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Development and Validation of a Rapid RP-HPLC Method for Simultaneous Estimation of Benfotiamine and Metformin

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Abstract: A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validation of Benfotiamine and Metformin, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Phenomenex Gemini C18 (4.6×250mm) 5µ column using a mixture of Methanol: TEA Buffer (65:35 v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 230nm. The retention time of the Benfotiamine and Metformin was 2.121, 3.643 ±0.02min respectively. The method produce linear responses in the concentration range of 10-50mg/ml of Benfotiamine and 20-100mg/ml of Metformin. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: Benfotiamine, Metformin, RP-HPLC, validation.

1. INTRODUCTION

Analytical chemistry is a scientific discipline used to study the chemical composition, structure and behaviour of matter. The purposes of chemical analysis are together and interpret chemical information that will be of value to society in a wide range of contexts. Quality control in manufacturing industries, the monitoring of clinical and environmental samples, the assaying of geological specimens, and the support of fundamental and applied research are the principal applications. Analytical chemistry involves the application of a range of techniques and methodologies to obtain and assess qualitative, quantitative and structural information on the nature of matter.[1-8]

- **Qualitative analysis** is the identification of elements, species and/or compounds present in sample.
- **Quantitative analysis** is the determination of the absolute or relative amounts of elements, species or compounds present in sample.

Structural analysis is the determination of the spatial arrangement of atoms in an element or molecule or the identification of characteristic groups of atoms (functional groups). An element, species or compound that is the subject of analysis is known as analyte. The remainder of the material or sample of which the analyte(s) form(s) a part is known as the matrix.

The gathering and interpretation of qualitative, quantitative and structural information is

essential to many aspects of human endeavour, both terrestrial and extra-terrestrials. The maintenance of an improvement in the quality of life throughout the world and the management of resources heavily on the information provided by chemical analysis. Manufacturing industries use analytical data to monitor the quality of raw materials, intermediates and finished products. Progress and research in many areas is dependent on establishing the chemical composition of man-made or natural materials, and the monitoring of toxic substances in the environment is of ever-increasing importance. Studies of biological and other complex systems are supported by the collection of large amounts of analytical data. Analytical data are required in a wide range of disciplines and situations that include not just chemistry and most other sciences, from biology to zoology, butte arts, such as painting and sculpture, and archaeology. Space exploration and clinical diagnosis are two quite desperate areas in which analytical data is vital. Important areas of application include the following.

Quality control (**QC**) in many manufacturing industries, the chemical composition of raw materials, intermediates and finished products needs to be monitored to ensure satisfactory quality and consistency. Virtually all consumer products from automobiles to clothing, pharmaceuticals and foodstuffs, electrical goods, sports equipment and horticultural products rely, in part, on chemical analysis. The food, pharmaceutical and water industries in particular have stringent requirements backed by legislation for major components and permitted levels of impurities or contaminants. The electronic industry needs analyses at ultra-trace levels (parts per billion) in relation to the manufacture of semi-conductor materials. Automated, computer-controlled procedures for process-stream analysis are employed in some industries.

Monitoring and control of pollutants The presence of toxic heavy metals (e.g., lead, cadmium and mercury), organic chemicals (e.g., polychlorinated biphenyls and detergents) and vehicle exhaust gases (oxides of carbon, nitrogen and sulphur, and hydrocarbons) in the environment are health hazards that need to be monitored by sensitive and accurate methods of analysis, and remedial action taken. Major sources of pollution are gaseous, solid and liquid wastes that are discharged or dumped from industrial sites, and vehicle exhaust gases.

Clinical and biological studies The levels of important nutrients, including trace metals (e.g., sodium, potassium, calcium and zinc), naturally produced chemicals, such as cholesterol, sugars and urea, and administered drugs in the body fluids of patients undergoing hospital treatment require monitoring. Speed of analysis is often a crucial factor and automated procedures have been designed for such analyses.

Geological assays The commercial value of ores and minerals are determined by the levels of particular metals, which must be accurately established. Highly accurate and reliable analytical procedures must be used for this purpose, and referee laboratories are sometimes employed where disputes arise.

Fundamental and applied research The chemical composition and structure of materials used in or developed during research programs in numerous disciplines can be of significance. Where new drugs or materials with potential commercial value are synthesized, a complete chemical characterization maybe required involving considerable analytical work. Combinatorial chemistry is an approach used in pharmaceutical research that generates very large numbers of new compounds requiring confirmation of identity and structure.

Analytical techniques There are numerous chemical or physico-chemical processes that can be used to provide analytical information. The processes are related to a wide range of atomic and molecular properties and phenomena that enable elements and compounds to be detected and/or quantitatively measured under controlled conditions. The underlying processes define the various *analytical techniques*. The more important of these are listed in Table.No.1 together with their suitability for qualitative, quantitative or structural analysis and the levels of analyte(s) in a sample that can be measured. *Atomic, molecular spectrometry* and *chromatography*, which together comprise the largest and most widely used groups of techniques, can be further subdivided according to their physico-chemical basis. *Spectrometric techniques* may involve either the *emission or absorption* of *electromagnetic radiation* over a very wide range of energies, and can provide qualitative, quantitative and structural information for

analytes from major components of a sample down to ultra-trace levels.

Chromatographic techniques provide the means of separating the components of mixtures and simultaneous qualitative and quantitative analysis, as required. The linking of chromatographic and spectrometric techniques, called *hyphenation*, provides a powerful means of separating and identifying unknown compounds.

Electrophoresis's another separation technique with similarities to chromatography that is particularly useful for this parathion of charged species.

Analytical methods[9-14]

An analytical method consists of a detailed, stepwise list of instructions to be followed in the qualitative, quantitative or structural analysis of a sample for one or more analytes and using a specified technique. It will include a summary and lists of chemicals and reagents to be used, laboratory apparatus and glassware, and appropriate instrumentation. The quality and sources of chemicals, including solvents, and the required performance characteristics of instruments will also be specified as will the procedure for obtaining a representative sample of the material to be analyzed. This is of crucial importance in obtaining meaningful results. The preparation or pre-treatment of the sample will be followed by any necessary standardization of reagents and/or calibration of instruments under specified conditions. Qualitative tests for the analyte(s) or quantitative measurements under the same conditions as those used for standards complete the practical part of the method. The remaining steps will be concerned with data processing, computational methods for quantitative analysis and the formatting of the analytical report. The statistical assessment of quantitative data is vital in establishing the reliability and value of the data, and the use of various statistical parameters and tests is widespread. Many *standard analytical methods* have been published as papers in analytical journals and other scientific literature, and in textbook form. Collections by trades associations representing, for example, the cosmetics, food, iron and steel, pharmaceutical, polymer plastics and paint, and water industries are available standards organizations and statutory authorities, instrument manufacturer's applications notes, the Royal Society of Chemistry and the US Environmental Protection Agency are also valuable sources of standard methods. Often, laboratories will develop their own *in-house methods* or adapt existing ones for specific purposes.

Method development forms a significant part of the work of most analytical laboratories, and *method validation and* periodic revalidation is a necessity. Selection of the most appropriate analytical method should take into account the following factors:

- The purpose of the analysis, the required time scale and any cost constraints;
- The level of Analyte(s) expected and the detection limit required;
- The nature of the sample, the amount available and the necessary sample preparation procedure;
- The accuracy required for a quantitative analysis;
- The availability of reference materials, standards, chemicals and solvents, instrumentation and any special facilities;
- Possible interference with the detection or quantitative measurement of the analyte(s) and the possible need for sample clean-up to avoid matrix interference;
- The degree of selectivity available − methods may be selective for a small number of analytes or specific for only one.
- Quality control and safety factors.

Table 1: Analytical techniques and principal applications

Technique	Property measured	Principal areas of application
Gravimetry	Weight of pure analyte or compound of known as stoichiometry	Quantitative for major or minor components
Titrimetry	Volume of standard reagent solution reacting with the analyte	Quantitative for major or minor Component

Atomic molecular spectrometry	Wavelength and intensity of electromagnetic radiation emitted/ absorbed by the analyte	Qualitative, quantitative or structural or for major down to trace level components
Mass spectrometry	Mass of analyte or fragments of it	Qualitative or structural for major down to trace level components isotope ratios
Chromatography and electrophoresis	Various physicochemical properties of separated analytes	Qualitative and quantitative separations of mixtures at major to trace levels
Thermal analysis	Chemical/physical changes in the analyte when heated or cooled	Characterization of single or mixed major/minor compounds
Electrochemical analysis	Electrical properties of the analyte in solution	Qualitative and quantitative for major to trace level components
Radiochemical analysis	Characteristic ionizing nuclear radiation emitted by the analyte	Qualitative and quantitative at major to trace levels

Table 2: Spectrometric Techniques and Principal Applications

Technique	**Basis**	**Principal applications**
Plasma emission spectrometry	Atomic emission after excitation in high temperature gas plasma	Determination of metals and some non-metals mainly at trace levels
Flame emission spectrometry	Atomic emission after flame excitation	Determination of alkali and alkaline earth metals
Atomic absorption spectrometry	Atomic absorption after atomization by flame or electro thermal means	Determination of trace metals and some non-metals
Atomic fluorescence spectrometry	Atomic fluorescence emission after flame excitation	Determination of mercury and hydrides of non-metals at trace levels
X-ray emission spectrometry	Atomic or atomic fluorescence emission after excitation by electrons or radiation	Determination of major and minor elemental components of metallurgical and geological samples
γ-spectrometry	γ-ray emission after nuclear excitation	Monitoring of radioactive elements in environmental samples
Ultraviolet/visible spectrometry	Electronic molecular absorption in solution	Quantitative determination of unsaturated organic
Infrared spectrometry	Vibrational molecular absorption	Identification of organic compounds
Nuclear magnetic resonance spectrometry	Nuclear absorption (change of spin states)	Identification and structural analysis of organic compounds
Mass spectrometry	Ionization and fragmentation of molecules	Identification and structural analysis of organic compounds

1.2 Chromatography[15-18]

1.2.1 Introduction

The chromatography was discovered by Russian Chemist and botanist *Micheal Tswett* (1872-1919) who first used the term chromatography (colour writing derived from Greek for colour – Chroma , and write – graphein) to describe his work on the separation

of coloured plant pigments into bands on a column of chalk and other material such as polysaccharides, sucrose and insulin.

" *Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system*".

The adsorbent material, or stationary phase, first described by Russian scientist named Tswett in 1906, has taken many forms over the years, including paper, thin layers of solids attached to glass plates, immobilized liquids, gels, and solid particles packed in columns. The flowing component of the system, or mobile phase, is either a liquid or a gas. Concurrent with development of the different adsorbent materials has been the development of methods more specific to particular classes of analytes. In general, however, the trend in development of chromatography has been toward faster, more efficient.

"In his early papers of Tswett (1906) stated that chromatography is a method in which the component of a mixture are separated on an adsorbent column in a flowing system. Chromatography has progressed considerably from Tswett's time and now includes a number of variations on the basic separation process".

"Chromatography is a physical method of separation in which the component to be separated are distributed between two phases of which in stationary while other moves in a definite direction (IUPAC)"

1.2.2. Chromatographic Process[19]

Chromatographic separations are based on a forced transport of the liquid (mobile phase) carrying the analyte mixture through the porous media and the differences in the interactions at analytes with the surface of this porous media resulting in different migration times for a mixture components. In the above definition the presence of two different phases is stated and consequently there is an interface between them. One of these phases provides the analyte transport and is usually referred to as the mobile phase, and the other phase is immobile and is typically referred to as the stationary phase. A mixture of components, usually called analytes, are dispersed in the mobile phase at the molecular level allowing for their uniform transport and interactions with the mobile and stationary phases. High surface area of the interface between mobile and stationary phases is essential for space discrimination of different components in the mixture. Analyte molecules undergo multiple phase transitions between mobile phase and adsorbent surface. Average residence time of the molecule on the stationary phase surface is dependent on the interaction energy. For different molecules with very small interaction energy difference the presence of significant surface is critical since the higher the number of phase transitions that analyte molecules undergo while moving through the chromatographic column, the higher the difference in their retention. The nature of the stationary and the mobile phases, together with the mode of the transport through the column, is the basis for the classification of chromatographic methods.

1.2.3. Types of Chromatography[20]

The mobile phase could be either a liquid or a gas, and accordingly we can subdivide chromatography into Liquid Chromatography (LC) or Gas Chromatography (GC). Apart from these methods, there are two other modes that use a liquid mobile phase, but the nature of its transport through the porous stationary phase is in the form of either (a) capillary forces, as in planar chromatography (also called Thin-Layer Chromatography, TLC), or (b) electro osmotic flow, as in the case of Capillary Electro Chromatography (CEC).

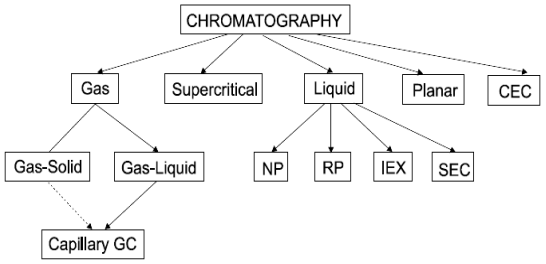


Fig.1: Showing flow chart for classification of chromatography

EXPERIMENTAL WORK

INSTRUMENTS USED

Instruments and Glasswares	Model
HPLC	WATERS, software: Empower 2, Alliance 2695 separation module. 996 PDA detector.
pH meter	LabIndia
Weighing machine	Sartorius
Volumetric flasks	Borosil
Pipettes and Burettes	Borosil
Beakers	Borosil
Digital ultra sonicator	Labman

CHEMICALS USED:

S.No	Chemical	Brand names
1	Benfotiamine	Provided by Sura Pharma labs
2	Metformin	Provided by Sura Pharma labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

HPLC METHOD DEVELOPMENT:

TRAILS

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Benfotiamine and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.3 ml of Benfotiamine and 0.6ml of Metformin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Water, Methanol: Phosphate buffer and ACN: Water with varying proportions. Finally, the mobile phase was optimized to TEA buffer (pH 4.0), Methanol in proportion 65:35 v/v respectively.

Optimization of Column:

The method was performed with various C18columns like Symmetry, X terra and ODS column. Phenomenex Gemini C18 (4.6×250mm) 5µ was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used	:	Waters Alliance 2695 HPLC with PDA Detector 996 model.
Temperature	:	40°C
Column	:	Phenomenex Gemini C18 (4.6×250mm) 5µ
Mobile phase	:	Methanol: TEA Buffer (65:35 v/v)
Flow rate	:	1ml/min
Wavelength	:	230nm
Injection volume	:	10µl
Run time	:	6minutes

VALIDATION

PREPARATION OF BUFFER AND MOBILE PHASE:

Preparation of Triethylamine buffer (pH-4.0):

Take 6.0ml of Triethylamine in to 750ml of HPLC water in a 1000ml volumetric flask and

mix well. Make up the volume up to mark with water and adjust the pH to 4.0 by using Orthophosphoric acid, filter and sonicate.

Preparation of mobile phase:

Accurately measured 350 ml (35%) of TEA buffer and 650 ml of HPLC Methanol (65%) were mixed and degassed in a digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

VALIDATION PARAMETERS

SYSTEM SUITABILITY

Accurately weigh and transfer 10 mg of Benfotiamine and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette out 0.3 ml of Benfotiamine and 0.6ml of Metformin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

SPECIFICITY STUDY OF DRUG:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Benfotiamine and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette out 0.3 ml of Benfotiamine and 0.6ml of Metformin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution:

Take average weight of one Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Benfotiamine and Metformin sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Filter the sample solution by using injection filter which contains 0.45µ pore size.

Further pipette out 0.3 ml of Benfotiamine and 0.6ml of Metformin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

PREPARATION OF DRUG SOLUTIONS FOR LINEARITY:

Accurately weigh and transfer 10 mg of Benfotiamine and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (10ppm of Benfotiamine and 20ppm of Metformin):

Pipette out 0.1ml of Benfotiamine and 0.2ml of Metformin in to a 10ml volumetric flask and make the volume upto mark by using diluent and sonicate for air entrapment.

Preparation of Level – II (20ppm of Benfotiamine and 40ppm of Metformin):

Pipette out 0.2ml of Benfotiamine and 0.4ml of Metformin in to a 10ml volumetric flask and make the volume upto mark by using diluent and sonicate for air entrapment.

Preparation of Level – III (30ppm of Benfotiamine and 60ppm of Metformin):

Pipette out 0.3ml of Benfotiamine and 0.6ml of Metformin in to a 10ml volumetric flask and make the volume upto mark by using diluent and sonicate for air entrapment.

Preparation of Level – IV (40ppm of Benfotiamine and 80ppm of Metformin):

Pipette out 0.4ml of Benfotiamine and 0.8ml of Metformin in to a 10ml volumetric flask and make the volume upto mark by using diluent and sonicate for air entrapment.

Preparation of Level – V (50ppm of Benfotiamine and 100ppm of Metformin):

Pipette out 0.5ml of Benfotiamine and 1ml of Metformin in to a 10ml volumetric flask and make the volume upto mark by using diluent and sonicate for air entrapment.

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

PRECISION

REPEATABILITY

Preparation of Benfotiamine and Metformin Product Solution for Precision:

Accurately weigh and transfer 10 mg of Benfotiamine and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette out 0.3 ml of Benfotiamine and 0.6ml of Metformin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

INTERMEDIATE PRECISION:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

DAY 1:

The standard solution was injected for Six times and measured the area for all Six injections in HPLC. The %RSD for the area of Six replicate injections was found to be within the specified limits.

DAY 2:

The standard solution was injected for Six times and measured the area for all Six injections in HPLC. The %RSD for the area of Six replicate injections was found to be within the specified limits.

Accuracy:

For preparation of 50% Standard stock solution:

Accurately weigh and transfer 10mg of Benfotiamine and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette out 0.15ml of Benfotiamine and 0.3ml of Metformin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution:

Accurately weigh and transfer 10 mg of Benfotiamine and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette out 0.3 ml of Benfotiamine and 0.6ml of Metformin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution:

Accurately weigh and transfer 10 mg of Benfotiamine and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette out 0.45ml of Benfotiamine and 0.9ml of Metformin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Benfotiamine and Metformin

and calculate the individual recovery and mean recovery values.

ROBUSTNESS:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard solution:

Accurately weigh and transfer 10 mg of Benfotiamine and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette out 0.3 ml of Benfotiamine and 0.6ml of Metformin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of flow conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected twice and chromatograms were recorded

Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. Methanol: TEA buffer 4pH was taken in the ratio and 60:40, 70:30 instead of 65:35 remaining conditions are same. 10µl of the above sample was injected twice and chromatograms were recorded.

RESULTS AND DISCUSSION

Optimized Chromatogram (Standard)

Mobile phase ratio	:	Methanol: TEA Buffer (65:35 v/v)
Column	:	Phenomenex Gemini C18 (4.6×250mm) 5µ
Column temperature	:	40°C
Wavelength	:	230nm
Flow rate	:	1ml/min
Injection volume	:	10µl
Run time	:	6minutes

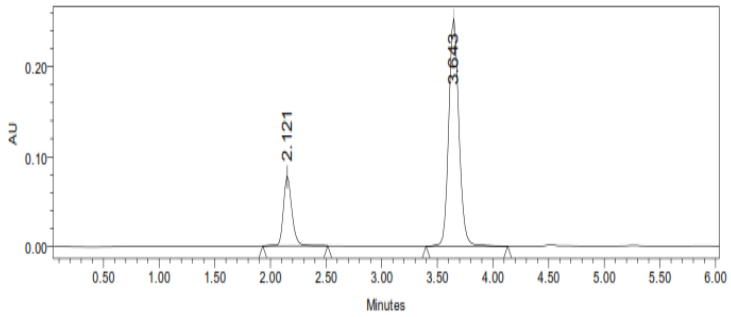


Fig 1: Optimized Chromatogram (Standard)

Table: Optimized Chromatogram (Standard)

S.no	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Benfotiamine	2.121	406433	77644	1.2	4009	
2	Metformin	3.643	1592811	251532	1.1	7849	9.8

Observation:

Optimized Chromatogram

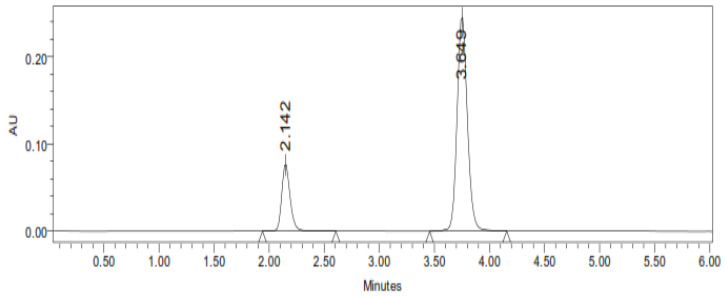


Fig 2: Optimized Chromatogram (Sample)

Table: Optimized Chromatogram (Sample)

S.no	Name	Rt	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Benfotiamine	2.142	403871	77464	1.2	4136	
2	Metformin	3.649	1573821	259361	1.1	7812	10.3

Acceptance criteria:

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

VALIDATION

Blank:

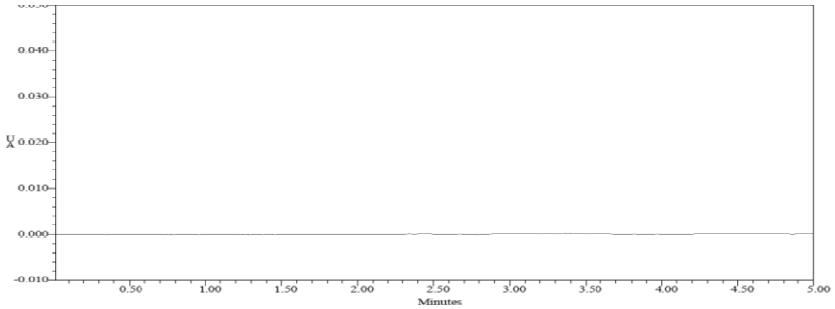


Fig 3: Chromatogram showing blank (mobile phase preparation)

System suitability:

Table: Results of system suitability for Benfotiamine

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Benfotiamine	2.152	382726	70725	5271	1.2
2	Benfotiamine	2.157	382621	70625	5928	1.2
3	Benfotiamine	2.141	389172	70617	5283	1.2
4	Benfotiamine	2.133	384152	70718	5763	1.2
5	Benfotiamine	2.166	389721	70172	6222	1.2
Mean			385678.4			
Std. Dev.			3497.932			
% RSD			0.906956			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

Table: Results of system suitability for Metformin

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Metformin	3.674	1562821	227365	5827	1.1	10.1
2	Metformin	3.631	1562726	226748	6183	1.1	10.1
3	Metformin	3.625	1567361	227163	5029	1.1	10.1
4	Metformin	3.692	1562811	226948	4920	1.1	10.1
5	Metformin	3.629	1563816	226452	5183	1.1	10.1
Mean			1563907				
Std. Dev.			1982.03				

% RSD			0.126736				

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

SPECIFICITY

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity to measure accurately quantitate Benfotiamine and Metformin in drug product.

Assay (Standard):

Table: Peak results for assay standard of Benfotiamine

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Benfotiamine	2.152	406538	77074	1.2	4009	1
2	Benfotiamine	2.198	409975	76001	1.2	4136	2
3	Benfotiamine	2.179	402283	76048	1.2	5263	3

Table: Peak results for assay standard of Metformin

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Metformin	3.646	1609924	251956	1.1	7849	1
2	Metformin	3.604	1601840	246020	1.1	7819	2
3	Metformin	3.610	1602832	248287	1.1	7826	3

Assay (Sample):

Table: Peak results for Assay sample of Benfotiamine

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Benfotiamine	2.152	406538	77074	1.2	4009	1
2	Benfotiamine	2.150	409975	76001	1.2	4136	2
3	Benfotiamine	2.187	402911	77823	1.2	5173	3

Table: Peak results for Assay sample of Metformin

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Metformin	3.646	1609924	251956	1.1	7849	1
2	Metformin	3.651	1601840	246020	1.1	7819	2
3	Metformin	3.601	1603821	240291	1.1	6812	3

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

= 1605195 /1604865 ×10/60×60/0.0254×99.5/100×0.0382/15×100

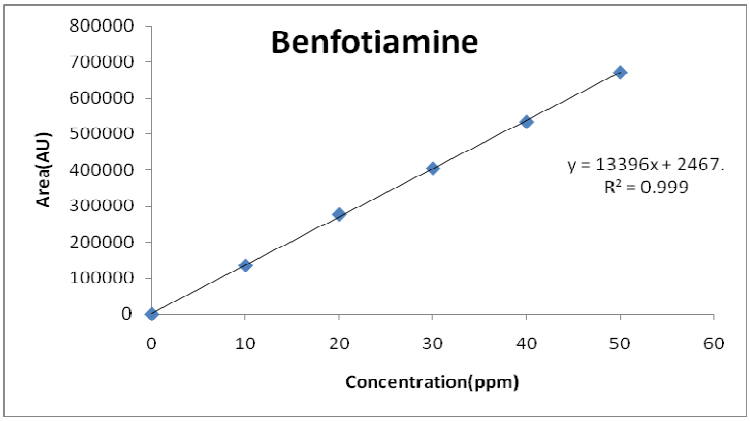
= 99.7%

The % purity of Benfotiamine and Metformin in pharmaceutical dosage form was found to be 99.7%

LINEARITY

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY OF BENFOTIAMINE:

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33	10	135005
66	20	277120
100	30	405128
133	40	534643
166	50	672357



LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of Benfotiamin is a straight line.

Y = mx + c

Slope (m) =13396

Intercept (c) = 2467

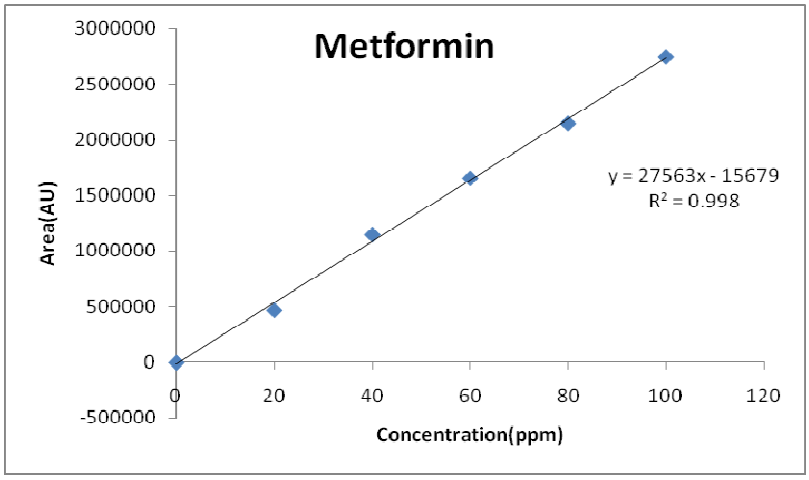
Correlation Coefficient (r) = 0.99

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 2467. These values meet the validation criteria.

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY OF METFORMIN:

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33	20	469094
66	40	1149397
100	60	1657592
133	80	2150412
166	100	2748444



LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of Metformin is a straight line.

Y = mx + c

Slope (m) =27563

Intercept (c) =15679

Correlation Coefficient (r) = 0.99

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 15679. These values meet the validation criteria.

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

REPEATABILITY

Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table: Results of repeatability for Benfotiamine:

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing	%Assay
1	Benfotiamine	2.157	400459	70717	1.2	4987	99%
2	Benfotiamine	2.159	402118	71819	1.2	5019	99.4%
3	Benfotiamine	2.186	405412	73930	1.2	5126	100%
4	Benfotiamine	2.160	406506	73333	1.3	4999	100%
5	Benfotiamine	2.170	407673	72623	1.2	5214	100%
Mean			404433.6				
Std. dev			2716.809				
%RSD			0.671757				

Acceptance criteria:

- %RSD for sample should be NMT 2

- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table: Results of repeatability for Metformin:

S. No	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	%Assay
1	Metformin	3.603	1617864	226985	1.1	7045	98.7%
2	Metformin	3.608	1618493	234764	1.1	7399	98.8%
3	Metformin	3.600	1628262	227712	1.2	7159	99.4%
4	Metformin	3.696	1615796	235459	1.1	7896	98.6%
5	Metformin	3.629	1619626	242158	1.1	7965	98.8%
Mean			1620008				
Std.dev			4310.623				
%RSD			0.266086				

Intermediate precision:

Day 1:

Table: Results of Intermediate precision for Benfotiamine

S.No	PeakName	RT	Area (µV*sec)	Height (µV)	USPP late count	USP Tailing	%Assay
1	Benfotiamine	2.198	405262	70572	5672	1.2	100%
2	Benfotiamine	2.196	405637	70516	5639	1.2	100%
3	Benfotiamine	2.160	405628	70572	6183	1.2	100%
4	Benfotiamine	2.160	405647	70372	5923	1.2	100%
5	Benfotiamine	2.160	405948	70592	6739	1.2	100%
6	Benfotiamine	2.186	408732	70526	5837	1.2	100%
Mean			406142.3				
Std. Dev.			1287.197				
% RSD			0.316933				

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2

Table: Results of Intermediate precision for Metformin

S.No	PeakName	Rt	Area (µV*sec)	Height (µV)	USPPlate count	USP Tailing	Resolution	%Assay
1	Metformin	3.623	1608292	235473	5372	1.1	10.1	98%
2	Metformin	3.611	1609283	235938	5927	1.1	10.1	98.2%
3	Metformin	3.696	1617836	235738	6129	1.1	10.1	98.7%
4	Metformin	3.696	1619743	235963	5284	1.1	10.1	99.7%
5	Metformin	3.696	1614262	231938	5284	1.1	10.1	98.5%
6	Metformin	3.642	1608471	235948	6347	1.1	10.1	98.2%

Mean			1611315					
Std. Dev.			6077.093					
% RSD			0.377151					

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2

Day 2:

Table: Results of Intermediate precision Day 2 for Benfotiamine

S.No	Peak Name	RT	Area	Height	USP Plate count	USP Tailing	%Assay
1	Benfotiamine	2.198	405423	70572	5672	1.2	100%
2	Benfotiamine	2.196	405927	70516	5639	1.2	100%
3	Benfotiamine	2.178	405029	70572	6183	1.2	100%
4	Benfotiamine	2.142	405432	70372	5923	1.2	100%
5	Benfotiamine	2.177	405062	70592	6739	1.2	100%
6	Benfotiamine	2.177	408417	70526	5837	1.2	101%
Mean			405881.7				
Std. Dev.			1283.857				
% RSD			0.316313				

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2

Table: Results of Intermediate precision Day 2 for Metformin

S. No	PeakName	RT	Area (µV*sec)	Height (µV)	USPPlate count	USPTailing	Resolution	%Assay
1	Metformin	3.611	1638732	244384	5363	1.1	10.1	100%
2	Metformin	3.623	1637438	235827	6282	1.1	10.1	100%
3	Metformin	3.684	1638474	236382	5938	1.1	10.1	100%
4	Metformin	3.697	1634273	239183	6194	1.1	10.1	99.7%
5	Metformin	3.684	1636372	231931	5402	1.1	10.1	99.8%
6	Metformin	3.684	1639283	234356	5837	1.1	10.1	100%
Mean			1637429					
Std. Dev.			1860.366					
% RSD			0.113615					

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2

ACCURACY:

Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated.

The accuracy results for Benfotiamine

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	201472.3	15	14.8	98.6	99.7%
100%	406193	30	30.1	100.3	
150%	607144	45	45.1	100.2	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The accuracy results for Metformin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	826527.7	30	30.5	101.6	99.6%
100%	1622241	60	59.4	99	
150%	2422702	90	88.4	98.2	

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

LIMIT OF DETECTION

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD = $3.3 \times \sigma / s$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

BENFOTIAMINE

Result: = 3.3×4269.822/13396

= 1.05μg/ml

METFORMIN

Result: =3.3×57796.93/27563

= 6.9μg/ml

QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

BENFOTIAMINE

Result: =10×4269.822/13396

=3.1μg/ml

METFORMIN

Result: =10×57796.93/27563

=20.9μg/ml

Robustness

Table: Results for Robustness

BENFOTIAMINE

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	406433	2.121	4009	1.2
Less Flow rate of 0.9 mL/min	398841	2.210	3800.8	0.9
More Flow rate of 1.1 mL/min	389947	2.184	4800.8	
Less organic phase	413898	2.200	4890.8	0.9
More Organic phase	389578	2.172	4190.8	0.7

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

METFORMIN

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	1592811	3.643	7849	1.1
Less Flow rate of 0.9 mL/min	1613422	4.498	3312.2	0.9
More Flow rate of 1.1 mL/min	1619138	3.505	4312.2	0.8
Less organic phase	1616104	4.504	4392.2	0.9
More organic phase	1623185	3.512	4292.2	0.9

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

SUMMARY

Summary of validation data for Benfotiamine:

S. No	Parameter	Observation	Acceptance criteria
1	**System suitability** Theoretical plates Tailing % RSD	 4009 1.2 0.9	 Not less than 2000 Not more than 2 Not more than 2.0%
2	**Specificity** %Assay	 99%	 98-102%
3	**Method Precision** (%RSD)	0.7	Not more than 2.0%
4	**Linearity** Slope Correlation coefficient(r^2)	10-50 µg/ml 13396 0.99	 ≤0.99
5	**Accuracy** Mean % recovery	 99.7	 98 - 102%
6	**Robustness** a) Flow rate variation b) Organic phase variation	All the system suitability parameters are within the limits.	

Summary of validation data for Metformin:

S.No	Parameter	Observation	Acceptance criteria
1	**System suitability** Theoretical plates Tailing %RSD	 7849 1.1 0.1	 Not less than 2000 Not more than 2 Not more than 2.0%

2	**Specificity** %Assay	 99%	 98-102%
3	**Method Precision** (%RSD)	0.7	Not more than 2.0%
4	**Linearity** Slope Correlation coefficient(r^2)	20-100 µg/ml 27563 0.99	 ≤0.99
5	**Accuracy** Mean % recovery	 99.6	 98 - 102%
6	**Robustness** a) Flow rate variation b) Organic phase variation	All the system suitability parameters are within the limits.	

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Benfotiamine and Metformin in bulk drug and pharmaceutical dosage forms.

This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps.

Benfotiamine and Metformin are freely soluble in ethanol, methanol and sparingly soluble in water.

Methanol: Triethylamine Buffer was chosen as the mobile phase. The solvent system used in this method was economical.

The %RSD values were within 2 and the method was found to be precise.

The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.

This method can be used for the routine determination of Benfotiamine and Metformin in bulk drug and in Pharmaceutical dosage forms.

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