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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF BENFOTIAMINE AND METFORMIN IN COMBINED DOSAGE FORM

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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.*

INTRODUCTION

Analytical chemistry[1]

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.

- **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.

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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.

MATERIALS AND METHODS

Benfotiamine Provided by Sura labs, Metformin Provided by Sura labs, Water and Methanol for HPLC from LICHROSOLV (MERCK), Acetonitrile for HPLC from 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.

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:

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

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)

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:

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: 40ºC

Wavelength: 230nm

Flow rate: 1ml/min

Injection volume: 10µl

Run time: 6minutes

Fig 1: Optimized Chromatogram (Standard)

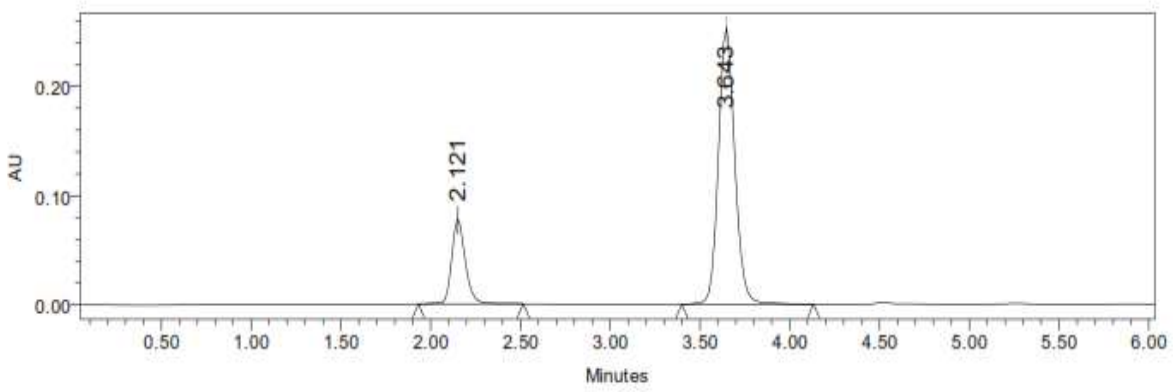

Table 1: Optimized Chromatogram (Standard)

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 (Sample)

Fig 2: Optimized Chromatogram (Sample)

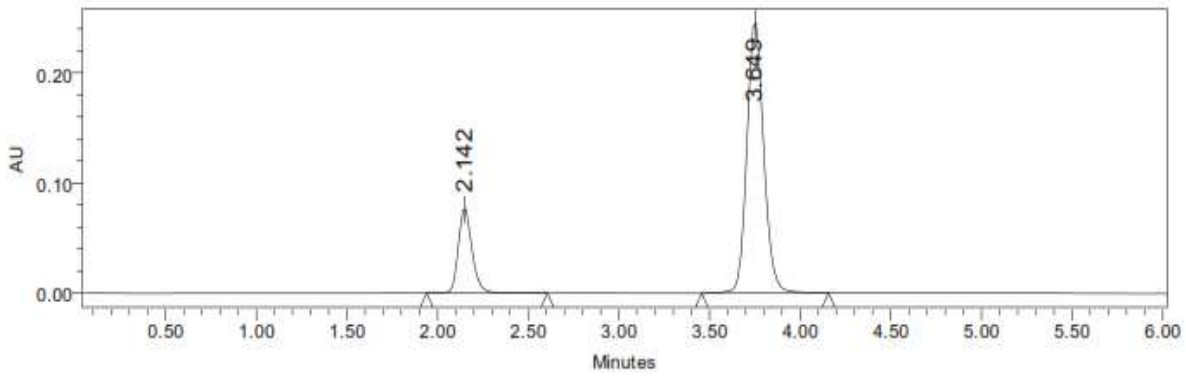

Table 2: Optimized Chromatogram (Sample)

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

VALIDATION

System suitability:

Table 3: 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			

Table 4: 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				

SPECIFICITY

Assay (Standard):

Table 5: 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 6: 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 7: 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 8: 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

LINEARITY

Table 9: 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

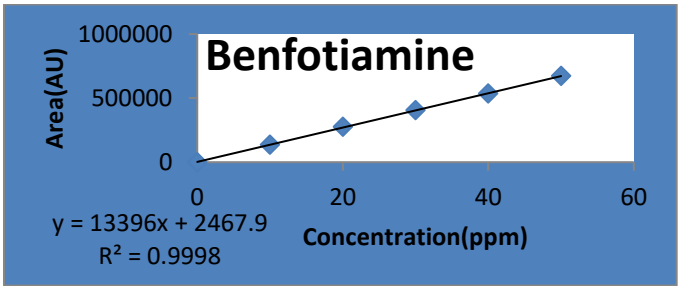

Figure 3: calibration graph for Benfotiamine

Table 10: 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

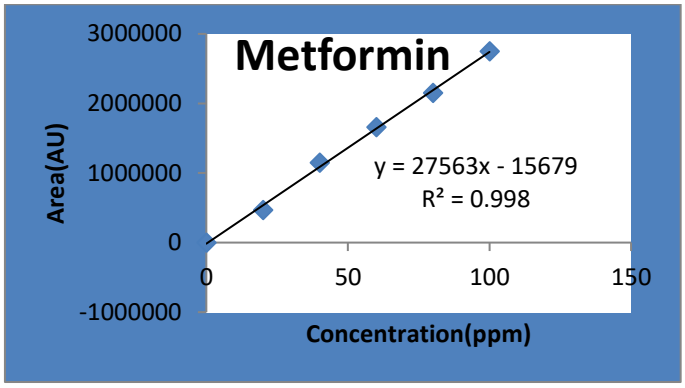

Figure 4: calibration graph for Metformin

REPEATABILITY

Table 11: 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				

Table 12: 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 13: Results of Intermediate precision for Benfotiamine

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate 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				

Table 14: Results of Intermediate precision for Metformin

S.No	Peak Name	Rt	Area (µV*sec)	Height (µV)	USP Plate 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					

Day 2:

Table 15: Results of Intermediate precision Day 2 for Benfotiamine

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	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				

Table 16: Results of Intermediate precision Day 2 for Metformin

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	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					

ACCURACY:

Table 17: 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	

Table 18: The accuracy results for Metformin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	826527.7	30	14.8	101.6	99.6%
100%	1622241	60	30.1	99	
150%	2422702	90	45.1	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 × σ / 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 19: 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

Table 19: Results for Robustness 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

SUMMARY

Table 20: 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.	

Table 21: 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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