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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR BOSENTAN HCl ESTIMATION IN BULK.

[1]Mrs.Akshada.S.Divekar, [2] Dr. Narendra.M.Gowekar, [3]Mrs.Vanita.N.Gade,[4]Mrs.Trupti.S.Kajale , [5]Mr.Mahesh.D.Gade,

[1]Assistant Professor, [2]Advisor-Approval Bureau, [3]Associate Professor, [4]Associate Professor, [5]Assistant Professor

[1,3,4,5]Department of Pharmaceutical Quality Assurance ,

[1, 3,4]Siddhant College of Pharmacy,Pune,India, [2]Pharmacy Council of India(PCI)

[5]Dr.Kolpe Institute of Pharmacy,Kopargoan,India,

Abstract: The present study focuses on the development and validation of a robust and precise Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the quantitative estimation of Bosentan Hydrochloride (HCl) in bulk form. Bosentan HCl, a dual endothelin receptor antagonist, is widely used in the treatment of pulmonary arterial hypertension. To ensure the quality and efficacy of this pharmaceutical compound, a reliable analytical method is essential.

The RP-HPLC method was optimized by systematically varying chromatographic conditions, including the selection of the mobile phase, flow rate, and detection wavelength. The finalized method utilized a [insert specific column type here], with a mobile phase consisting of [insert mobile phase composition here], and detection was carried out at [insert wavelength] nm. The method demonstrated excellent linearity in the concentration range of [insert range], with a correlation coefficient (R^2) greater than 0.999.

Validation of the developed method was conducted in accordance with ICH guidelines, assessing key parameters such as accuracy, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ), and robustness. The method exhibited high precision, with intra-day and inter-day variations within acceptable limits. Recovery studies confirmed the accuracy of the method, with percentage recoveries close to 100%.

The proposed RP-HPLC method was successfully applied for the estimation of Bosentan HCl in bulk, proving to be simple, rapid, and reliable for routine quality control analysis.

This abstract provides a concise summary of the key aspects of your analytical method development and validation study. Be sure to customize the specific details, such as the column type, mobile phase composition, and concentration range, based on your actual experimental setup.

I. INTRODUCTION

INTRODUCTION TO CHROMATOGRAPHY

Chromatography is a technique for breaking down mixtures of substances into their constituents based on their molecular structure and composition. A stationary phase (a solid or a liquid supported on a solid) and a mobile phase are involved (a liquid or a gas). The mobile phase flows through the stationary phase, carrying the mixture's components with it. Sample components with stronger interactions with the stationary phase will move through the column more slowly than components with weaker interactions. The separation of various components is caused by the difference in rates. Chromatographic separations can be performed using a variety of stationary phases, including immobilised silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography), and liquids (liquid chromatography) (liquid chromatography).

HPLC

HPLC is an abbreviation for High Performance Liquid Chromatography. Prior to the availability of HPLC, LC analysis was carried out using gravitational flow of the eluent (the solvent used for LC analysis), which took several hours to complete. Even improvements made later in time were able to slightly shorten the analysis time. Those early/classical LC systems were known as "low pressure chromatography" or "column chromatography."

In the 1970s, Jim Waters founded Waters Corporation and began selling HPLC instruments in the United States. This boosted the use of HPLC in practical analysis applications. Waters Corporation developed LC systems that used a high-pressure pump to generate a rapid-flow of eluent, resulting in a dramatic improvement in analysis time. In comparison to "low pressure chromatography," the newer types were referred to as "high pressure liquid chromatography." As a result, it was previously assumed that HPLC stood for High Pressure Liquid Chromatography; however, it is now widely accepted that HPLC stands for High Performance Liquid Chromatography. Another significant difference from Tswett's date was the data acquisition methods. Instead of observing the changes in layers with the naked eye, the detector system was connected to the LC, and the output was recorded on a paper chart. Tswett's analysis result would look like figure 3 if we were to display it on a chart (chromatogram).

Waters Corporation's system was initially referred to as the HPLC system. Waters Corporation is still the HPLC pioneer, but there are a number of other companies that manufacture and sell HPLC systems. Technically, the term LC refers to all types of liquid chromatography, including low pressure LC; however, because most LC systems used today are HPLC, the term LC is frequently used interchangeably with HPLC.

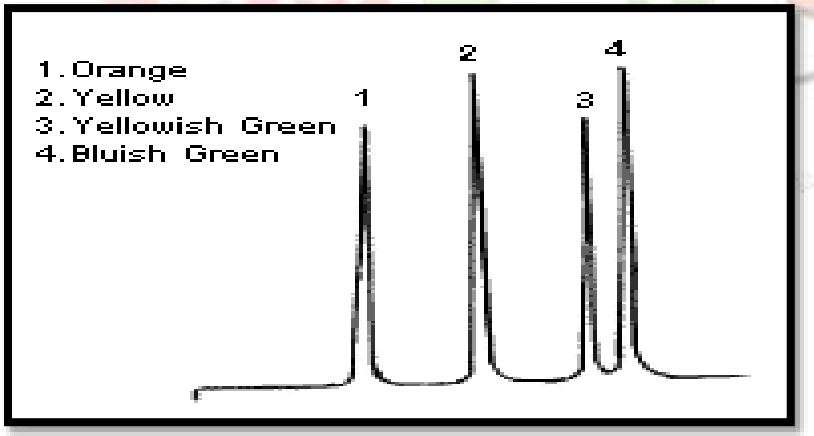

Fig No. 1.1 Representation of Tswett's LC analysis

Types of HPLC:

Depending on the phase system (stationary) in the process, the following HPLC variants exist:

- **Normal Phase HPLC**:

This method separates analytes based on their polarity. Polar stationary phase and non-polar mobile phase are used in NP-HPLC. As a result, silica is typically used as the stationary phase, with hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these as the mobile phase. As a result, polar samples are retained on the polar surface of the column packing for a longer period of time than less polar materials.

- **Reverse Phase HPLC:**

The stationary phase is nonpolar (hydrophobic), whereas the mobile phase is a polar liquid, such as water-methanol or acetonitrile mixtures. It operates on the basis of hydrophobic interactions, so the more nonpolar the material, the longer it will be retained.

- **Size-exclusion HPLC:**

The column is filled with material with precisely controlled pore sizes, and particles are separated based on their molecular size. Larger molecules are washed through the column quickly, while smaller molecules penetrate the porous packing particles and elute later.

- **Ion-Exchange HPLC:**

The stationary phase has an ionically charged surface that is charged in the opposite direction as the sample ions. This technique is almost always used with ionic or ionizable samples. The stronger the charge on the sample, the more it will be attracted to the ionic surface and thus take longer to elute. The mobile phase is an aqueous buffer in which elution time is controlled by both pH and ionic strength..

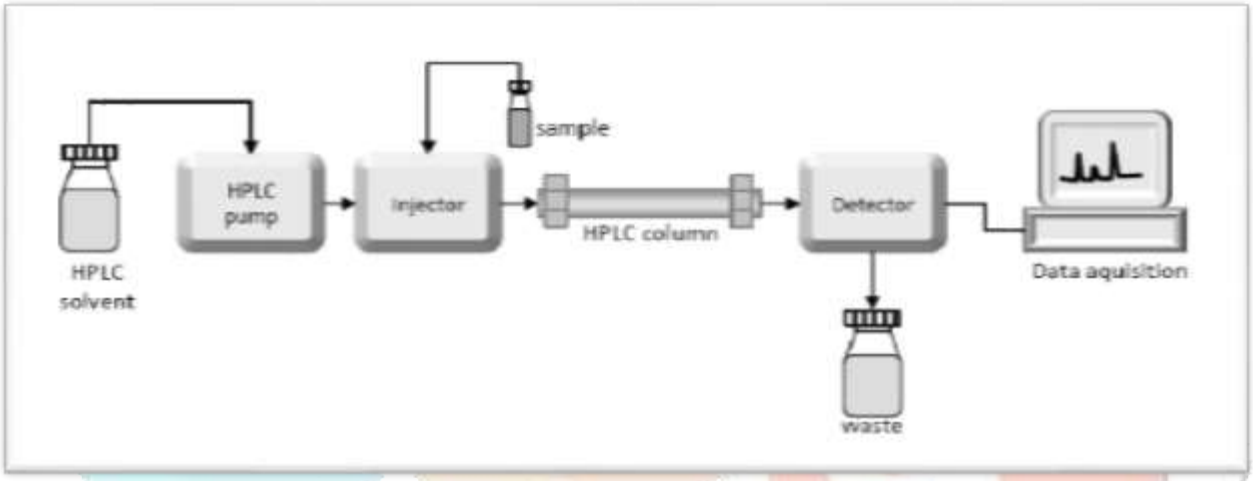

Fig No. 1.2 HPLC Components

4.1 Pump

The pump was the most important component of the system in the early stages of HPLC development. The development of HPLC can be described as a pump system development. The pump is located in the LC system's upper stream and generates a flow of eluent from the solvent reservoir to the system. One of the most important system requirements during the early stages of LC development was the ability to generate high pressure.

4.2 Injector

Next to the pump is an injector. The simplest method is to use a syringe to introduce the sample into the flow of eluent. Because the reproducibility of sample injection has a large impact on the precision of LC measurement, the injector design is critical. The sampling loops are the foundation of the most widely used injection method. The use of an autosampler (auto-injector) system, which allows for repeated injections at predetermined intervals, is also common.

4.3 Column

Because the separation occurs within the column, it is possible to refer to the column as the "heart" of an LC system. Since Tswett's time, the theory of chromatography columns has not changed; however, column development has improved continuously. Instead of the glass columns used in Tswett's experiment, recent columns are frequently prepared in stainless steel housing. In contrast to Tswett's use of calcium carbonate, the packing material most commonly used is silica or polymer gels.

4.4 Detector

Analytes are separated within the column, and the obtained separation is monitored using a detector. When no analyte is present, the eluent composition is consistent. While the presence of analyte alters the eluent's composition. These differences are measured by the detector. This difference is tracked as an electronic signal. There are various types of detectors available.

4.5 Recorder

The change in eluent detected by a detector is in the form of an electronic signal, which is not visible to our eyes. Pen (paper) chart recorders were commonly used in the past. In today's world, computer-based data processors (integrators) are more common.

4.6 Degasser

The eluent used for LC analysis may contain gases that are not visible to our eyes, such as oxygen. When there is gas in the eluent, it is detected as noise and causes an unstable baseline. Sparging (bubbling of inert gas), the use of an aspirator, a distillation system, and/or heating and stirring are all common methods. However, the method is inconvenient, and when the solvent is left for an extended period of time (e.g., during a long analysis), the gas gradually dissolves back. To remove gases, a degasser employs special polymer membrane tubing. The numerous very small pores on the surface of the polymer tube allow air to pass through while preventing liquid from passing through. By placing this tubing in a low-pressure container, pressure differences were created inside and outside the tubing (higher inside the tubing). This difference allowed the dissolved gas to move through the pores and out. In comparison to traditional batch degassing, the degasser can be used on-line, making it more convenient and efficient. Many new HPLC unit systems include a degasser.

Application of HPLC:

HPLC can provide information such as compound resolution, identification, and quantification. It also aids in the separation and purification of chemicals. Other HPLC applications include:

- **Pharmaceutical Applications**

  1. To control drug stability.
  2. Tablet dissolution study of pharmaceutical dosages form.
  3. Pharmaceutical quality control.

- **Environmental Applications**

  1. Detection of phenolic compounds in drinking water.
  2. Bio-monitoring of pollutants.

- **Applications in Forensics**

  1. Quantification of drugs in biological samples.
  2. Identification of steroids in blood, urine etc.
  3. Forensic analysis of textile dyes.
  4. Determination of cocaine and other drugs of abuse in blood, urine etc.

- **Food and Flavour**

  1. Measurement of Quality of soft drinks and water.
  2. Sugar analysis in fruit juices.
  3. Analysis of polycyclic compounds in vegetables.
  4. Preservative analysis.

- **Applications in Clinical Tests**

  1. Urine analysis, antibiotics analysis in blood.
  2. Analysis of bilirubin, biliverdin in hepatic disorders.
  3. Detection of endogenous Neuropeptides in extracellular fluid of brain etc.

GUIDELINES FOR ANALYTICAL METHOD VALIDATION

Validation

The process of validating an analytical method is the process of establishing, through laboratory studies, that the method's performance characteristics meet the requirements for the intended analytical applications.

Validation is defined as follows by different agencies.

Food and Drug administration (FDA)

Creating documentation Evidence that provides a high level of assurance that a specific process will consistently produce a product that meets its predetermined specifications and quality attributes.

World Health Organization (WHO)

action of ensuring that any procedure, process, equipment, material, activity, or system produces the desired results.

European Committee

Action of ensuring that any procedure, process, equipment, material, activity, or system produces the expected results in accordance with the principles of good manufacturing practise. In a nutshell, validation is a critical process for effective quality assurance.

Reasons for Validation

In the pharmaceutical industry, there are two major reasons for validating assays. The first, and most importantly, is that assay validation is an essential component of the quality-control system.

The second reason is that current GMP regulations require assay validation. **Steps followed for validation procedures**

1. Proposed protocols or parameters for validations are established.
2. Experimental studies are conducted.
3. Analytical results are evaluated.
4. Statistical evaluation is carried out.
5. Report is prepared documenting all the results.

Objective and Parameters of Analytical Method Validation

The primary goal of validation is to provide a foundation for written procedures for production and process control that are designed to ensure that the products have the identity, strength, quality, and purity that they purport or are represented to have. Quality, safety, and efficacy must be designed into the product. To maximise the likelihood that the finished products meet all quality and design specifications, each step of the manufacturing process must be controlled.

Analytical Method Development

When there are no official methods for a new product, methods are developed. Alternative methods for existing (non-Pharmacopoeia) products are being developed in order to reduce costs and time while improving precision and ruggedness. Trial runs are carried out, and the method is optimised and validated. When an alternate method is proposed to replace an existing procedure, comparative laboratory data, including benefits and drawbacks, is made available.

Steps of method development

Documentation begins at the very beginning of the development process, with the establishment of a system for full documentation of development studies. All data from these studies must be documented in a laboratory notebook or an electronic database.

1. Analyte standard characterization

a. All known information about the analyte and its structure, including physical and chemical properties, is gathered.

b. The standard analyte (purity 100%) is obtained. The necessary preparations are made for its proper storage (refrigerator, desiccators and freezer).

c. When analysing multiple components in the sample matrix, the number of components is noted, data is assembled, and the availability of standards for each one is determined.

d. Only methods that are compatible with sample stability (spectroscopic, MS, GC, HPLC, etc.) are considered.

2. Method requirement

The analytical method's goals or requirements that must be developed are considered, and analytical figures of merit are defined. The detection limits, selectivity, linearity, range, accuracy, and precision required are specified.

3. Literature search and prior methodology

The literature is searched for all types of information about the analyte. Synthesis, physical and chemical properties, solubility, and relevant analytical methods are examples. Books, periodicals, chemical manufacturers' compendia, and regulatory agency publications such as USP / NF, AOAC, and ASTM are all reviewed. The automated computerised literature searches provided by the Chemical Abstracts Service (CAS) are convenient.

4. Choosing a method

a) Methodology is adapted using information from the literature and prints. Modifications are made to the methods as needed. It is sometimes necessary to purchase additional instrumentation in order to replicate, modify, improve, or validate existing methods for in-house analytes and samples.

b) If there are no prior methods for the analyte in the literature, analogy is used to investigate and work out compounds that are similar in structure and chemical properties. Typically, there is one compound for which an analytical method exists that is similar to the analyte of interest.

5. Instrumental setup and initial studies

The necessary instrumentation has been installed. The installation, operational, and performance qualification of instrumentation are verified using laboratory Standard Operating Procedures (SOPs). New consumables (such as chemicals, solvents, and gases) are always used.

The analyte standard is prepared in a suitable injection / introduction solution and in known concentrations and solvents. It is critical to begin with an authentic, well-known standard rather than a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then work can begin with the actual sample.

6. Optimization

Rather than using a trial and error approach, one parameter is changed at a time during optimization, and a set of conditions is isolated. Work was completed according to a methodical plan, and each step was documented (in a lab notebook) in case of a dead end.

7. Documentation of analytical figures of merit

The originally determined analytical figures of merit, limit of quantitation (LOQ), limit of detection (LOD), linearity, time per analysis, cost, sample preparation, and so on are documented.

8. Evaluation of method development with actual samples

The sample solution should result in the unequivocal, absolute identification of the analyte peak of interest, independent of all other matrix components.

9. Determination of percent recovery of actual sample and demonstration of quantitative sample analysis

The percentage recovery of a spiked, authentic standard analyte into a sample matrix that is shown to be analyte-free is calculated. It has been demonstrated that recovery is reproducible (average +/- standard deviation) from sample to sample and that recovery has been optimised. It is not necessary to achieve 100 percent recovery as long as the results are reproducible and known with high certainty. Only laboratory studies can confirm the validity of an analytical method. As a result, proof of successful completion of such studies is a basic requirement for determining whether a method is appropriate for its intended application.

Method Validation

This procedure consists of establishing the performance characteristics and the method's limitations.

Method Performance Parameters are Determined using Equipment that is:

1. Within specification
2. Working correctly
3. Adequately calibrated

Method Validation is required when:

1. A new method is being developed
2. Revision of the established method
3. When established method are used in different laboratories and by different analysts etc.
4. Comparison of method
5. When quality control indicates method changes

Performance characteristics examined when carrying out method validation are:

1. Accuracy
2. Precision
3. Specificity
4. Selectivity
5. Sensitivity
6. Limit of detection.
7. Limit of quantification
8. Linearity and Range
9. Ruggedness
10. Robustness
11. System suitability

1. Accuracy

The accuracy of the measurement refers to how close the measured value is to the true value for the sample. According to the ICH documents, accuracy should be evaluated using a minimum of nine determinations over a minimum of three concentration levels within the specified range (i.e., three concentrations and three replicates of each concentration). Accuracy was tested (percentage recovery and percent RSD of individual measurements) by analysing samples at least in triplicate, at each level (80,100, and 120 percent of label claim). Fresh samples were prepared for each determination, and the assay value was calculated. The linearity study's regression equation was used to calculate recovery. The mean relative error for a set of replicate analyses (i.e. the difference between measured and nominal concentration) for spiked samples was used to determine accuracy.

2. Precision

The precision of an analytical procedure expresses the degree of agreement between a series of measurements obtained from multiple samplings of the same homogeneous sample under specified conditions. The precision of an analytical method is typically expressed as the standard deviation, relative standard deviation, or coefficient of variation of a set of measurements. According to the ICH documents, repeatability should be assessed using a minimum of nine determinations covering a specified range of procedures. Precision is a measure of the reproducibility or repeatability of an analytical method under normal operating conditions.

$$\boldsymbol{Stanadard\ Deviation} = \sqrt{\frac{\sum(\boldsymbol{x} - \overline{\boldsymbol{x}})^2}{\boldsymbol{n} - \boldsymbol{1}}}$$

Where,

$x = sample,$

$\bar{x} = mean\ value\ of\ samples,$

$n = number\ of\ samples$

$$\boldsymbol{Relative\ Standard\ Deviation} = \frac{\boldsymbol{Standard\ Deviation}}{\overline{\boldsymbol{x}}} \times \boldsymbol{100}$$

Repeatability:

Repeatability expresses precision over a short time interval under the same operating conditions. Repeatability is also known as intra-assay precision.

Intermediate Precision:

Intermediate precision is expressed through variations in laboratories: different days, different analysts, and different equipment.

Reproducibility:

When the procedure is performed by different analysts in different laboratories with varying equipment, regents, and laboratory settings. Repeatability and intermediate precision were used to determine reproducibility. An inter-laboratory trial is used to assess reproducibility.

3. Specificity

During the validation of identification tests and the determination of impurities, specificity should be investigated. An ICH document defines specificity as the ability to assess the analyte unequivocally in the presence of compounds that may be expected to be present in products and matrix components. The following are the implications of the definition:

Identification test:

Appropriate identification tests should be able to distinguish between compounds with closely related structures that are likely to be present. To ensure an analyte's identity, the analyte should be free of interference from other extraneous components and well separated from them.

Purity Test:

To ensure that all analytical procedures performed allow an accurate statement of an analyte's impurity content, such as related substances tests, heavy metals, residual solvents, and so on.

Assay:

This allows an accurate statement on the content or potency of the analyte in a sample to be provided in order to provide an exact result.

4. Selectivity:

It is a procedure for detecting the analyte qualitatively in the presence of compounds that are expected to be present in the sample matrix, or the ability of a separative method to resolve different compounds. It is a measure of the distance between two peaks or the relative method location.

Determination of selectivity: The selectivity of an analyte is determined by comparing the test results obtained with and without the addition of potentially interfering material. When such components are unknown or unavailable, selectivity can be determined by determining the recovery of a standard addition of pure analyte to a material containing a constant level of the other compounds.

5. Sensitivity Sensitivity refers to the test procedure's ability to detect small changes in concentration. It is the calibration curve's slope.6. Limit of detection (LOD)

It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantities as an exact value, under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte (percentage parts per million) in the sample.

Determination of detection limit

For both instrumental and non-instrumental methods, the detection limit is generally determined by analysing samples with known analyte concentrations and determining the minimum level at which the analyte can be reliably detected.

$$LOD = \frac{3.3 \times Standard\ Deviation}{Slope}$$

7. Limit of quantification (LOQ)

Under the specified experimental conditions, it is the smallest amount of analyte in a sample that can be determined with acceptable precision and accuracy. The quantification limit is expressed as the analyte concentration (e.g., percent ppm) in the sample.

Determination of quantification limit

The quantitation limit for both instrumental and non-instrumental methods is generally determined by analysing samples with known analyte concentrations and establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

$$LOD = \frac{10 \times Standard\ Deviation}{Slope}$$

Fig No. 1.8 Limit of detection and Limit of quantification via signal to noise

8. Linearity and Range

The ability of an analytical procedure to produce test results that are directly proportional to the concentration of analyte in samples is referred to as its linearity. The intervals between the upper and lower concentrations of analyte in a sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity are referred to as an analytical range.

$$r^2 = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{(n\sum x^2) - (\sum x)^2\,(n\sum y^2) - (\sum y)^2}}$$

Determination of linearity and range:

These characteristics are determined by applying the procedure to a series of samples with analyte concentrations spanning the procedure's claimed range. When

Standardization may be provided by means of a calibration curve if the relationship between response and concentration is not linear.

The ICH recommends that a minimum of five commonly used concentrations be used to establish linearity.

9. Ruggedness

The degree of reproducibility of test results obtained by analysing the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, and so on, which is typically expressed as the lack of influence on test results of operational and environmental variables of the analytical method.

Ruggedness is a measure of the reproducibility of test results under normal variation in conditions from laboratory to laboratory and analyst to analyst. The degree to which test results are representative is then determined as a function of the assay variable.

By analysing aliquots from homogeneous lots in various laboratories, by various analysts, and using ope.

$$\boldsymbol{Stanadard\ Deviation} = \sqrt{\frac{\sum(\boldsymbol{x} - \overline{\boldsymbol{x}})^2}{\boldsymbol{n-1}}}$$

Where,

$x = sample,$

$\bar{x} = mean\ value\ of\ samples,$

$n = number\ of\ samples$

$$\boldsymbol{Relative\ Standard\ Deviation} = \frac{\boldsymbol{Standard\ Deviation}}{\overline{\boldsymbol{x}}} \times \mathbf{100}$$

10. Robustness

The robustness of an analytical method is a measure of its ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Changing some or all of the conditions:• Solvent

- Column temperature
- pH of buffer in mobile phase
- Flow rate
- Wave length

$$\boldsymbol{Stanadard\ Deviation} = \sqrt{\frac{\sum(\boldsymbol{x} - \overline{\boldsymbol{x}})^2}{\boldsymbol{n-1}}}$$

Where,

$x = sample,$

$\bar{x} = mean\ value\ of\ samples,$

$n = number\ of\ samples$

$$\boldsymbol{Relative\ Standard\ Deviation} = \frac{\boldsymbol{Standard\ Deviation}}{\overline{\boldsymbol{x}}} \times \mathbf{100}$$

11. System Suitability

System suitability tests are based on the idea that the equipment, electronics, analytical operations, and samples all work together to form an integrated system that can be evaluated as a whole. According to the USP, system suitability is an essential component of chromatographic methods. These tests ensure that the system's resolution and reproducibility are adequate for the analysis. As a result of the robustness and ruggedness evaluation, a set of system suitability parameters should be established to ensure that the analytical method's validity is maintained whenever it is used.

The parameters that are affected by the changes in chromatographic conditions are,

- Capacity factor (k')
- Peak asymmetry / tailing factor (As)
- Column efficiency (N)
- Selectivity (α)
- Dead volume
- Retention time (Rt)
- Retention volume (Rv)

1. Capacity factor (k')

The capacity factor, K', is related to retention time and reflects the proportion time of a specific solute residue in the stationary phase as opposed to the mobile phase. Long retention times result in high K' valves. Using the following equations, the capacity factor K' can be calculated for each peak defined in a chromatogram.

$$\boldsymbol{K} = \frac{\boldsymbol{Moles\ of\ solute\ in\ stationary\ phase\ Capacity\ factor}}{\boldsymbol{Moles\ of\ solute\ in\ mobile\ phase}}$$

$$K' = \frac{(t_R - t_0)}{t_0}$$

The capacity factor is a measure of an analyte's retention relative to an unretained peak, where tR is the sample peak's retention time and to is the unretained peak's retention time.

Recommendations

The peak should be clearly separated from the other peaks and the void volume. In general, the value of k' is greater than two.

Precision / Injection repeatability (RSD) of 1% for 'n' > 5 is preferred.

2. Tailing factor (T)

A measure of a peak's symmetry, given by the following equation, where W0.05 is the peak width at 5% height and f is the distance from the peak front to the apex point at 5% height. Peaks should ideally be Gaussian in shape or completely symmetrical.

$$T = \frac{W0.05}{2f}$$

Because of the difficulties encountered by the integrator in determining where/when the peak ends and thus calculating the area under the peak, quantitation accuracy decreases as peak tailing increases. The analyst sets the integrator variables for the best calculation of the area for the peak of interest.

Recommendations

T of </= 2 is preferred

3. Theoretical plate number / Efficiency (N)

Peak band spreading as measured by various methods, some of which are sensitive to peak asymmetry. The most common are depicted here, with the one exception**.**

4-sigma / tangential

$$N = 16(\frac{t_R}{W})^2 = \frac{L}{H}$$

Half height

$$N = 5.54(\frac{t_R}{W})^2 = \frac{L}{H}$$

Theoretical plate number is a column efficiency metric. Theoretical plate number is a measure of column efficiency, or how many peaks can be located per unit run-time of the chromatogram, where tR is the sample peak retention time and W is the peak width.

For each peak on a chromatogram with a fixed set of operating conditions, N is fairly constant. The column efficiency per unit length (L) of the column is measured by H, or HETP, the height equivalent of a theoretical plate. Peak position, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and analyte molecular weight are all variables that can affect N or H.

Recommendations

The theoretical plate number varies with elution time but should be greater than 2000 in general.

4. Resolution (Rs)

A column's ability to separate chromatographic peaks, Resolution can be improved by lengthening the column, decreasing particle size, raising the temperature, or changing the eluent or stationary phase. It can also be expressed as the distance between the apexes of two peaks divided by the average tangential width of the peaks.

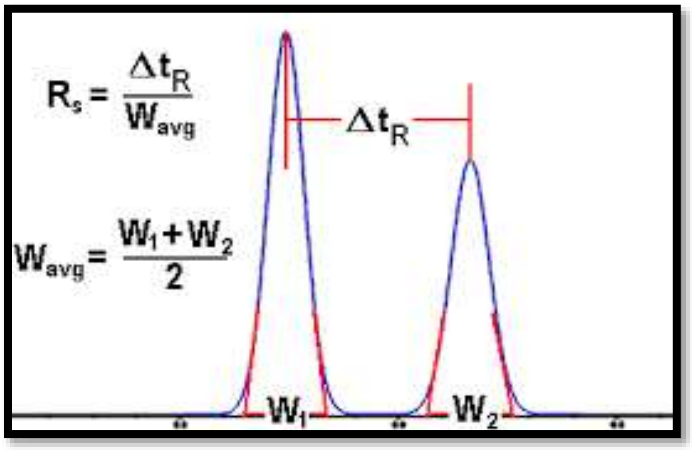

Fig No. 1.3 Resolution Chromatogram

$$RS = \frac{(t_{R2} - t_{R1})}{0.5(w_1 + w_2)}$$

Where tR1 and tR2 are the retention times of the two compounds and w1 and w2 are their widths. Well-separated peaks are required for quantitation to be reliable.

Recommendations

Rs greater than two between the peak of interest and the nearest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) are preferred.

5. **Dead Volume**

The term "dead volume" refers to any empty space or unoccupied volume, the presence of which can result in disastrous efficiency losses. There will be dead volume in the column itself, which is the space that the stationary phase does not occupy.

The injection unit, the tubing and fittings at each end of the column, and the detector cell are also sources of dead volume.

6. **Retention time (Rt)**

The retention time is the time elapsed between the point of injection and the appearance of peak maxima. The retention time is the amount of time required for 50% of a component to be eluted from a column. The duration of retention is measured in minutes and seconds. Retention time is also proportional to the distance moved on chart paper, which can be measured in centimetres or millimetres.

7. **Retention volume (Rv)**

The retention volume is the amount of mobile required to elute 50% of the component from the column. It is the sum of the retention time and the flow rate.

$$Retention\ Volume = Retention\ time\ x\ flow\ rate$$

BOSENTAN

The drug is not official in any pharmacopoeia. Few analytical techniques havebeen described in the literature for the determination of BST in pharmaceutical dosage forms and biological fluids. Das *et al.,* (2010), Annapurna *et al.,* (2011), Ashok kumar *et al.,* (2011) and Narendra *et al.,* (2012) reported UV-spectrophotometric methods employing different solvents for the determination of BST in pharmaceutical dosage forms.

Three UV spectrophotometric methods using three different approaches weredeveloped by Das *et al.,* (2010) for the determination of BST in pharmaceuticalformulations. First method (Zero-derivative spectrometry) is based on themeasurement of absorbance of BST in 0.1N NaOH at 272 nm. The second method(First-derivative spectrometry) is based on the derivatisation of the zero-orderspectrums obtained in the first method to get first-order derivative spectra. Theamplitudes of the corresponding troughs were measured at 284.65 nm. For the areaunder curve technique (third method), the area under curve of BST in 0.1N NaOH solution in the wavelength ranges 260-280 nm was selected for determination of BST. Determination of BST by three different approaches is proposed by Annapurna *et al.,* (2011). Measurement of the absorbance of methanolic solution ofBST at 269 nm has served as the basis of the first method. The second method is thefirst derivative technique, in which the zero crossing point of methanolic solution ofBST was selected at 291 nm. Third method is area under curve technique where the absorbance of the BST in methanol was recorded in the wavelength region, 259nm - 279 nm

.

In a method reported by Ashok kumar *et al.,* (2011), the absorbance of BST inmethanol: water (60:40) mixture was measured at 270 nm. Narendra *et al.,* (2012) method is based on the measurement absorbance of the BST in methanol and octane 1-sulfonic acid mixture (50:50 *v/v*) at 273 nm. Though the reported methods UV spectrophotometric methods are simple, but they are not much sensitive and suffered from the lack of selectivity. Hence they are not suggested for the routine analysis of BST in tablet dosage forms.

Two narrow-bore liquid chromatography with ion spray tandem mass spectrometric detection methods were reported by Lausecker *et al.,* (1995; 2000). Thefirst method (Lausecker *et al.,* 1995) is applied for the determination of BSN in human plasma whereas the second method (Lausecker *et al.,* 2000) is useful for simultaneous determination of BSN and its three main metabolites in plasma, serum, bile, and liver samples from man, dog and rat. A bioanalytical method (Parekh *et al.,* 2012) has been developed for the simultaneous determination of bosentan and its active metabolite, hydroxybosentan, in human plasma by solid phase extraction - liquid chromatography-tandem mass spectrometry. The reported liquidchromatography - mass spectrometric detection methods (Lausecker *et al.,* 1995; Lausecker *et al.,* 2000; Parekh *et al.,* 2012) require expensive detector, tedious sample preparation steps, require a skilled person to operate the instrument. Further more these methods were not applied for tablet dosage forms. These features make them unattractive to routine analysis of BST in tablet dosage forms.

Among various chromatographic and spectrophotometric methods available for the determination of the BST, HPLC with UV detection and visible spectrophotometry seem to be the most suitable for a routine analysis of BST, due to their simplicity, reasonable sensitivity, precise, accurate and significant economical advantage. A few HPLC methods with UV detection have been reported earlier for the estimation of BST in pharmaceutical dosage forms.

In a method reported by Karnakerreddy *et al.,* (2010) separation and quantification were achieved on a C18 hypersil (250 x 4.6 mm, 5µm) column with mobile phase consisting of methanol: potassium dihydrogen orthophosphate buffer pH 7.8 (60:40 *v/v*) and UV-detection at 220 nm. An HPLC method (Muralidharan & Rajakumar, 2012) with UV detection at 265 nm has been carried out with a C18 column using a mobile phase consisting of acetonitrile: 10 Mm ammonium acetate buffer pH 4.5 (70:30 *v/v*).

Assay of BST in bulk and tablets by HPLC has been reported by Nageswararao *et al.,* (2012). The separation was achieved on a Zorbax SB-Phenyl (150×4.6 mm), 3.5 µm make: Aglient column using 0.02M sodium dihydrogen phosphate and methanol (30:70 *v/v*) as mobile phase and quantification was achieved by measuring UV absorption at 225 nm. Sujatha *et al,* (2012) have reported an HPLC method using an analytical column Kromasil C18 (150 x 4.6 mm; 5 µm) with a mobile phase consisting of phosphate buffer (pH 4.0) and acetonitrile (30:70 *v/v*) and UV detection at 270 nm. A method developed for determination of BST by Kalaichelvi & Jayachandran (2013) was carried out using an Agilent XDB C18column (150 mm × 4.6 mm, i.d., 5 µm) with mobile phase containing phosphatebuffer (pH-5) and acetonitrile in 45:55 v*/v* ratio. The column effluents were monitored at 270 nm**.**

Annapurna *et al.,* (2013) have developed a HPLC method using a C18 column(250 mm × 4.6 mm, 5 µm) and a mixture of tetra butyl ammonium hydrogen sulphate-acetonitrile (35: 65, v/v) as a mobile phase with UV detection at 268 nm. Paresh & Lipi (2013) reported an RP-HPLC method in which the separation was carried out using mobile phase consisting of buffer (pH-2.5): methanol: acetonitrile (30:40:30 v/v) in Inertsil-ODS-3, (150 mm x 4.6 mm i.d., 3 µm) column using PDA detection at 273 nm. In the method developed by Suganthi *et al.,* (2014), the RP- HPLC separation was achieved on pre-packed RP-18 column (250×4.6 mm, 5 µm) using mobile phase methanol: 0.1% formic acid pH-6.4 (70:30 *v/v*) and quantificationwas achieved with photodiode array detection at 275 nm

Two stability indicating HPLC methods (Sanjay *et al.,* 2011; Khan *et al.,* 2012) were reported in the literature for BST quantification. In the Sanjay *et al.,* (2011) method, an RP-HPLC method was developed determination of process and degradation related impurities formed under the stress conditions in Bosentan monohydrate. Chromatographic separation was achieved on Zorbax SB-Phenyl column under gradient elution by a binary mixture of solvent A (60% phosphate buffer, pH 2.5, and 40% methanol) and solvent B (acetonitrile). The detection was carried out at a wavelength of 220 nm. In the Khan *et al.,* (2012) method, four impurities (impurity A, B, C & D) were isolated from the BST API and were characterized by spectroscopic studies. The separation was carried out on a Zorbax SB-C8 (250 x 4.6mm, 5µm) column. Mobile phase A consisted of buffer (1gm of octane-1-sulfonic acid sodium salt and 1 ml triethylamine in 1 liter of water, pH 2.5) and methanol was used as mobile phase B. The compounds were detected at 220 nm.

Most of the reported methods, however, suffer from such disadvantages as poor narrow range of linear response (Khan *et al.*, 2012; Nageswararao *et al.*, 2012; Paresh *et al.*, 2013; Suganthi *et al.*, 2014), poor sensitivity (Muralidharan & Raja Kumar, 2012; Annapurna *et al.*, 2013; Kalaichelvi & Jayachandran, 2013; Paresh *et al.*, 2013), more flow rate (Nageswararao *et al.*, 2012; Annapurna *et al.*, 2013), lack of accuracy and precision (Karnakerreddy *et al.*, 2010; Sanjay et al., 2011; Muralidharan & Raja kumar, 2012; Nageswararao *et al.*, 2012; Sujatha *et al*, 2012; Annapurna *et al.*, 2013; Paresh *et al.*, 2013; Suganthi *et al.*, 2014). Moreover, in allthe earlier reported HPLC with UV detection methods the retention time of the BST is more which leads to a longer runtime for a single sample. Because of all these reasons, the earlier reported methods are not considered suitable for the routineanalysis of BST in tablet dosage forms. In the existing literature there is a lack of information regarding the use of visible spectrophotometry for the quantification of BST in bulk and tablet dosage forms.

Hence the need arises to develop certain simple, precise, accurate, economical HPLC Method.

Aim:

Analytical Method Development and Validation for Bosentan HCl Estimation in Bulk.

Objectives:

- To Develop novel qualitative and quantitative analytical methods for estimating Bosentan HCl in bulk in accordance with ICH guidelines.
- To validate novel qualitative and quantitative analytical methods for estimating Bosentan HCl in bulk in accordance with ICH guidelines.

Bosentan HCl

Bosentan is a dual endothelin receptor antagonist marketed under the trade name Tracleer by Actelion Pharmaceuticals. Bosentan is used to treat pulmonary hypertension by blocking the action of endothelin molecules that would otherwise promote narrowing of the blood vessels and lead to high blood pressure.

Chemical Formula:

$C_{27}H_{29}N_5O_6S$

Melting Point:

112-116°C

Solubility:

Bosentan monohydrate (Tracleer, $C_{27}H_{29}N_5O_6S.H_2O$, MW 569.63) occurs as a white to yellowish powder that is poorly soluble in water (1.0 mg/100 mL) and in aqueous solutions at low pH (0.1 mg/100 mL at pH 1.1 and 4.0; 0.2 mg/100 mL at pH 5.0).

Pharmacodynamics

Bosentan belongs to a class of drugs known as endothelin receptor antagonists (ERAs). Patients with PAH have elevated levels of endothelin, a potent blood vessel constrictor, in their plasma and lung tissue. Bosentan blocks the binding of endothelin to its receptors, thereby negating endothelin's deleterious effects.

Mechanism of Action

Endothelin-1 (ET-1) is a neurohormone, the effects of which are mediated by binding to ET_A and ET_B receptors in the endothelium and vascular smooth muscle. It displays a slightly higher affinity towards ET_A receptors than ET_B receptors. ET-1 concentrations are elevated in plasma and lung tissue of patients with pulmonary arterial hypertension, suggesting a pathogenic role for ET-1 in this disease. Bosentan is a specific and competitive antagonist at endothelin receptor types ET_A and ET_B.

1. **Selection of Drugs**

Online journals, chemical and analytical abstracts were studied to identify drugs for which reported QbD methods or methods were observed; many methods were found to be costly and time consuming. A market survey was conducted to determine the availability of these drugs. Individual drug profiles explain the drug selection criteria.

2. **Drug: Bosentan HCl**

3. **Selection of analytical techniques**:

- RP-HPLC method

4. **Method development and validation:**

Development and validation of analytical methods are:

A. HPLC Method:

- Selection of different mobile phase.
- Selection of range of proportion of mobile phase with appropriate retention time.
- Selection of suitable detection wavelength.

- Optimization
- To develop calibration curve of optimized result for Bosentan HCl.
- Method Validation as per ICH guidelines.

EXPERIMENTAL WORK:
MATERIAL AND METHODS:

➢ **Reagents and chemicals:**

- Acetonitrile (HPLC Grade),
- Disodium Hydrogen Phosphate (AR Grade)
- HPLC grade water.

All chemicals and reagents that is Acetonitrile, Phosphoric acid, were purchased from LOBA CHEME PVT. LTD., Mumbai.

➢ **Instruments:**

1. HPLC:
   - Borwin chromatography software (version 1.50)
   - Model PU 2080 Plus Intelligent HPLC pump
   - Rheodyne sample injection port with 50µl loop
   - Grace C_{18} column,(150 x 4.6 mm, 3.5µm)
   - JASCO UV-2075 UV-VIS detector
2. Shimadzu (model AY-120) Electronic weighting balance
3. Sonicator: PRAMA solutions for laboratory
4. Extrapure lab link water purification system.
5. Electronic pH meter
6. Calibrated Glassware's.

Development and validation of HPLC Method for estimation of Bosentan HCl

1 Experimental, Results and Discussion:

Table 1: Trials of mobile phase for HPLC method development of Bosentan HCl:

Sr. No	Mobile phase	Observations	Chromatogram
1.	MeOH: water (70:30 v/v)	Peak shape was not proper. RT = 1.717 min.	
2.	Acetonitrile: 10 mM disodium hydrogen Phosphate Buffer, pH-6.8 (20:80 v/v)	Peak shape not proper. Fronting RT = 6.849 min.	
3.	Acetonitrile : disodium hydrogen phosphate buffer (pH 3) in proportion 30:70 v/v	Good Peak Shape, RT at 6.233 min.	

A. Selection of mobile phase and chromatographic conditions:

Chromatographic separation studies were carried out on the working standard solution of Bosentan HCl10 µg/ml. Initially, trials were carried out using Acetonitrile and buffer in various proportions of varying pH, to obtain the desired system suitability parameters. After few trials, Acetonitrile: 20 mM disodium hydrogen phosphate buffer (pH 3) in the ratio of 30:70 v/v was chosen as the mobile phase, which gave good resolution and acceptable peak parameters.

B. Preparation of Sodium Hydrogen Phosphate buffer and mobile phase:

Disodium hydrogen phosphate buffer (20mM) was prepared by dissolving 2.84 g of disodium hydrogen phosphate in 1000 ml of HPLC grade water and pH was adjusted to 3 by orthophosphoric acid. Mobile phase was prepared by mixing Acetonitrile and disodium hydrogen phosphate buffer in the ratio of 30:70 v/v. It was then filtered through 0.45 µm membrane filter paper using filtration assembly and then sonicated on ultrasonic water bath for 10 min.

C. Preparation of Standard stock solution:

Standard stock solution of drug was prepared by dissolving 10 mg of drug in 10 ml of Acetonitrile to get concentration of 1000 µg/ml. From the corresponding standard stock solution, working standard solution was prepared containing 100 µg/ml of Bosentan HCl in acetonitrile. From this further dilution was made in acetonitrile to get final solution of Bosentan HCl (10 µg/ml).

D. Selection of Detection Wavelength:

From the standard stock solution further dilutions were done using acetonitrile and scanned over the range of 200 - 400 nm and the spectra was obtained (Fig. 1). It was observed that drug showed considerable absorbance at 243 nm.

Fig1: UV-VIS Spectra of Bosentan HCl (10 µg/ml)

E. Preparation of sample solution:(Tablet formulation)

A tablet containing 20 mg of Bosentan HCl(Jupiros-20, Alkem Laboratories Ltd, Label Claim: Each film tablet contains Bosentan HCl … 20 mg) was weighed and powdered. Powder equivalent to 10 mg of drug was transferred to 10 ml volumetric flask and volume was made up with acetonitrile to get concentration (1000µg/ml) and was sonicated for 10 min. Solution was filtered, from this solution 1 ml ofdrug was taken in 10 ml volumetric flask and volume was made up with acetonitrile. Further dilution in mobile phase was done to get final concentration 4 µg/ml.

F. Chromatogram and system suitability parameter of drug:

The column was equilibrated with the mobile phase (indicated by constant back pressure at desired flow rate). Working standard solution of drug (10 µg/ml) was injected on system. The retention time for the drug was found 6.238 ± 0.277 min.
Chromatogram ofBosentan HCl shown in Fig.2
A)

B)

Fig 2: Chromatogram of A) Blank B) Bosentan HCl (10 µg/ml)

Table2: System suitability parameters for Bosentan HCl

Drug	Concentration (µg/ml)	RT ± SD (Min)	Area	Plates	Asymmetry
BOSENTAN HCL	4	6.238 ± 0.277	459281.889	3526.58	0.97

Summary of Chromatographic parameters selected:
Table3: Summary of Chromatographic parameters

Sr. No.	Parameter	Conditions used for Analysis

1	Column	Grace C_{18} column, (150 x 4.6 mm, 3.5 µm)
2.	Mobile phase	Acetonitrile:10 mM disodium hydrogen phosphate buffer (pH – 3) (30:70 v/v)
3.	Flow rate	0.8 ml/min
4.	Detection Wavelength	243 nm
5.	Sample injector	20 µl loop
6.	Column temperature	Ambient

Validation of Analytical Method

A. Linearity

From the standard stock solution (1000 µg/ml) of Bosentan HCl, solution was prepared containing 100 µg/ml of Bosentan HCl withacetonitrile. This solution was further used to prepare range of solutions containing six different concentrations. The linearity (relationship between peak area and concentration) was determined by analyzing six solutions over the concentration range of2-12µg/ml.The results obtained are shown in Table4.Linearity curve of Bosentan HCl is shown in Fig. 3 and calibration curve shown in Fig. 4.

Table4: Linearity study of Bosentan HCl

Replicates	Concentrations of Bosentan HCl					
	2 µg/ml	4 µg/ml	6 µg/ml	8 µg/ml	10 µg/ml	12 µg/ml
	Peak Area					
1	259874.711	459281.889	631369.499	821552.732	1021762.409	1218825.1
2	260980.604	453777.399	633596.874	821628.674	1030719.624	1208835.1
3	261058.527	459281.889	633837.853	825149.138	1023248.016	1218825.0
4	259893.692	459052.019	631459.418	823550.695	1022249.516	1228814.9
5	259870.715	455864.31	641967.621	823850.472	1022273.801	1240803.1
6	264053.754	459511.76	644346.234	824150.172	1020944.181	1230813.8
Mean	260955.334	457794.878	636096.250	823313.647	1023532.925	1224486.2
Std. Dev.	1708.905	2557.551	5720.888	1287.647	3906.309	12202.614
%RSD	0.655	0.559	0.899	0.156	0.382	0.997

Fig 3: Linearity curve of Bosentan HCl (2-12 µg/ml)

Fig 4: Calibration curve of Bosentan HCl

B. Range:

2-12 µg/ml.

C. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ are calculated from the formula: -

Where,
σ = standard deviation of Y intercept= 3291.056
S = slope of the calibration curve= 95744.087
LOD = 0.113 µg/ml

LOQ = 0.344 µg/ml

D. Precision:

The precision of the method was demonstrated by intra-day and inter-day variation studies. In the Intra-day studies, 3 replicates of 3different concentrations were analyzed in a day and percentage RSD was calculated. For the inter day variation studies, 3 different concentrations were analyzed on 3 consecutive days and percentage RSD were calculated. The results obtained for Intraday and Inter day variationsare shown in Table5and Table6, respectively.

Table 5: Intra-day Precision Results

Conc (µg/ml)	Area	Amount recovered (µg/ml)	% Recovery	Average % Recovery	SD	%RSD
6	639372.105	5.973	99.551			
6	648623.123	6.070	101.161	100.413	0.811	0.808
6	644972.998	6.032	100.526			
8	834375.067	8.010	100.122			
8	830623.123	7.971	99.6322	99.628	0.496	0.498
8	826772.998	7.930	99.130			
10	1041241.114	10.170	101.704			
10	1034325.004	10.098	100.981	101.171	0.468	0.462
10	1032852.114	10.083	100.828			

Table 6: Inter-day Precision Results

Conc (µg/ml)	Area	Amount recovered (µg/ml)	% Recovery	Average % Recovery	SD	%RSD
6	638219.985	5.961	99.350			
6	643349.003	6.015	100.243	99.895	0.478	0.478
6	642481.128	6.006	100.092			
8	842683.127	8.097	101.207			
8	841218.987	8.081	101.016	101.163	0.131	0.129
8	843136.138	8.101	101.266			
10	1021130.084	9.960	99.603			
10	1028316.904	10.035	100.354	100.568	1.087	1.081
10	1041640.499	10.175	101.746			

E. Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 996, indicating the no interference of any other peak of degradation product, impurity or matrix. (Table 7).

Table7: Peak purity of Bosentan HCl

Drug	Purity tail	Purity front
Bosentan HCl	996.146	998.428

F. Assay

Jupiros-20tablet formulation analysis was carried out as mentioned under sample solution preparation. Procedure was repeated for six times. Sample solution was injected and area was recorded. Concentration and % recovery was determined from linear equation. (Table 8)

Table8: Assay of marketed formulation

Sr. No.	Peak area	Amount Recovered (µg/ml)	% Recovery
1	457820.123	4.077	101.921
2	454919.111	4.047	101.163
3	452232.908	4.018	100.462
4	449071.111	3.985	99.636
5	456991.76	4.068	101.704
6	450069.093	3.996	99.897
Mean	453517.351	4.032	100.797
SD	3629.315	0.038	0.948
%RSD	0.800	0.940	0.940

A. Accuracy

To check accuracy of the method, recovery studies were carried by spiking the standard drug to the Jupirostablet sample solution, at three different levels around 50, 100 and 150 %.Basic concentration of sample solution chosen was 4 µg/ml. % recovery was determined from linearity equation. The results obtained are shown in (Table 9)

Table9: Recovery studies ofBosentan HCl

Level	Conc. of Sample solution (µg/ml)	Conc. of Standard solution spiked (µg/ml)	Area	Amount recovered (µg/ml)	Mean % recovery ± RSD
50%	4	2	644741.631	6.029	100.531 ± 0.694
			641134.006	5.991	
			649134.604	6.075	
100%	4	4	831129.603	7.976	100.379 ± 0.825
			843402.123	8.104	
			834489.987	8.011	
150%	4	6	1030098.320	10.054	100.978 ± 0.547
			1032542.980	10.080	
			1040226.876	10.160	

B. Robustness

Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase composition, detection wavelength, flow rate was altered and the effects on the area were noted. The results obtained are shown in Table10.

Table10: Robustness study

% RSD Found For Robustness Study(peak area)								
MP COMPOSITION			**DETECTION WAVELENGTH (± 1 nm)**			**FLOW RATE (± 0.05 ml/min)**		
32:68	30:70	28:72	242	243	244	0.75	0.8	0.85
1.787	0.523	1.898	0.389	0.136	0.572	0.190	0.472	0.935

Summary of validation study:

Table 11: Summary of validation study by HPLC method

Sr. No.	**Validation Parameter**	**Bosentan HCl**
1.	Linearity	y = 95744 x + 67488

		R^2 = 0.999
2.	Range	2-12 µg/ml
3.	Precision	(%RSD)
	A) Intraday precision	0.462 – 0.808
	B) Interday precision	0.129 – 1.081
4.	Assay ± %RSD	100.797 ± 0.940
5.	Accuracy	Mean % recovery ± %RSD
	50 %	100.531 ± 0.694
	100 %	100.379 ± 0.825
	150 %	100.978 ± 0.547
6.	Limit of Detection	0.133 µg/ml
7.	Limit of Quantitation	0.344 µg/ml
8.	Robustness	Robust
9.	Specificity	Specific

It is always necessary to develop methods capable of analysing a large number of samples in a short period of time with due accuracy and precision for routine analytical purposes. Bosentan HCl is listed in the Indian Pharmacopoeia.

HPLC, HPTLC, and UV- Visible spectrophotometric methods are among the few analytical methods that have been published in the literature for the determination of Bosentan HCl. In light of the foregoing, some simple analytical methods with sensitivity, accuracy, precision, and economy were planned to be developed. In the current study, an HPLC method was used to quantify Bosentan HCl in bulk according to ICH guidelines. HPLC methods were validated as linearity, precision, accuracy, specificity, system suitability, and robustness exceeded the limit. When compared to the previously reported method, the HPLC method is more sensitive, accurate, and precise. There was no excipient interference in the recovery study. The low percent RSD and molar extinction coefficient (L mol-1 cm-1) values indicated that the developed methods were sensitive. The proposed high-performance liquid chromatographic method was also evaluated for accuracy, precision, and robustness, and it was found to be convenient and effective for Bosentan HCl quality control. The developed method was found to be simple and cost effective for the quail.

Furthermore, the reduced solvent consumption results in a more cost-effective and environmentally friendly spectroscopic procedure. As a result, the proposed methodology is quick and selective, requires only a simple sample preparation procedure, and is suitable for Bosentan HCl.

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