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DEVELOPMENT & VALIDATION OF RP-HPLC METHOD FOR QUANTITATIVE ESTIMATION OF BRINZOLAMIDE AND ITS IMPURITIES IN DRUG SUBSTANCE AS PER ICH GUIDELINES

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ABSTRACT
The analysis of improved RP-HPLC method for the separation and quantification of Brinzolamide and its impurities are described. Samples are analysed by means of reverse phase (RP-HPLC) using an Inertsil ODS-3V, 250 x 4.6 mm, 5µm, and the mobile phase consists of two Channels A and B. Channel-A pH 5.50 buffer: Acetonitrile (80:20 %v/v) and Channel-B: pH Acetonitrile: Methanol: Water (80:10:10%v/v). The flow rate is 1.0 ml/min. The column temperature was maintained at 25°C and sample temperature was maintained at (15°C) and wavelength fixed at 230nm UV-detection. It is found that the method of RP-HPLC with UV-detection system for the analysis of Brinzolamide impurities are straight forward and applied in qualitative and quantitative analysis. The developed LC method was validated with respect to specificity, precision, linearity, ruggedness and robustness. Validation study compared as per ICH guideline.

KEYWORDS: Brinzolamide, estimation of related substances, liquid chromatography.

1.0 INTRODUCTION

Brinzolamide chemically is (R)-4-(Ethylamino)-3, 4-dihydro-2-(3-methoxypropyl)-2H-thieno [3,2-e]-1,2-thiazine-6-sulfonamide 1,1-dioxide.[1-2] Brinzolamide is a Carbonic anhydrase inhibitor. It is a highly specific, non-competitive, reversible carbonic anhydrase inhibitor. Brinzolamide act as a highly specific inhibitor of CAII, which is the main CA isoenzyme involved in the secretion of aqueous humor. Inhibition of CA in the ciliary process of the eye slows the formation of bicarbonate, and reduces sodium and fluid transport. This results in a reduction in the rate of aqueous humor secretion and the intraocular pressure. Very Soluble in Ethanol but Soluble in Water and Methanol. It is official in USP30-NF25.[3] The chemical structure of Brinzolamide shown in **(Fig. 1.1).**

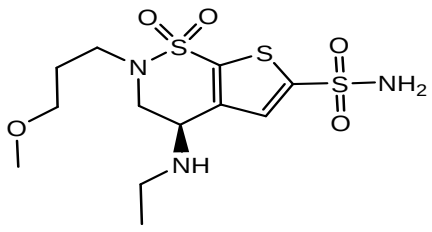


Figure: 1.1. Chemical Structure of Brinzolamide.

There are no extensive data available concerning the identification and/or quantification of the process related impurities. USP-NF has published the monographs of Brinzolamide with its S-isomer as "related substance impurity-A" and "R- isomer as related substance impurity B".

Literature review reveals several methods developed has been reported for Brinzolamide estimation in biological fluids and there are some methods reported by voltametry[4], spectroscopy[5], HPTLC & HPLC, UPLC and capillary electrophoresis.[6-8]

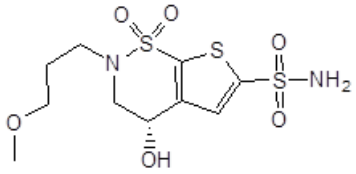
The objective of the present work is to develop a stability indicating HPLC method and validated as per ICH and USP validation guidelines for the estimation of Brinzolamide in applied for routine analysis in laboratories and is suitable for the quality control of the raw materials.

Impurity profiling of active pharmaceutical ingredients (API) in both bulk material and formulations is one of the most challenging tasks. The presence of unwanted or in certain cases unknown chemicals, even in small amounts, may influence not only the therapeutic efficacy but also the safety of the pharmaceutical products. For

these reasons, all major international pharmacopoeias have established maximum allowed limits for related compounds for both bulk and formulated APIs. As per the requirements of various regulatory authorities, the impurity profile study of drug substances and drug products has to be carried out using a suitable analytical method in the final product.

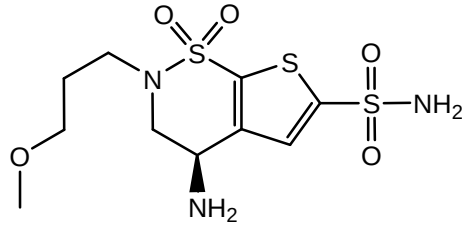
Brinzolamide Impurity-A

Chemical Name: (S)-3, 4-Dihydro-4-hydroxy-2-(3-methoxypropyl)-2H-thieno [3, 2-e]-1, 2-thiazine-6-sulfonamide 1,1-dioxide. Molecular formula: $C_{10}H_{16}N_2O_6S_3$, Molecular weight. 356.44.



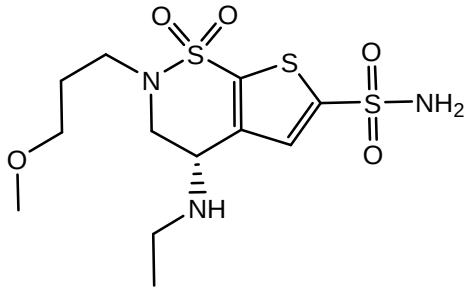
Brinzolamide Impurity-B

Chemical Name: (4R)-4-Amino-3, 4-dihydro-2-(3-methoxypropyl)-2H-thieno [3,2-e]-1,2-thiazine-6-sulfonamide 1,1-Dioxide. Molecular formula: $C_{10}H_{17}N_3O_5S_3$, Molecular weight: 355.46.



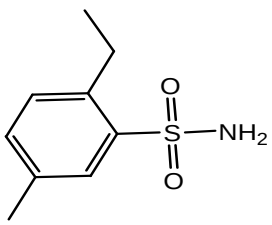
Brinzolamide Impurity-C

Chemical Name: (S)-4-(Ethylamino)-3, 4-dihydro-2-(3-methoxypropyl)-2H-thieno [3,2-e]-1,2-thiazine-6-sulfonamide 1,1-Dioxide. Molecular formula: $C_{12}H_{21}N_3O_5S_3$, Molecular weight: 383.51.



Brinzolamide Impurity-D

Chemical Name: N-Ethyl-p-toluene sulfonamide. Molecular formula: $C_9H_{13}NO_2S$, Molecular weight: 199.27



2.0 EXPERIMENTAL

2.1 Reagents and chemicals

Triethyl amine, Octane 1-sulfonic acid sodium salt, Orthophosphoric acid, Acetonitrile and Methanol was procured from Merck. Water (Milli-Q). All chemicals were of an analytical grade and used as received.

2.2 Instrumentation

Chromatographic separation was achieved by using an Agilent-1200, Open-lab software using, Inertsil ODS-3V, 250 x 4.6 mm, 5µm, and the mobile phase consists of two Channels A and B. Channel-A pH 5.50 buffer: Acetonitrile (80:20 %v/v) and Channel-B: pH Acetonitrile: Methanol: Water (80:10:10%v/v). The flow rate is 1.0 ml/min. The column temperature was maintained at 25°C and sample temperature was maintained at (15°C) and wavelength fixed at 230nm UV-detection. The overall run time was 60 minutes. 15 µl of sample was injected into the HPLC system. Retention times of impurities were 6.45 for impurity-A, 5.15 for Impurity-B, 12.05 for Impurity-C, 14.05 for Impurity-D and 8.35 for Brinzolamide.

2.3 Preparation of mobile phase and standard and sample solution

Preparation of Buffer

Weigh accurately about 0.25g of Octane 1-sulfonic acid sodium salt in 1000 ml of water. Add 10 ml of triethyl amine and adjust the pH to 5.5 ± 0.1 with dilute orthophosphoric acid. Filter through 0.45µ membrane filter paper.

Mobile phase-A: Transfer 800mL of buffer and 200mL of acetonitrile into 1000mL beaker mixed well. Filter through 0.45µ membrane filter and degas.

Mobile phase-B: Transfer 800mL of acetonitrile, 100mL of methanol and 100mL of water into 1000mL beaker mixed well. Filter through 0.45µ membrane filter and degas.

Diluent preparation: Mobile phase-A and Mobile phase-B in the ratio of (75:25 v/v).

Preparation of Test solution

Weigh accurately and transfer about 100mg of test sample into a 100ml volumetric flask, dissolve in and dilute to the volume with diluent.

Preparation of Standard solution

Weigh accurately and transfer about 50mg of standard into a 50ml volumetric flask, added 15 ml of diluent sonicated for 5 minutes to dissolve and then dilute to the volume with diluent. Further transferred 5.0 ml of this solution in to 50 ml of volumetric flask containing 15 ml of diluent mixed well then made up to the mark with diluent.

3.0 RESULTS AND DISCUSSION

3.1 Method optimization parameters

An understanding of the nature of API (functionality, acidity, or basicity), the synthetic process, related impurities, the possible degradation pathways and their degradation products are needed for successful method development in reverse-phase HPLC. In addition, successful method development should result a robust, simple and time efficient method that is capable of being utilized in manufacturing setting.

3.2 Selection of wavelength

The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for related substances and the drugs to be detected. The wavelength for measurement was selected as 230 nm from the absorption spectrum.

3.3. Selection of stationary phase

Proper selection of the stationary phase depends up on the nature of the sample and chemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography. From literature survey, it was found that different C18 columns could be appropriately used for the separation of related substances for Brinzolamide.

3.4. Selection of mobile phase

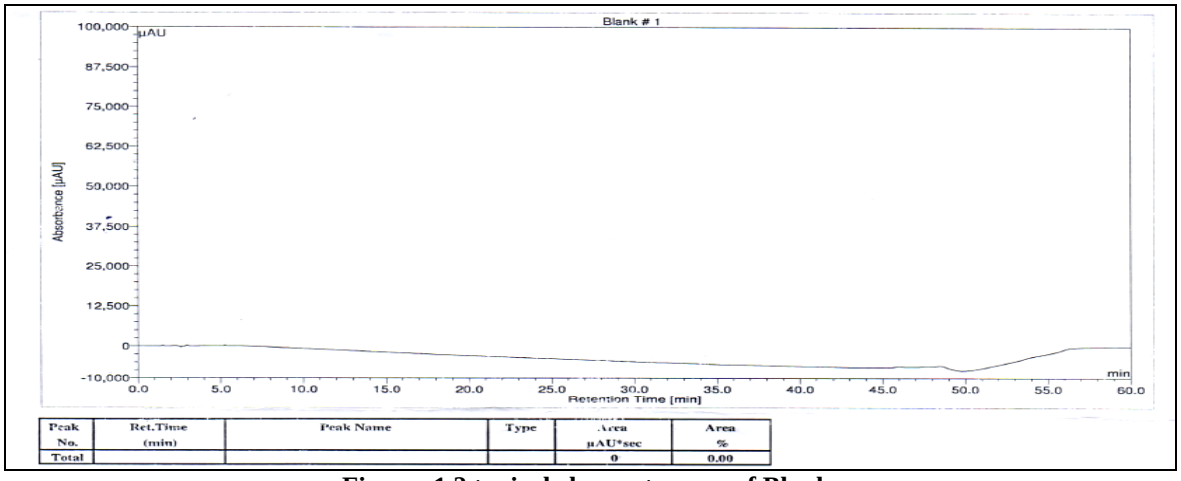
Different mobile phase and stationary phases were employed to develop a suitable LC method for the quantitative determination of impurities in Brinzolamide. A number of column chemistries supplied by different manufacturers and different mobile phase composition were tried to get good peak shapes and selectivity for the impurities present in Brinzolamide.

4.0 METHOD VALIDATION

4.1 Specificity

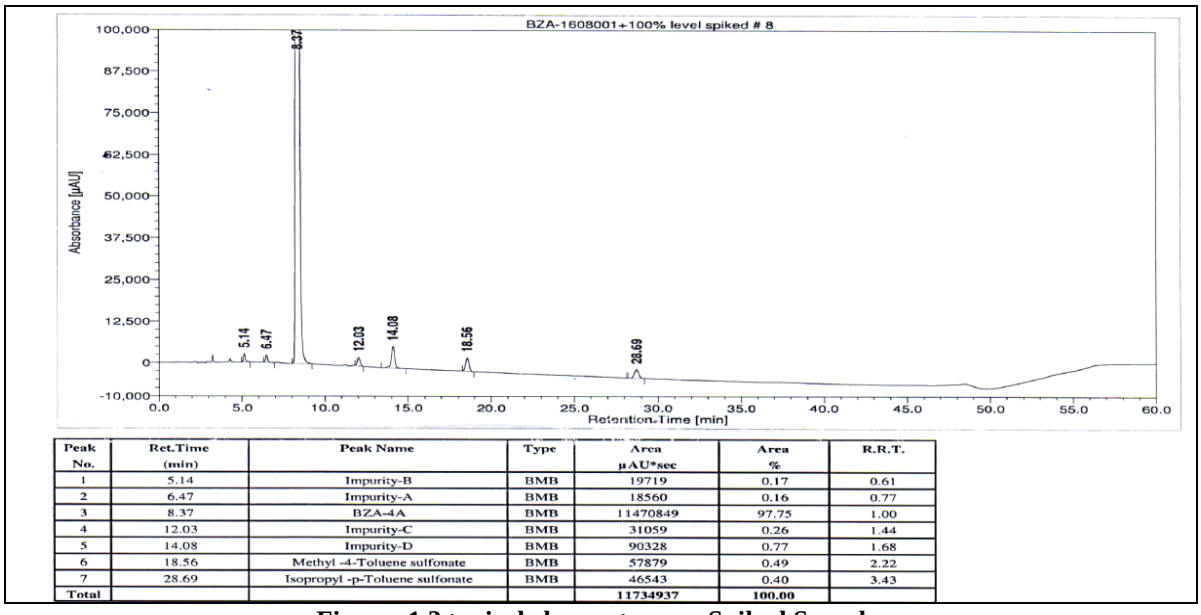
Blank interference

A study to establish the interference of blank was conducted. Diluent was injected as per the test method.



Peak No.	Ret.Time (min)	Peak Name	Type	Area µAU*sec	Area %
Total				0	0.00

Figure: 1.2 typical chromatogram of Blank.



Peak No.	Ret.Time (min)	Peak Name	Type	Area µAU*sec	Area %	R.R.T.
1	5.14	Impurity-B	BMB	19719	0.17	0.61
2	6.47	Impurity-A	BMB	18560	0.16	0.77
3	8.37	BZA-4A	BMB	11470849	97.75	1.00
4	12.03	Impurity-C	BMB	31059	0.26	1.44
5	14.08	Impurity-D	BMB	90328	0.77	1.68
6	18.56	Methyl -4-Toluene sulfonate	BMB	57879	0.49	2.22
7	28.69	Isopropyl -p-Toluene sulfonate	BMB	46543	0.40	3.43
Total				11734937	100.00	

Figure: 1.3 typical chromatogram Spiked Sample.

Table: 1.1 Impurity interference data

Peak Name	Retention Time	Relative retention time(RRT)
Impurity-A	6.45	0.77
Impurity-B	5.15	0.61
Impurity-C	12.05	1.44
Impurity-D	14.10	1.68
Brinzolamide	8.35	1.00

It was observed that known impurities are not co eluting with each other and main analyte peak. Brinzolamide standard solution preparation and in spiked test preparation was calculated and found to be within the acceptable limit.

4.2 Precision

4.2.1 System Precision

System precision

Perform the analysis of reference solution (Diluted standard) six times and determine the percentage relative standard deviation of peak area of replicate injections of Brinzolamide.

Table 1.3: System Precision data for Brinzolamide.

Injection No	Brinzolamide
1	1174695
2	1173089
3	1173301
4	1173399
5	1173862
6	1173427
Mean area	**1173629**
%RSD	**0.05**

The %RSD of peak area for Brinzolamide was found to be 0.05% which is below 5.0% indicates that the system gives precise result.

4.2.2 Method Precision

Precision of the impurities and degradants method was determined by injecting six sample solutions spiked with impurities (Impurity-A, B, C and D) at specification level. The samples were prepared as per the method and the result for precision study is tabulated in **Table: 1.4.**

Table: 1.4 Results of method precision.

Inj. No	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Individual Unknown impurity
1	0.16	0.13	0.14	0.15	0.06
2	0.16	0.15	0.14	0.13	0.06
3	0.15	0.13	0.14	0.14	0.06
4	0.16	0.13	0.15	0.14	0.05
5	0.16	0.14	0.14	0.14	0.06
6	0.16	0.13	0.13	0.14	0.05
Mean (%)	0.16	0.13	0.14	0.15	0.06
% RSD	2.58	6.20	4.52	4.52	9.11

The method precession was performed with six replicate solutions of standard solutions prepared and the system suitability parameters found were within the acceptance criteria.

4.3 Limit of Quantitation (LOQ) & Limit of detection (LOD)

A solution containing 0.13 µg/ml of Brinzolamide standard, 0.12 µg/ml of impurity-A, 0.10 µg/ml of impurity-B, 0.12 µg/ml of impurity-C and 0.05 µg/ml of impurity-D was injected six times.

Table: 1.7 LOQ for Brinzolamide and impurities.

Component Name	LOQ (%)	S/N
Impurity–A	0.0127	10.475
Impurity–B	0.0101	9.121
Impurity–C	0.0116	10.341
Impurity–D	0.0050	10.906
Brinzolamide	0.0130	10.115

A solution containing 0.04 µg/ml of Brinzolamide standard, 0.04 µg/ml of impurity-A, 0.03 µg/ml of impurity-B, 0.04 µg/ml of impurity-C and 0.016 µg/ml of impurity-D was injected three times.

Table: 1.8 LOD for Brinzolamide and impurities.

Component Name	LOD (%)	S/N
Impurity–A	0.0042	3.187
Impurity–B	0.0033	3.444
Impurity–C	0.0038	3.185
Impurity–D	0.0016	3.200
Brinzolamide	0.0043	3.344

The limit of limit of quantitation and detection of quantitation values obtained for each impurity and Brinzolamide are within the acceptance criteria.

4.4 Linearity and Range

The linearity is determined by injecting the solutions in duplicate containing known impurities and Brinzolamide and impurities ranging from LOQ to 150% of the specified limit. Perform the regression analysis and determine the correlation coefficient and residual sum of squares. Determine the response factor for each impurity with respect to Brinzolamide. Report the linearity range as the range for determining the impurities. Results obtained are in the tables & figures show the line of best fit for peak area versus concentration for each impurity. **(Table 1.8, Table 1.9, Table 1.10. Table 1.11 and Table 1.12) &** figures show the line of best fit for peak area versus concentration for each impurity.

Table1.8: Linearity of detector response Impurity-A.

Level	Concentration (%)	Mean Area
LOQ	0.0127	1780
25%	0.0365	4940
50%	0.0731	10022
75%	0.1096	15141
100%	0.1461	19843
125%	0.1827	24556
150%	0.2192	29538
Correlation coefficient		0.9980
R^2 Value		0.9990
% Y-intercept		0.76
Slope		134329
Intercept		151.0

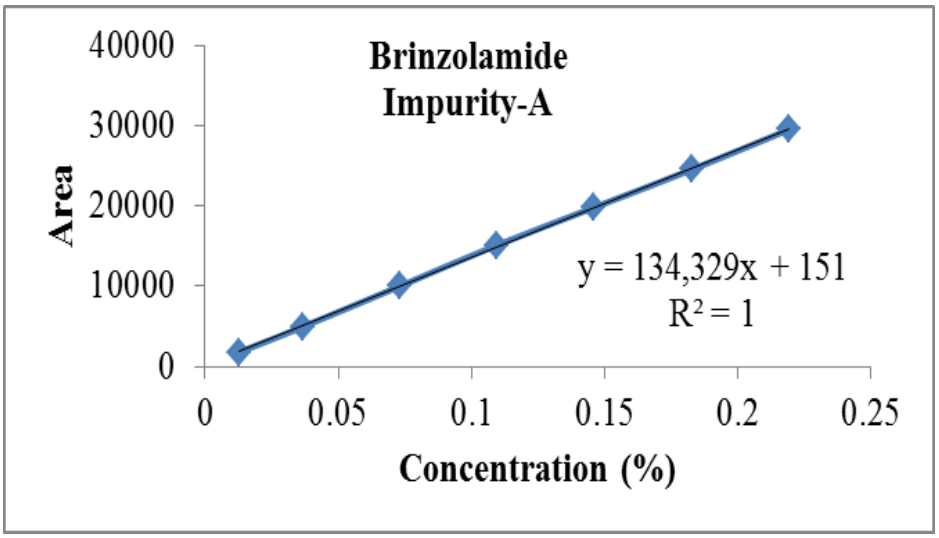


Fig. 1.4: linearity of detector response for Impurity-A.

Table 1.9: Linearity of detector response Impurity-B.

Level	Concentration (%)	Mean Area
LOQ	0.0101	1458
25%	0.0271	3993
50%	0.0543	7889
75%	0.0814	11716
100%	0.1085	15772
125%	0.1357	19520
150%	0.1628	23314
Correlation coefficient		0.9980
R^2 Value		0.9990
% Y-intercept		0.55
Slope		143217
Intercept		87.00

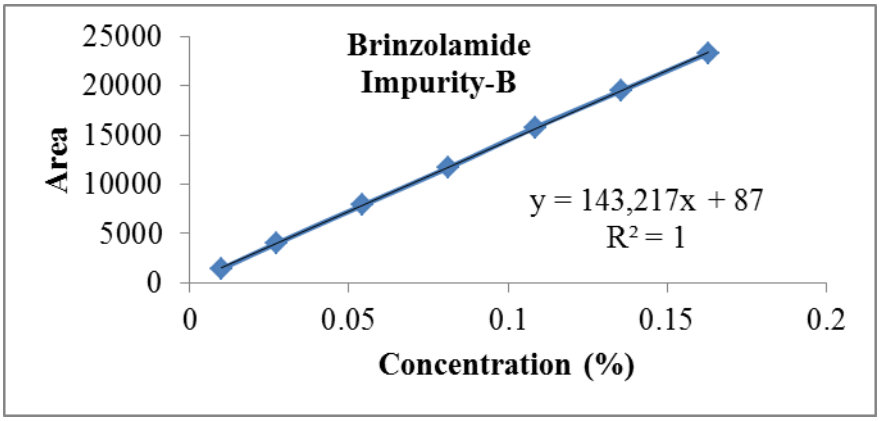


Fig. 1.5: linearity of detector response for Impurity-B.

Table 1.10: Linearity of detector response Impurity-C.

Level	Concentration (%)	Mean Area
LOQ	0.0116	2804
25%	0.0361	8772
50%	0.0722	17552
75%	0.1083	26675
100%	0.1444	35374
125%	0.1805	43676
150%	0.2166	52379
Correlation coefficient		0.9980
R^2 Value		0.9990
% Y-intercept		0.35
Slope		242125
Intercept		124.0

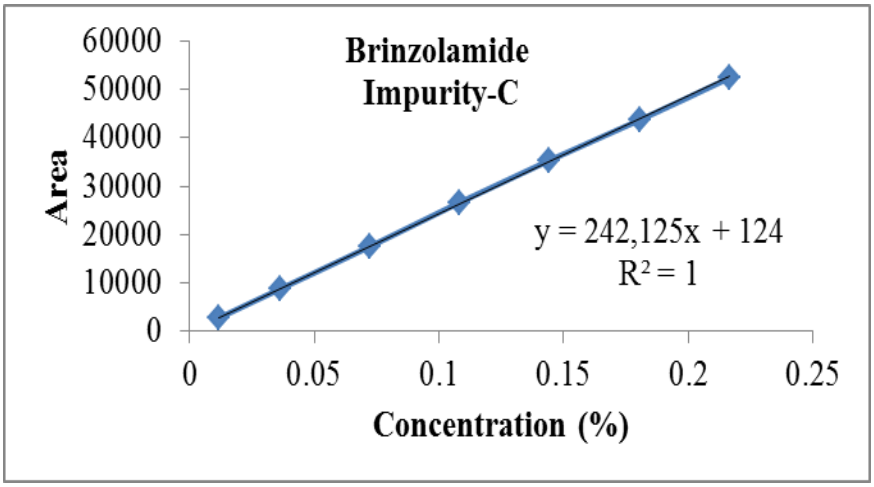


Fig. 1.6: linearity of detector response for Impurity-C.

Table: 1.11 Linearity of detector response Impurity-D.

Level	Concentration (%)	Mean Area
LOQ	0.0050	2839
25%	0.0374	20174
50%	0.0748	40888
75%	0.1122	60828
100%	0.1496	80990
125%	0.1870	100761
150%	0.2243	120502
Correlation coefficient		0.9980
R^2 Value		0.9990
% Y-intercept		0.44
Slope		537105
Intercept		358.0

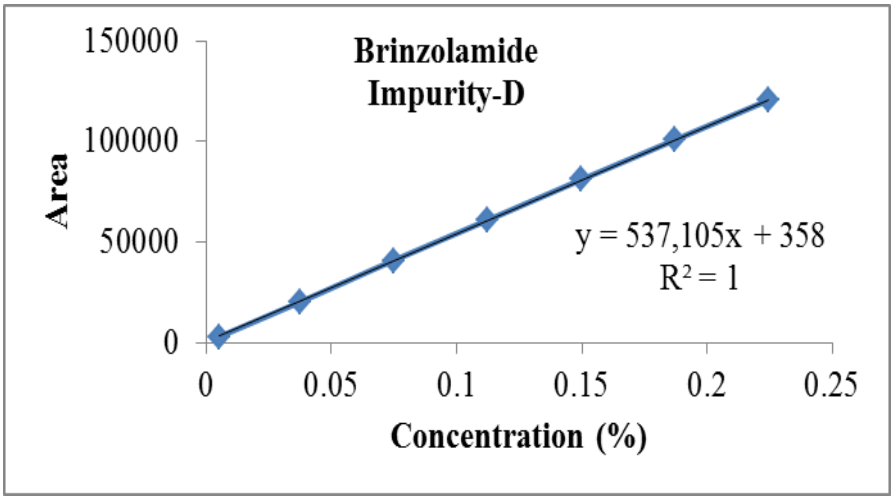


Fig. 1.6: linearity of detector response for Impurity-D.

Table 1.12: Linearity of detector response Brinzolamide.

Level	Concentration (%)	Mean Area
LOQ	0.0130	1794
25%	0.0376	4658
50%	0.0751	9509
75%	0.1127	13686
100%	0.1502	18162
125%	0.1878	22890
150%	0.2253	27080
Correlation coefficient		0.9980
R^2 Value		0.9990
% Y-intercept		
Slope		119478
Intercept		285.0

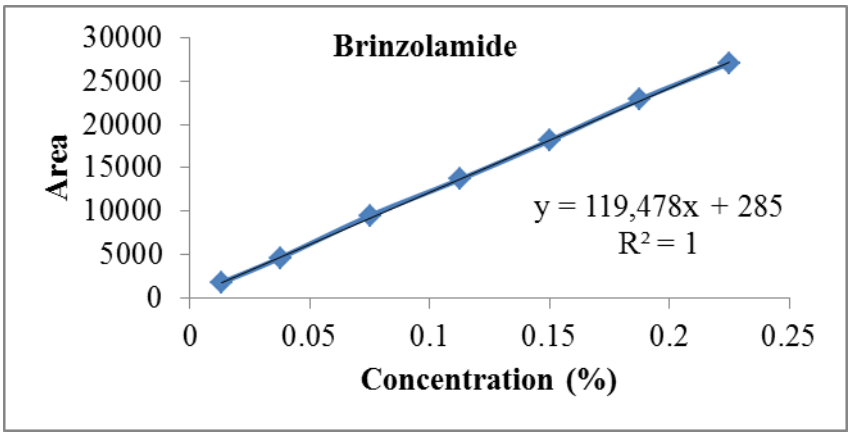


Fig. 1.7: linearity of detector response for Brinzolamide.

The linearity results for Brinzolamide and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.

4.5 Accuracy

Recovery of Brinzolamide impurities in Brinzolamide was performed. The sample was taken and varying amounts of Brinzolamide impurities representing LOQ to 150 % of specification level were added to the flasks. The spiked samples were prepared as per the method and the results are tabulated in **Table 1.13.**

Table 1.13: Accuracy study of Brinzolamide.

S.No.	Theoretical (%)	% Mean Recovery			
		Impurity-A	Impurity-B	Impurity-C	Impurity-D
1	LOQ	114.4	85.4	93.8	114.4
2	50	107.0	104.2	98.9	95.3
3	100	104.4	106.7	97.6	92.4
4	150	102.2	107.1	97.7	94.9

5.0 RESULTS AND DISCUSSION

A simple, economic, accurate and precise HPLC method was successfully developed. The results obtained were accurate and reproducible. The method developed was statistically validated in terms of Selectivity, accuracy, linearity, precision, robustness, and stability of solution.

For Selectivity, the chromatograms were recorded for standard and sample solutions of Brinzolamide and its related substances. Selectivity studies reveal that the peak is well separated from each other. Therefore the method is selective for the determination of related substances in Brinzolamide. There is no interference of diluent at Brinzolamide and impurities peaks. The elution order and the retention times of Impurities and Brinzolamide obtained from individual standard preparations and mixed standard Preparations are comparable.

The limit of detection (LOD) and limit of quantitation (LOQ) for Brinzolamide standard 0.04&0.13µg/mL, impurity-A 0.04&0.12µg/mL, impurity-B 0.03&0.10 µg/Ml, impurity-C 0.04 & 0.12 µg/mL and impurity-D 0.016&0.05 respectively.

The linearity results for Brinzolamide and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.Calibration curve was plotted and correlation co-efficient for Brinzolamide and its impurities found to be 0.9980, 0.9980, 0.9980, 0.9980 and 0.9980 respectively.

The accuracy studies were shown as % recovery for Brinzolamide and its impurities at specification level. The limit of % recovered shown is in the range of 80 and 120% and the results obtained were found to be within the limits. Hence the method was found to be accurate.

For Precision studies six (6) replicate injections were performed. %RSD was determined from the peak areas of Brinzolamide and its impurities. The acceptance limit should be not more than 10, and the results were found to be within the acceptance limits.

Hence, the chromatographic method developed for Brinzolamide and its related substances are rapid, simple, sensitive, precise, and accurate. Therefore, the proposed method can be successfully applied for the routine analysis of the active pharmaceutical ingredients for assurance of its quality during its formulation.

6.0. CONCUSIONS

The new HPLC method developed and validated for determination of Brinzolamide pharmaceutical dosage forms and assured the satisfactory precision and accuracy and also determining lower concentration of drug in its solid dosage form by RP-HPLC method. The method was found to be simple, accurate, economical and rapid and they can be applied for routine analysis in laboratories and is suitable for the quality control of the raw materials.

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