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Research

ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF AMOXICILLIN AND BROMOHEXINE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

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Abstract

A new, simple, rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validation of Amoxicillin and Bromohexine, in its pure form as well as in combined marketed formulation. Chromatography was carried out on a Phenomenex Luna C18 (4.6mm×250mm) 5µm particle size column using a mixture of : Methanol and glacial acetic acid (50:50 v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 254nm. The retention time of the Amoxicillin and Bromohexine was found to be was 2.133, 3.692 ± 0.02min respectively. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The method produce linear responses in the concentration range of 20-60mg/ml of Amoxicillin and 10-30mg/ml of Bromohexine. The inter-day and intra-day precisions were found to be within limits. 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: Amoxicillin and Bromohexine, RP-HPLC, Validation, Accuracy.

1. INTRODUCTION

Analysis may be defined as the science and art of determining the composition of materials in terms of the elements or compounds contained in them. In fact, analytical chemistry is the science of chemical identification and determination of the composition (atomic, molecular) of substances, materials and their chemical structure.

Chemical compounds and metallic ions are the basic building blocks of all biological structures and processes which are the basis of life. Some of these naturally occurring compounds and ions (endogenous species) are present only in

very small amounts in specific regions of the body, while others such as peptides, proteins, carbohydrates, lipids and nucleic acids are found in all parts of the body. The main object of analytical chemistry is to develop scientifically substantiated methods that allow the qualitative and quantitative evaluation of materials with certain accuracy. Analytical chemistry derives its principles from various branches of science like chemistry, physics, microbiology, nuclear science and electronics. This method provides information about the relative amount of one or more of these components. [1]

Every country has legislation on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called "Pharmacopoeia" (e.g. IP, USP, and BP). Quantitative chemical analysis is an important tool to assure that the raw material used and the intermediate products meet the required specifications. Every year number of drugs is introduced into the market. Also quality is important in every product or service, but it is vital in medicines as it involves life.

There is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, report of new toxicities and development of patient resistance and introduction of better drugs by the competitors. Under these conditions standard and analytical procedures for these drugs may not be available in Pharmacopoeias. In instrumental analysis, a physical property of the substance is measured to determine its chemical composition. Pharmaceutical analysis comprises those procedures necessary to determine the identity, strength, quality and purity of substances of therapeutic importance. [2]

Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and quality of medicament depends. The quality of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations.

Quality control is a concept which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production. The decision to release or reject a product is based on one or more type of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation. Providing simple analytical procedure for complex formulation is a matter of most importance. So, it becomes necessary to develop new analytical methods for such drugs. In brief the reasons for the development of newer methods of drugs analysis are:

1. The drug or drug combination may not be official in any pharmacopoeias.
2. A proper analytical procedure for the drug may not be available in the literature due to Patent regulations.
3. Analytical methods for a drug in combination with other drugs may not be available.
4. Analytical methods for the quantitation of the drug in biological fluids may not be available.
5. The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable. [1, 2]

1.1 DIFFERENT METHODS OF ANALYSIS

The following techniques are available for separation and analysis of components of interest.

Spectral methods

The spectral techniques are used to measure electromagnetic radiation which is either absorbed or emitted by the sample.

E.g. UV-Visible spectroscopy, IR spectroscopy, NMR, ESR spectroscopy, Flame photometry, Fluorimetry.2

Electro analytical methods

Electro analytical methods involved in the measurement of current voltage or resistanceas a property of concentration of the component in solution mixture.

E.g. Potentiometry, Conductometry, Amperometry.[2]

Chromatographic methods

Chromatography is a technique in which chemicals in solutions travel down columns or over surface by means of liquids or gases and are separated from each other due to their molecular characteristics.

E.g. Paper chromatography, thin layer chromatography (TLC), High performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), Gas chromatography (GC). [2]

Miscellaneous Techniques

Mass Spectrometry, Thermal Analysis.

Hyphenated Techniques

GC-MS (Gas Chromatography – Mass Spectrometry), LC-MS (Liquid Chromatography – Mass Spectrometry), ICP-MS (Inductivity Coupled Plasma- Mass Spectrometry), GC-IR (Gas Chromatography – Infrared Spectroscopy), MS-MS (Mass Spectrometry – Mass Spectrometry).

Analytical techniques that are generally used for drug analysis also include biological and microbiological methods, radioactive methods and physical methods etc.

MATERIALS AND METHODS

INSTRUMENTS USED

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

CHEMICALS USED:

Amoxicillin	Sura labs
Bromohexine	Sura labs
Water and Methanol for HPLC	LICHROSOLV (MERCK)
Acetonitrile for HPLC	Merck
Potassium Dihydrogen Phosphate	Merck

RESULTS AND DISCUSSION

Optimized Chromatogram (Standard)

Mobile phase ratio : Methanol and glacial acetic acid (50:50 v/v)

Column : Phenomenex Luna C18 (4.6mm×250mm) 5µm particle size

Column temperature : 35ºC

Wavelength : 254nm

Flow rate : 1ml/min

Injection volume : 10µl

Run time : 6minutes

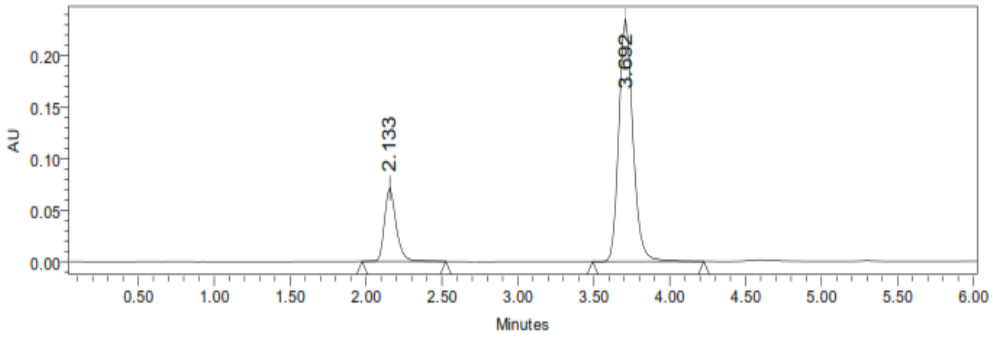

Figure-: Optimized Chromatogram (Standard)

Table-: Optimized Chromatogram (Standard)

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Amoxicillin	2.133	526389	86756	1.56	5679	
2	Bromohexine	3.692	1687285	367532	1.79	8685	9.8

Observation: From the above chromatogram it was observed that the Amoxicillin and Bromohexine peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Optimized Chromatogram

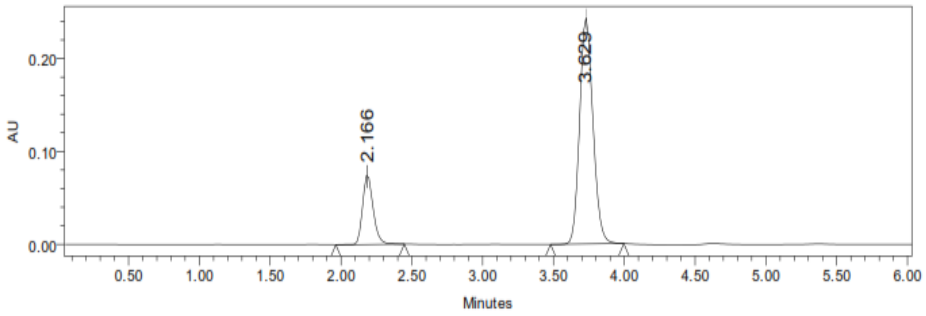

Figure-: Optimized Chromatogram (Sample)

Table-: Optimized Chromatogram (Sample)

S.No.	Name	Rt	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Amoxicillin	2.166	536587	77464	1.57	5789	
2	Bromohexine	3.629	1695846	378564	1.80	8795	10.01

VALIDATION

Blank:

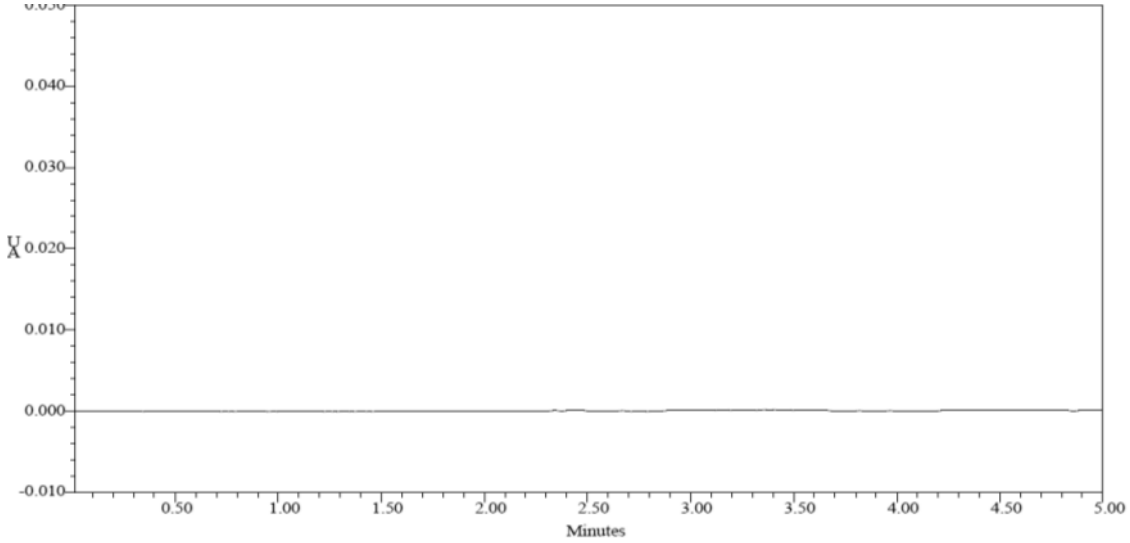

Fig-: Chromatogram showing blank Solution (Mobile Phase Preparation)

System Suitability:

Table-: Results of system suitability for Amoxicillin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Amoxicillin	2.152	526358	86598	5695	1.56
2	Amoxicillin	2.157	526548	86254	5652	1.57
3	Amoxicillin	2.141	526854	86598	5627	1.56
4	Amoxicillin	2.133	526598	86245	5692	1.57
5	Amoxicillin	2.166	524874	86521	5641	1.56
Mean			526246.4			
Std. Dev.			787.353			
% RSD			0.149617			

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 Bromohexine

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Bromohexine	3.674	1682821	1686958	8659	1.56	9.8
2	Bromohexine	3.631	1682726	1685745	8675	1.57	9.9
3	Bromohexine	3.625	1687361	1685421	8692	1.56	9.8
4	Bromohexine	3.692	1682811	1685242	8642	1.57	9.8

5	Bromohexine	3.629	1683816	1685364	8635	1.58	9.8
Mean			1683907				
Std. Dev.			1982.03				
% RSD			0.117704				

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 Amoxicillin and Bromohexine in drug product.

Assay (Standard):

Table-: Peak results for assay standard of Amoxicillin

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Amoxicillin	2.152	526358	86598	1.56	5698	1
2	Amoxicillin	2.198	526584	86784	1.57	5687	2
3	Amoxicillin	2.179	529658	86253	1.56	5639	3

Table-: Peak results for assay standard of Bromohexine

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Bromohexine	3.646	1687589	365879	1.80	8659	1
2	Bromohexine	3.604	1685987	365854	1.79	8697	2
3	Bromohexine	3.610	1685974	369854	1.80	8675	3

Assay (Sample):

Table-: Peak results for Assay sample of Amoxicillin

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection

1	Amoxicillin	2.152	536859	87584	1.58	5789	1
2	Amoxicillin	2.150	532654	87965	1.59	5784	2
3	Amoxicillin	2.187	532685	87465	1.58	5769	3

Table-: Peak results for Assay sample of Bromohexine

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Bromohexine	3.646	1698568	378562	1.81	8759	1
2	Bromohexine	3.651	1698574	375847	1.80	8795	2
3	Bromohexine	3.601	1698547	376584	1.81	8745	3

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

= 99.89%

The % purity of Amoxicillin and Bromohexine in pharmaceutical dosage form was found to be 99.89%

LINEARITY

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY OF AMOXICILLIN:

Table-: Chromatographic Data for Linearity Study of Amoxicillin

Concentration μg/ml	Average Peak Area
20	272897
30	402986
40	526389
50	649785
60	769287

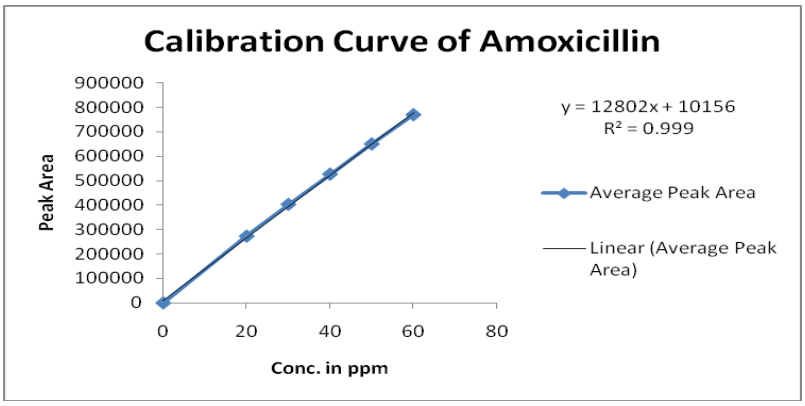

Fig-: Calibration Curve of Amoxicillin

LINEARITY PLOT:

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

Y = mx + c

Slope (m) =12802

Intercept (c) = 10156

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 10156. These values meet the validation criteria.

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY OF BROMOHEXINE:

Table-: Chromatographic Data for Linearity Study of Bromohexine

Concentration µg/ml	Average Peak Area
10	1000237
15	1448768
20	1887285
25	2365897
30	2826845

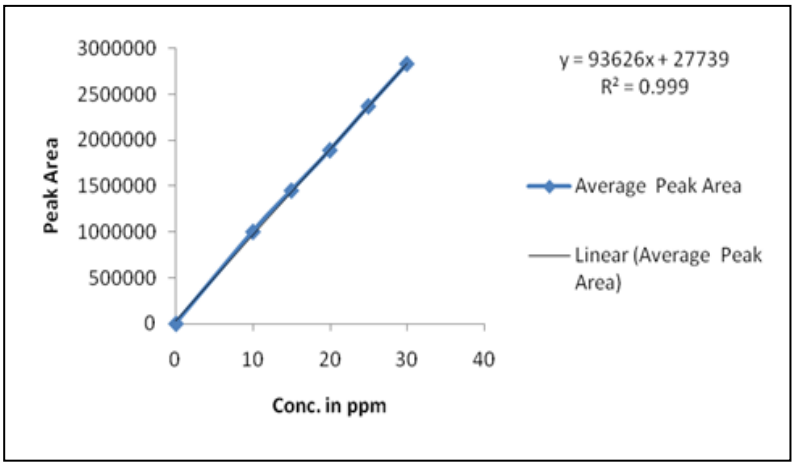

Fig-: Calibration Curve of Bromohexine

LINEARITY PLOT:

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

Y = mx + c

Slope (m) = 93626

Intercept (c) = 27739

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 27739. 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 Amoxicillin:

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Amoxicillin	2.157	526358	86598	5689	1.56
2	Amoxicillin	2.159	524856	86542	5687	1.57
3	Amoxicillin	2.186	526985	86578	5684	1.56
4	Amoxicillin	2.160	528654	86354	5689	1.56
5	Amoxicillin	2.170	528457	86958	5639	1.56
Mean			527062			
Std.dev			1569.114			
%RSD			0.297709			

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 Bromohexine:

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Bromohexine	3.603	1687589	367859	8659	1.79
2	Bromohexine	3.608	1685987	368547	8679	1.80
3	Bromohexine	3.600	1685987	367985	8645	1.80
4	Bromohexine	3.696	1685754	365874	8695	1.79
5	Bromohexine	3.629	1685985	364589	8625	1.79
Mean			1686260			
Std.dev			749.493			
%RSD			0.044447			

Intermediate precision:

Day 1:

Table-: Results of Intermediate precision for Amoxicillin

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	%Assay
1	Amoxicillin	2.198	546585	87589	5898	1.58	100%
2	Amoxicillin	2.196	548758	87985	5879	1.59	100%
3	Amoxicillin	2.160	549854	87452	5868	1.58	100%
4	Amoxicillin	2.160	548798	87421	5847	1.59	100%
5	Amoxicillin	2.160	542659	87963	5896	1.58	100%
6	Amoxicillin	2.186	548754	87254	5874	1.59	100%
Mean			547568				
Std. Dev.			2631.576				
% RSD			0.480593				

Acceptance criteria:

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

Table: Results of Intermediate precision for Bromohexine

S.No	Peak Name	Rt	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	Resolution	%Assay
1	Bromohexine	3.623	1698587	385482	8789	1.81	9.8	98%
2	Bromohexine	3.611	1698574	385698	8759	1.80	9.8	98.2%
3	Bromohexine	3.696	1698532	385748	8754	1.81	9.9	98.7%
4	Bromohexine	3.696	1698574	386958	8754	1.81	10.01	99.7%
5	Bromohexine	3.696	1698532	385755	5798	1.80	9.98	98.5%
6	Bromohexine	3.642	1698547	386558	8762	1.80	10.02	98.2%
Mean			1698558					
Std. Dev.			23.77113					
% RSD			0.001399					

Acceptance criteria:

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

Day 2:

Table-: Results of Intermediate precision Day 2 for Amoxicillin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Amoxicillin	2.198	536854	8758	5789	1.58
2	Amoxicillin	2.196	536985	8795	5726	1.59
3	Amoxicillin	2.178	536587	8746	5742	1.58
4	Amoxicillin	2.142	532546	8754	5746	1.59
5	Amoxicillin	2.177	534587	8725	5798	1.58
6	Amoxicillin	2.177	538598	8726	5785	1.59

Mean			536026.2			
Std. Dev.			2131.492			
% RSD			0.397647			

Acceptance criteria:

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

Table-: Results of Intermediate precision Day 2 for Bromohexine

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	Resolution
1	Bromohexine	3.611	1678598	356875	8875	1.82	9.9
2	Bromohexine	3.623	1678985	358985	8856	1.83	10.01
3	Bromohexine	3.684	1678984	358754	8862	1.82	9.9
4	Bromohexine	3.697	1678985	352412	8849	1.83	10.01
5	Bromohexine	3.684	1678549	358987	8873	1.82	9.9
6	Bromohexine	3.684	1678984	358986	8842	1.83	10.01
Mean			1678848				
Std. Dev.			212.8048				
% RSD			0.012676				

Acceptance criteria:

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

6.3.4: ACCURACY:

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

Table-: The accuracy results for Amoxicillin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery

50%	267011.3	20	20.063	100.315%	100.28%
100%	523752.3	40	40.118	100.295%	
150%	778457.3	60	60.133	100.221%	

Acceptance Criteria:

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

Table-: The accuracy results for Bromohexine

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	972876.3	10	10.094	100.94%	100.48%
100%	1900122	20	19.998	99.99%	
150%	2851152	30	30.156	100.52%	

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.

$$\mathbf{LOD = 3.3 \times \sigma / s}$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

AMOXICILLIN

Result:

= 1.04µg/ml

BROMOHEXINE

Result:

= 3.12µg/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

AMOXICILLIN

Result:

=2.1µg/ml

BROMOHEXINE

Result:

=6.3µg/ml

Robustness

Table-: Results for Robustness

AMOXICILLIN

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	526389	2.133	5679	1.56
Less Flow rate of 0.9 mL/min	542685	2.210	5264	1.54
More Flow rate of 1.1 mL/min	526483	2.184	5426	1.52
Less organic phase	516854	2.200	5163	1.57
More Organic phase	506898	2.172	5098	1.51

Acceptance criteria:

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

BROMOHEXINE

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	1687285	3.692	8685	1.79

Less Flow rate of 0.9 mL/min	1725468	4.498	8265	1.68
More Flow rate of 1.1 mL/min	1652847	3.505	8415	1.59
Less organic phase	1687485	4.504	8326	1.62
More organic phase	1674524	3.512	8415	1.63

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 Amoxicillin:

S.No	Parameter	Observation	Acceptance criteria
1	**System suitability** Theoretical plates Tailing %RSD	 5679 1.56 0.14	 Not less than 2000 Not more than 2 Not more than 2.0%
2	**Specificity** %Assay	 99.89%	 98-102%
3	**Method Precision** (%RSD)	0.29	Not more than 2.0%
4	**Linearity** Slope Correlation coefficient(r^2)	20-60 µg/ml 12802 0.999	 ≤0.99
5	**Accuracy** Mean % recovery	 100.28%	 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 Bromohexine:

S.No	Parameter	Observation	Acceptance criteria
1	**System suitability** Theoretical plates Tailing %RSD	 8685 1.79 0.11	 Not less than 2000 Not more than 2 Not more than 2.0%
2	**Specificity** %Assay	 99.89%	 98-102%
3	**Method Precision** (%RSD)	0.044	Not more than 2.0%
4	**Linearity** Slope Correlation coefficient(r^2)	10-30 µg/ml 93626 0.999	 ≤0.99
5	**Accuracy** Mean % recovery	 100.48%	 98 - 102%
6	**Robustness** a) Flow rate variation	All the system suitability	

	b) Organic phase variation	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 Amoxicillin and Bromohexine 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.
Amoxicillin was found to be slightly soluble in water, very slightly soluble in ethanol (96 per cent), soluble in organic solvents such as DMSO and dimethyl formamide. Bromohexine was found to be soluble in water. Methanol and glacial acetic acid (50:50 v/v) 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 Amoxicillin and Bromohexine in bulk drug and in Pharmaceutical dosage forms.

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