ABSTRACT

Objective: The objective of the study was to develop a new, simple, accurate, precise and reproducible RP-UPLC method for the estimation of bumetanide in bulk and pharmaceutical dosage form.

Methods: Acuity SR C18, 2 x 100 mm, 1.8 µm, 5 µ particle size column with the mobile phase consisting of water: acetonitrile in the ratio of 30:70 v/v were used. The effluents were monitored at 254 nm and the flow rate was 1.0 ml/minute.

Results: The retention time was 0.852 min. Quantitative linearity was obeyed in the concentration range of 12.5 to 75 µg/ml. The correlation coefficient for bumetanide was found to be 0.999. Recovery and assay studies of bumetanide were within 99 to 102%, indicating that the proposed method can be adaptable for quality control analysis of bumetanide.

Conclusion: Proposed method was found to be simple, accurate, precise, and quick and can be used for regular analysis. This condition was applied to tablet dosage form. The statistical parameters and recovery studies were reported.

Keywords: UPLC, Bumetanide, Method development, ICH Guidelines

INTRODUCTION

The best way to illustrate the quality of a drug substance is to verify its purity. The basic task of pharmaceutical studies includes how to assure the quality safety and efficacy of drugs [1]. The quality of the drugs encompasses the potency, uniformity, purity, pharmacological action, stability etc. Therefore, it becomes essential for the manufacturer to maintain the quality and produce effective safe and non-toxic forms of drugs by developing newer analytical methods.

The methods of identification, estimation of drug substances and drug products are divided into physical, chemical, physicochemical and biological. Physical methods of analysis involve the use of physical reactions between the analyte and reagent. Physicochemical methods are used to study the physical phenomena that take place as a result of chemical reactions [2]. The techniques are usually based on the study of optical (emission, e. g., flurometry), absorption (UV, visible, IR spectrophotometry), electrochemical (potentiometry, amperometry, polarography) characteristics and chromatographic mode (HPLC, GLC, HPTLC) which include separation and quantification (e. g. photodiode array detector). The chromatography is very popular technique and it is frequently used analytically. There are various types of chromatographic techniques namely 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]. High-performance liquid chromatography which is also called as high-pressure liquid chromatography. It is a popular analytical technique used for the separation, identification, and quantification of each constituent of the mixture. HPLC is a highly developed technique for column liquid chromatography. The solvent generally flows through a column with the assist of gravity but in HPLC technique the solvent is forced under high pressures up to 400 atmospheres. Hence that sample can be separated into different constituents with the assist of difference in relative affinities. In HPLC, pumps are used to pass pressurized liquid solvent. It also includes the sample mixture which is permitted to enter into a column filled with solid absorbent material. The interaction of every sample component varies which causes a difference in flow rates of each component and finally leads to separation of components in column [4]. UPLC is a modern technique which gives a new approach for liquid chromatography. UPLC develops primarily in three areas: speed, resolution, and sensitivity [5]. UPLC shows an extraordinary development in speed, resolution as well as the sensitivity of analysis by using particle size less than 2 µm and the system is operational at higher pressure. The mobile phase can be able to run at better linear velocities as compared to HPLC [6]. This practice is considered as a new central point in the field of liquid chromatographic studies [7].

Bumetanide is a loop diuretic used to treat heart failure. The world anti-doping agency (WADA) and national football league (NFL) consider the supplement a banned ingredient for athletes. Its alleged use is to disguise steroids by increasing urine output. Bumetanide [3-{(Aminosulfonfyl)-5-(butylamino)-4-phenox-y-benzoi} acid is a potent high-ceiling or loop diuretic that has an efficiency 40 to 60 times greater than furosemide [8]. The chemical formula and molecular weight of bumetanide are C17H13N5O5 and 364.416 respectively. This compound belongs to the sulfonamide family, although its structure differs considerably from furosemide and others of its class. The main aim and objective of this work was method development, validation and degradation studies of bumetanide in pharmaceutical dosage form by RP-UPLC.

MATERIALS AND METHODS

Instrumentation

UPLC instrument used was of WATERS UPLC 2965 SYSTEM with auto-injector and PDA detector. Software used was Empower 2. UV-VIS spectrophotometer PG Instruments T60 with special bandwidths of 2 mm and 10 mm and matched quartz was used for measuring absorbance for bumetanide solutions.

Chemicals

HPLC grade acetonitrile, orthophosphoric acid, and all other chemicals were purchased from Rankem chemical division, Hyderabad. HPLC grade water was used throughout the study.
Preparation of orthophosphoric acid (0.1%)

0.1 gms of orthophosphoric acid was accurately weighed and transferred to a 1000 ml volumetric flask. Then 1000 ml of water was added to the volumetric flask and degassed for removal of water bubbles.

Preparation of mobile phase

Buffer solution and HPLC grade acetonitrile were transferred to the volumetric flask in the ratio of 30:70. Prior to use, the mobile phase was degassed in an ultrasonic water bath for 5 min and the solution was filtered through 4.5μ filter paper under vacuum filtration.

Standard solution preparation

Bumetanide (5 mg) was accurately weighed and transferred into a 10 ml clean dry volumetric flask and 7 ml of diluents was added. The solution was sonicated for 30 min and made up to the final volume with diluents. From the above solution, 1 ml was transferred to 10 ml volumetric flask and then made up to final volume with diluents.

Sample preparation

Twenty tablets were weighed and calculated the average weight of each tablet. Then the weight equivalent to 1 tablet was transferred to a 100 ml volumetric flask. Diluents (80 ml) were added and sonicated for 25 min. Then the volume was made up of diluents and filtered. From the filtered solution, 1 ml was transferred to 10 ml volumetric flask and made up to final volume with diluents.

Method development

Initially, reverse phase liquid chromatography separation was tried to develop using various ratios of water: methanol and acetonitrile as mobile phases, in which the drug did not respond properly and the resolution was also poor. The organic content of mobile phase was also investigated to optimize the separation of the drug. Thereafter, orthophosphoric acid:acetonitrile were taken in 30:70% v/v at a flow rate of 0.4 ml/min. Acquity SB C18, 2 x 100 mm, 1.8 μm, 5μ particle size column was used as the stationary phase. It was selected to improve resolution and the tailing of both peaks which were reduced considerably and brought close to 1.5. Detection was tried at various wavelengths from 210 nm to 280 nm for drug analysis. The wavelength at which bumetanide showed maximum absorption at 254 nm was selected as the detection wavelength for PDA detector. The retention times were found to about 0.852 min. The obtained chromatogram was shown in fig. 5.

Method validation

System suitability

Sample solution and six replicate injections were injected from freshly prepared standard solutions. Each solute was analyzed for their peak area, theoretical plates (N), resolution (R) and tailing factors [9].

Linearity

Five solutions ranging from 12.5-75 μg/ml were prepared. Each experiment was performed in triplicate according to the optimized chromatographic situation. The peak area of the chromatograms was plotted against the concentration of bumetanide to obtain the calibration curve [10].

Precision

Precision was determined as repeatability and intermediate precision by analyzing the samples in accordance with ICH guidelines. Determinations were performed on the same day as well as on consequent days [11]. Each stage of precision was investigated by 3 sequential replicates of injections of concentrations of 50, 100 and 200 μg/ml. The precision was expressed as the relative standard deviation (RSD).

Repeatability

Bumetanide sample solutions of 10μg/ml concentration were spiked for repeatability of the method. The precision was examined by analyzing six replicates. The retention time and percentage relative standard deviation were calculated.

Intermediate precision

The intermediate precision was studied on the next day of sample preparation. Working standard solution (50 ppm) of bumetanide at three concentration levels (50 %, 100 %, and 150 %) was analyzed. The % RSD of the analytical responses was calculated.

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.

LOD and LOQ

A series of solutions were injected and the signal-to-noise ratio for each compound was calculated. This process was continued until the S/N ratio was 3 for LOD and 10 for LOQ.

Robustness

Robustness was carried by varying three parameters from the optimized chromatographic conditions such as flow rate (±0.1 ml/min), mobile phase composition (±5%) and column temperature (±5°C) [13].

Degradation studies

Oxidation

The Stock solution of bumetanide (1 ml) was mixed with 1 ml of 20% hydrogen peroxide (H₂O₂). The solutions were kept for 30 min at 60 °C. For UPLC study, the resultant solution was diluted to obtain 50μg/ml solution and 1.0 μl was injected into the system. The chromatograms were recorded to assess the stability of the sample.

Acid degradation studies

The Stock solution of bumetanide (1 ml) was mixed with 1 ml of 2N hydrochloric acid and refluxed for 30 min at 60 °C. The resultant solution was diluted to obtain 50μg/ml solution and injected into the system. The chromatograms were recorded to assess the stability of the sample.

Alkali degradation studies

The Stock solution of bumetanide (1 ml) was mixed with 1 ml of 2N sodium hydroxide and refluxed for 30 min at 60 °C. The resultant solution was diluted to obtain 50μg/ml solution and injected into the system. The chromatograms were recorded to assess the stability of the sample.

Dry heat degradation studies

The standard drug solution was placed in an oven at 105 °C for 6 h for dry heat degradation studies. For UPLC study, the resultant solution was diluted to 50μg/ml solution and 1.0 μl was injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photostability studies

50μg/ml solution was prepared and exposed to UV light by keeping the beaker in UV Chamber for 1 day. For UPLC study, the resultant solution was diluted to 50μg/ml solutions and was injected into the system. The chromatograms were recorded to assess the stability of the sample.

Neutral degradation studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 1 hour at a temperature of 60 °. For UPLC study, the resultant solution was diluted to 50μg/ml solution and injected into the system. The chromatograms were recorded to assess the stability of the sample.

Assay

Standard solution and sample solution were injected into the chromatographic system and the peak area response for the analytes was measured. Standard preparations are made from the API and sample preparations are prepared from the formulation. Both sample and standard preparations were analyzed in six
replicates. Standard was taken as the reference for the estimation of the drug in the formulation.

**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. 1-5.

**System suitability**

System suitability parameters such as retention factor (0.852), plate number (9177), tailing factor (1.5), RSD (1.0), standard deviation (907.8) and mean area (132667) were evaluated for six replicate injections of the drug.

The results were given in table 1.
Fig. 4: Fourth trail run of bumetanide

Fig. 5: Typical chromatogram of bumetanide by the proposed method

Table 1: System suitability data of bumetanide

| Parameter           | Result          |
|---------------------|-----------------|
| RT                  | 0.852 min/ml    |
| Area(mean)          | 132667          |
| USP plate count     | 9177            |
| USP tailing         | 1.5             |
| *Standard deviation | 907.8           |
| %RSD                | 1.0             |

*Number of experiments: 6, % RSD: Relative standard deviation

Fig. 6: Linearity plot of bumetanide

Table 2: Linearity concentration and response

| Linearity level (%) | Concentration (ppm) | Area   |
|---------------------|---------------------|--------|
| 0                   | 0                   | 0      |
| 25                  | 12.5                | 34131  |
| 50                  | 25                  | 66387  |
| 75                  | 37.5                | 96431  |
| 100                 | 50                  | 130931 |
| 125                 | 62.5                | 159408 |
| 150                 | 75                  | 187732 |
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 12.5 and 75µg/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. 6.

Precision
The % RSD for the repeatability and intraday precision was reported to be 0.1 and 0.7. The results of precision were shown in table 3 and 4.

| S. No. | Peak area |
|--------|-----------|
| 1      | 131625    |
| 2      | 134117    |
| 3      | 132905    |
| 4      | 134057    |
| 5      | 130849    |
| 6      | 131659    |
| *Average | 132535 |
| Standard deviation | 1370.7 |
| %RSD  | 1.0       |

*Number of experiments-6, %RSD: Relative standard deviation

Table 4: Intermediate precision

| S. No. | Peak area |
|--------|-----------|
| 1      | 131257    |
| 2      | 134055    |
| 3      | 132542    |
| 4      | 132537    |
| 5      | 133017    |
| 6      | 132505    |
| *Average | 132652 |
| Standard deviation | 904.8 |
| %RSD  | 0.7       |

*Number of experiments-6, % RSD: Relative standard deviation

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 5.

Robustness
The robustness method was evaluated by deliberately varying the chromatographic conditions. The % RSD of flow minus, flow plus, mobile phase minus, mobile phase plus, temperature minus and temperature plus were found to be 0.6, 1.6, 0.8, 0.7, 1.0, 0.4 respectively. The parameters like tailing factor and retention time showed adherence to the limits. The data obtained with these changes were mentioned in table 6.

LOD and LOQ
Ratios of 3:1 and 10:1 signal-to-noise were considered as acceptable criteria for estimation of the LOD and LOQ, respectively. So, the limit of detection and the limit of quantification were determined to be 0.84 and 0.27 µg/ml, respectively. Chromatograms showing LOD and LOQ were as shown in fig. 7 and 8.

Forced degradation studies
Degradation studies were performed with the formulation and degraded samples were injected.

The % degradation of the drug for acid, alkali, oxidation, thermal, UV and water were 4.54, 2.92, 1.81, 0.86, 0.89, 0.52, respectively. Data was recorded in table 7.

| Level  | Amount spiked (µg/ml) | % recovery | *Mean % recovery |
|--------|-----------------------|------------|------------------|
| 50%    | 25                    | 99.40      | 99.29%           |
|        | 25                    | 98.02      |                  |
|        | 25                    | 99.71      |                  |
| 100%   | 50                    | 99.51      |                  |
|        | 50                    | 99.14      |                  |
|        | 50                    | 98.96      |                  |
| 150%   | 75                    | 99.58      |                  |
|        | 75                    | 99.00      |                  |
|        | 75                    | 100.32     |                  |

*Number of experiments: 3, % RSD: Relative standard deviation
Table 6: Robustness data of bumetanide

| Parameter          | %RSD* |
|--------------------|-------|
| Flow Minus         | 0.6   |
| Flow Plus          | 1.6   |
| Mobile phase Minus | 0.8   |
| Mobile phase Plus  | 0.7   |
| Temperature minus  | 1.0   |
| Temperature plus   | 0.4   |

Number of experiments: 3, %RSD: Relative standard deviation

Fig. 7: LOD chromatogram of bumetanide

Fig. 8: LOQ chromatogram of bumetanide

Table 7: Forced degradation studies of bumetanide

| S. No. | Degradation condition | % drug degraded |
|--------|-----------------------|-----------------|
| 1      | Acid                  | 4.54            |
| 2      | Alkali                | 2.92            |
| 3      | Oxidation             | 1.81            |
| 4      | Thermal               | 0.86            |
| 5      | UV                    | 0.89            |
| 6      | Water                 | 0.52            |

Table 8: Assay of formulation of bumetanide

| Sample No. | % assay |
|------------|---------|
| 1          | 98.9    |
| 2          | 100.8   |
| 3          | 99.9    |
| 4          | 100.7   |
| 5          | 98.3    |
| 6          | 98.9    |
| Average    | 99.60   |
| Standard deviation | 1.03 |
| %RSD       | 1.03    |

*Number of experiments-6, % RSD: Relative standard deviation
Assay of marketed formulation

Standard solution and sample solution were injected separately into the system. The % RSD of the marketed formulation was found to be 1.03. The drug present in the sample was calculated and results were summarized in table 8.

DISCUSSION

Analytical method development helps to know the vital process parameters and to reduce their influence on accuracy and precision. Analytical methods must be used by following GMP and GLP guiding principles and must be developed using the protocols and acceptance criteria set out in the ICH guidelines Q2 (R1). Method validation helps to validate the analytical method for a diversity of concentrations so that the change in formulation or concentration does not need additional validation. Once the methods have been developed, qualified and validated the impact they have on out-of-specification rates and process capability needs to be quantified. Methods are evaluated to determine its effectiveness for future use [14]. Several mobile phase compositions were tried to develop a new RP-UPLC method. A satisfactory separation with good peak symmetry was obtained with acuity SB C18, 2x100 mm, 1.8 mm, 5µ column using mobile phase containing orthophosphoric acid: acetonitrile (30:70) (v/v) at a flow rate of 0.4 ml/min. Quantification was achieved at UV detection at 254 nm based on peak area. The retention time for bumetanide was found to be 0.852 min. The optimized method was validated as per ICH guidelines.

System suitability tests were an integral part of a liquid chromatographic technique. They were used to verify that the proposed method was able to produce good resolution between the peaks of interest with high reproducibility. Retention factor, plate number (N), tailing factor (T), and RSD evaluated were within the range of acceptable limits [15]. The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found [16]. The standard addition and recovery experiments were conducted to demonstrate the accuracy of the method. The values of % recovery were found to be 99.29%, indicates that the method was accurate.

The precision of an analytical method gives information on the random error. It expresses the agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Six injections at the unchanged concentration were analyzed on the same day and two different days for verifying the variation in the precision. The % RSD for bumetanide was within the acceptable limit of ±2. Hence the method is reproducible on different days with different analyst and column. This indicates that the method was precise. The linearity of the method was tested in order to demonstrate the proportional relationship of response versus analyte concentration over the working range [17]. It is usual practice to perform linearity experiments over a wide range of analyte. This gives confidence that the response and concentration are proportional. It also accordingly ensures that calculation can be performed using a single reference standard/working standard, relatively than the equation of a calibration line. Bumetanide showed a linearity of response between 12.5-75 µg/ml. These were represented by a linear regression calibration line. Bumetanide showed a linearity of response between standard/working standard, relatively than the equation of a line. This ensures that calculation can be performed using a single reference standard. Bumetanide showed a linearity of response between standard/working standard, relatively than the linearity of the method. The values of % recovery were found to be 99.60%. The assay of the marketed formulation was carried out. The LOD and LOQ were found to be 0.27µg/ml and 0.80µg/ml, respectively. By using above method, assay of the marketed formulation was carried out. The mean percentage of recovery of the formulation was 99.60%. The % RSD of the marketed formulation was found to be 1.03. The drug present in the sample was calculated and results were summarized in table 8.

CONCLUSION

Chromatographic conditions used were stationary phase equiity SB C18, 2 x 100 mm, 1.8µ column and a mobile phase consisting of 0.1% orthophosphoric acid: Acetonitrile in the ratio of 30:70. The flow rate was maintained at 1 ml/min. The detection wavelength in UV was 254 nm. The column temperature was 30 °C. System suitability parameters were reported by injecting the standard six times and results were well under the range of acceptance criteria. Linearity study was carried out between 25 % to 150 % levels were within the acceptable range. Precision had shown good results which are acceptable to carry out a regular analysis. LOD and LOQ were found to be 0.27µg/ml and 0.80µg/ml, respectively. By using above method, assay of the marketed formulation was carried out. The mean percentage of recovery of the formulation was 99.60%. The proposed method was found to be simple, accurate, precise, and quick and could be used for regular analysis. This condition was applied to tablet dosage form. The statistical parameters and recovery studies were reported. Degradation studies of bumetanide were done, in all conditions purity threshold was more than purity angle and within the acceptable range.

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AUTHORS CONTRIBUTIONS

The complete research work was suggested and designed by Raja Sundararajan. Method development and validation works were carried out by Chaitanya Boni. Further, the manuscript was drafted by Chaitanya Boni and it was edited by Raja Sundararajan. Authors read and approved the final manuscript.

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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