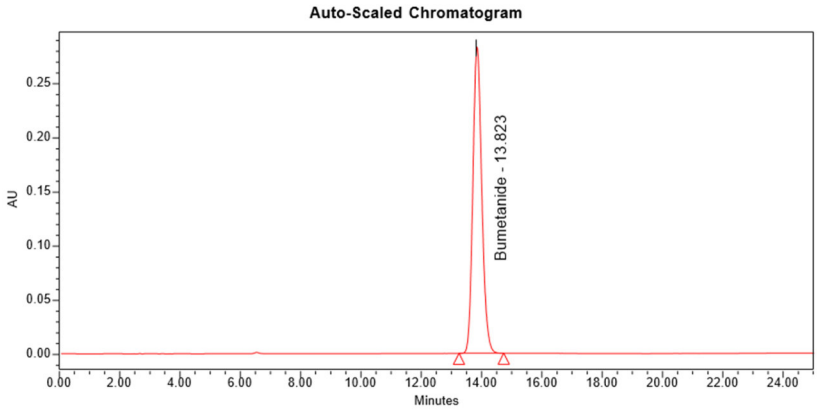


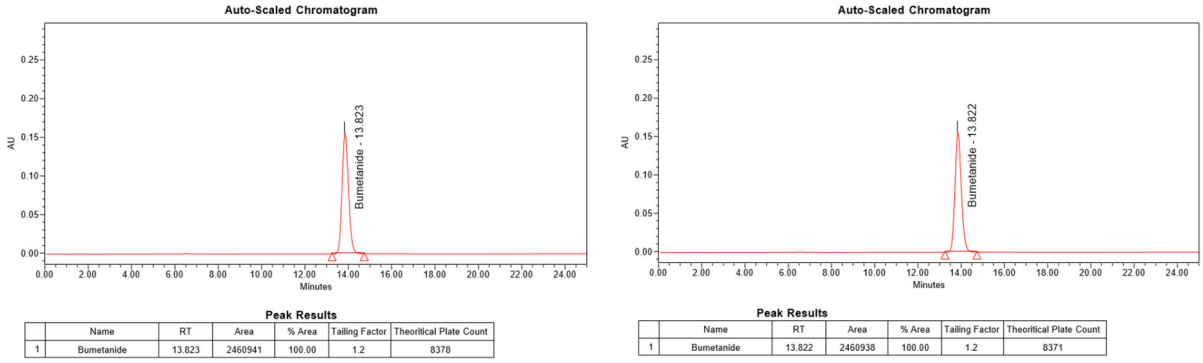

**Fig 1: Chromatograms for System Suitability**

**Linearity**

A linear correlation was obtained between the peak area used and the absorbance Vs concentrations of bumetanide. The calibration curve was linear for concentrations between 50 and 150µg/ml. The linearity of the calibration curves was validated by the values of the regression correlation coefficients ($r^2$). The correlation coefficient was found to be 0.999. The results of the linearity experiment were listed in table 2 and plot was presented in fig. 3.

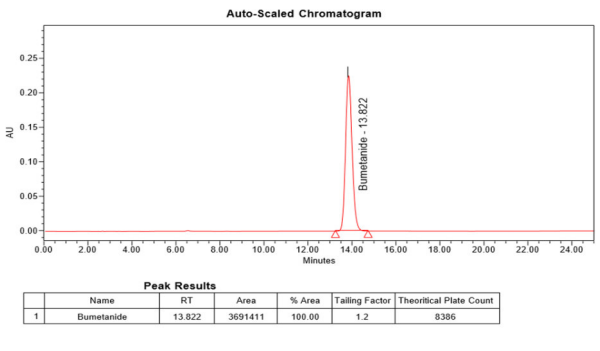
**Table 2: linearity concentration and response**

| S.No | Name | Linearity Level-50% | Linearity Level-75% | Linearity Level-100% | Linearity Level-125% | Linearity Level-150% |
|---|---|---|---|---|---|---|
| 1 | INJ-1 | 2461618 | 3692427 | 4923237 | 6154046 | 7384855 |
| 2 | INJ-2 | 2460938 | 3691433 | 4921860 | 6152321 | 7382858 |
| 3 | INJ-3 | 2460988 | 3691445 | 4921871 | 6152375 | 7382865 |

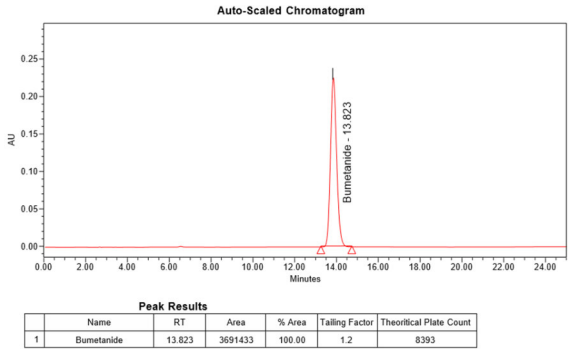



Chromatogram No 10-Linearity at 50% level-1 Chromatogram No 11-Linearity at 50% level-2

**Fig 2: Chromatograms for Linearity**

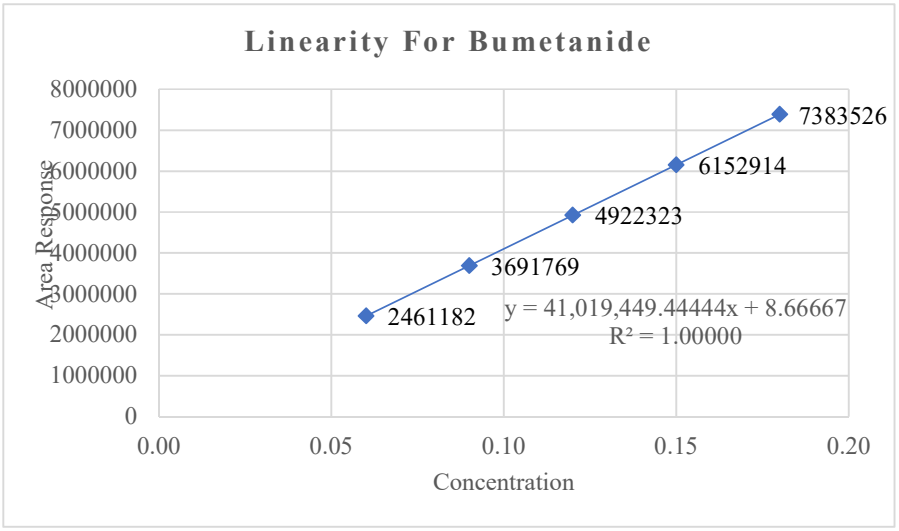



**Chromatogram No 12-Linearity at 75% level-1**




**Chromatogram No 13-Linearity at 75% level-2**

**Fig 3: Linearity plot of bumetanide**




**Precision**

The % RSD for the repeatability and intraday precision was reported to be 0.1 and 0.16. The results of precision were shown in table 3.

**Table 3: Intermediate precision**

| S.No | Acceptance Criteria | Result |
|---|---|---|
| 1 | The results should be within the specification limit. | Complies |
| 2 | The RSD calculated on 6 determinations for Bumetanide content should be NMT 2.0% | 0.16% |

**Accuracy**

The mean % recovery at concentrations ranging from (spike level) 50%, 100%, 150% was found to be 98.02 to 100.32 which were in the acceptance limit of 98.0 to 102.0 %. The RSD was not more than 2.0%. The results were shown in table 4.

**Table 4: Area Response of Accuracy Solutions**

| S.No | Name | Accuracy Level-50% | Accuracy Level-100% | Accuracy Level-150% |
|---|---|---|---|---|
| 1 | INJ-1 | 2542802 | 5075690 | 7628407 |
| 2 | INJ-2 | 2542823 | 5075611 | 7628490 |
| 3 | INJ-3 | 2542888 | 5075628 | 7628435 |

## DISCUSSIONS

In this study, we successfully developed a simple, rapid, specific, and economical chromatographic method for the assay of Bumetanide using High-Performance Liquid Chromatography (HPLC). The method's efficiency is underscored by using a mobile phase composed of a buffer and acetonitrile in a 35:65 ratio, which demonstrates the optimal conditions for the separation of Bumetanide from potential interferences.

The selected column, Inertsil ODS 3V (3.9 mm x 150 mm, 5 µm), proved effective for achieving the desired separation within the runtime of 25 minutes. This efficiency is crucial in the pharmaceutical industry where rapid results are necessary for both quality control and research purposes. The UV detection at 214 nm further enhances the method's sensitivity and specificity, allowing for the accurate quantification of Bumetanide without interference from impurities or degradation products.

Maintaining the auto-sampler temperature at 5°C is a vital aspect of the method, as it helps minimize thermal degradation of the analyte and ensures consistent peak area responses. The injection volume of 20 µL at a flow rate of 1.0 mL/min strikes a balance between sensitivity and ease of handling, facilitating routine analysis.

The methodological development incorporated guidelines set forth by the ICH for Analytical Method Validation (Q2 (R2)) and the United States Pharmacopeia (USP) standards, ensuring that the assay is robust, reproducible, and compliant with regulatory requirements. Specifically, adhering to the USP 40 guidelines for the validation and verification of compendial methods confirms the method's reliability for the analysis of pharmaceutical ingredients.

The successful application of this HPLC method for the determination of the Bumetanide active pharmaceutical ingredient highlights its practical utility in both research and quality assurance settings. Given the rigor of the validation process and alignment with established guidelines, this method is positioned not only for immediate use but also paves the way for further method validation studies.

## CONCLUSION

A simple, rapid, specific and economic chromatographic method have been verified for the assay of Bumetanide by HPLC. The HPLC determination was achieved with the mobile phase mixture in the ratio of 35:65 (Buffer: Acetonitrile). The HPLC separation was achieved with the column - Inertsil ODS 3V 3.9mm x 150mm x 5µm diameter. The response was recorded at the 214nm using UV detector without any interference and the runtime was 25mins.The Auto sampler temperature was maintained at 5°C.The injection volume was about 20µL at the flow rate of 1.0 mL/min. The method was developed with the reference of ICH guidelines for Analytical Method Validation Q2 (R2), USP 40< 1225> Validation of compendial Methods, USP 40< 1226> Verification of compendial Methods. The method was successfully applied for the determination of assay of Bumetanide Active Pharmaceutical ingredient. The Assay Method developed for Bumetanide active pharmaceutical ingredient using the HPLC method is appropriate for subsequent method validation.

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