DEVELOPMENT AND VALIDATION OF A RAPID AND SPECIFIC RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF BENAZEPRIL AND HYDROCHLOROTHIAZIDE IN PURE FORM AND IN THEIR MARKETED PHARMACEUTICAL DOSAGE FORM

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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 Benazepril and Hydrochlorothiazide 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: Phosphate Buffer (pH-4.2) (37:63% v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 275nm. The retention time of the Benazepril and Hydrochlorothiazide 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 Benazepril and 10-30mg/ml of Hydrochlorothiazide. 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: Benazepril and Hydrochlorothiazide, RP-HPLC, Validation, Accuracy, Precision.

INTRODUCTION

INTRODUCTION TO HPLC

HPLC is also called as high pressure liquid chromatography since high pressure is used to increase the flow rate and efficient separation by forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system. The development of HPLC from classical column chromatography can be attributed to the development of smaller particle sizes. Smaller particle size is important since they offer more surface area over the conventional large particle sizes. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

1. Improved resolution of separated substances
2. column packing with very small (3,5 and 10 µm) particles
3. Faster separation times (minutes)
4. Sensitivity
5. Reproducibility
6. continuous flow detectors capable of handling small flow rates
7. Easy sample recovery, handling and maintenance. [1]

INSTRUMENTATION OF HPLC

The basic liquid chromatograph consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

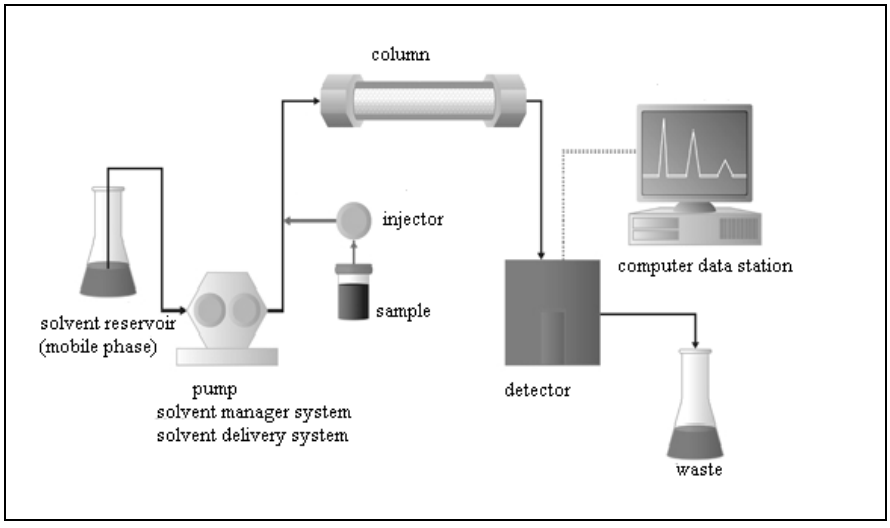

Fig.1.1: Components of HPLC instrument block diagram. [2]

METHOD DEVELOPMENT AND VALIDATION

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result .it should be simple as possible, yet it should allow the use of sophisticated tools such as computer modeling3

Table: Separation goals in HPLC method development.[7]

Goal	**Comment**
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures
Quantitation	≤2% for assays; ≤5% for less-demanding analyses ≤15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent consumption	Minimum mobile-phase use per run is desirable.

Table : Steps involved in development of HPLC method.[7]

Literature survey: To avoid duplication of the method and invention of new method. Information about the solubility, the structure of the drugs and their Physico- chemical properties for the known compounds and can be applied for the new compounds.

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Selection of chromatographic method: First reversed phase should be tried**.** If not successful, normal phase should be taken into consideration. For ion exchange or ion pair chromatography, first ion suppression by pH control and reversed phase chromatography should be tried.

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Selection of stationary phase: Matching the polarity of sample and stationary phase and using a mobile phase of different polarity achieves a successful separation.

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Selection of mobile phase: Reversed phase bonded packing, when used in conjunction with highly polar solvents. Mobile phase may be either single liquid or combination of liquids, which are compatible with sample, column and instrument

↓

Selection of suitable detector: Detector is the eye of HPLC system and measures the compounds after their separation on the column. There are basically two types of detectors: bulk property detectors; Solute property detectors.UV detector is the first choice because of its convenience and applicability in case of most of the samples. The latest version of equipments is available with photo diode-array detectors.

ANALYTICAL METHOD VALIDATION

Method validation as per ICH can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".[4]

Objective of validation

There are two important reasons for validating assays in the pharmaceutical industry. The first, and by for most important is that assay validation is an integral part of the quality control system. The second is that current good manufacturing practice regulation requires assay validation. In industry it would be difficult to confirm that the product being manufactured is uniform and that meet the standards set to assure fitness for use. The varying nature of the differences between the analytical development laboratory and quality control laboratory is a good reason for validation program.

Method validation study includes Specificity / Selectivity, Linearity, Accuracy, Precision, Limit of detection, Limit of Quantitation, Robustness, System suitability and Stability criteria.[2]

MATERIALS

Benazepril, Hydrochlorothiazide, Water and Methanol for HPLC, Acetonitrile for HPLC, Potassium Dihydrogen Phosphate from MERCK were provided by SURALABS at Dilsuknagar.

METHODOLOGY

HPLC Method Development

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Benazepril and Hydrochlorothiazide 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 0.4ml of Benazepril and 0.2ml of Hydrochlorothiazide from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

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 Benazepril and Hydrochlorothiazide 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 0.4ml of Benazepril and 0.2ml of Hydrochlorothiazide from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation Of Buffer And Mobile Phase:

Preparation of Potassium dihydrogen Phosphate (KH2PO4) buffer (pH-4.2):

Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 4.2 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration and ultra sonication.

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

Optimized Chromatographic Conditions:

Instrument used	:	Waters Alliance 2695 HPLC with PDA Detector 996 model.
Temperature	:	35ºC
Column	:	Phenomenex Luna C18 (4.6mm×250mm) 5µm particle size
Mobile phase	:	Methanol: Phosphate Buffer (pH-4.2) (37:63 v/v)
Flow rate	:	1ml/min
Wavelength	:	275nm
Injection volume	:	10µl
Run time	:	6minutes

Method Validation

System Suitability:

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:

Procedure:

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

%ASSAY =

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

Linearity:

Prepare various concentrations of the drugs (i.e., Benazepril in the range of 20-60 µg/ml, and Hydrochlorothiazide in the range of 10-30 µg/ml) using mobile [phase as the diluent.

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: 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:

Further pipette out 0.2ml of Benazepril and 0.1ml of Hydrochlorothiazide 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:

Further pipette out 0.4 ml of Benazepril and 0.2ml of Hydrochlorothiazide 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:

Further pipette out 0.6ml of Benazepril and 0.3ml of Hydrochlorothiazide 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 Benazepril and Hydrochlorothiazide 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. .

Further pipette out 0.4 ml of Benazepril and 0.2ml of Hydrochlorothiazide 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: Phosphate Buffer 4.2 pH was taken in the ratio and 42:58, 32:68 instead of 37:63 remaining conditions are same. 10µl of the above sample was injected twice and chromatograms were recorded.

RESULTS AND DISCUSSION

Optimized Chromatogram (Standard) and (Sample)

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

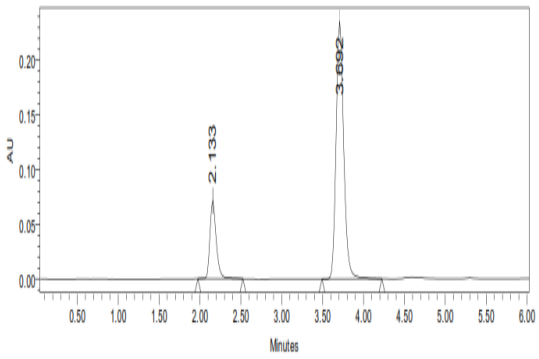

Figure-: Optimized Chromatogram (Standard)

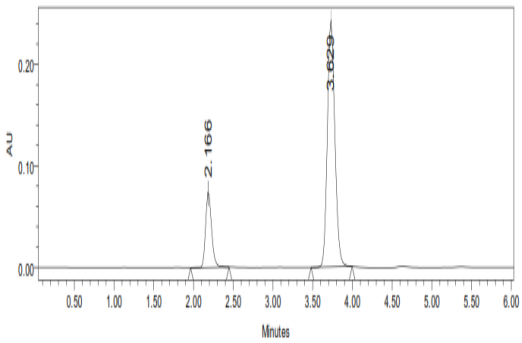

Figure-: Optimized Chromatogram (Sample)

It was found from above data that all the system suitability parameters for developed method were within the limit.

Method Validation

System Suitability:

Table-: Results of system suitability for Benazepril

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Benazepril	2.152	526358	86598	5695	1.56

2	Benazepril	2.157	526548	86254	5652	1.57
3	Benazepril	2.141	526854	86598	5627	1.56
4	Benazepril	2.133	526598	86245	5692	1.57
5	Benazepril	2.166	524874	86521	5641	1.56
Mean			526246.4			
Std. Dev.			787.353			
% RSD			0.149617			

Table-: Results of system suitability for Hydrochlorothiazide

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Hydrochlorothiazide	3.674	1682821	1686958	8659	1.56	9.8
2	Hydrochlorothiazide	3.631	1682726	1685745	8675	1.57	9.9
3	Hydrochlorothiazide	3.625	1687361	1685421	8692	1.56	9.8
4	Hydrochlorothiazide	3.692	1682811	1685242	8642	1.57	9.8
5	Hydrochlorothiazide	3.629	1683816	1685364	8635	1.58	9.8
Mean			1683907				
Std. Dev.			1982.03				
% RSD			0.117704				

Specificity :

%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 Benazepril and Hydrochlorothiazide in pharmaceutical dosage form was found to be 99.89%

Linearity:

Table-: Chromatographic Data for Linearity Study of Benazepril

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

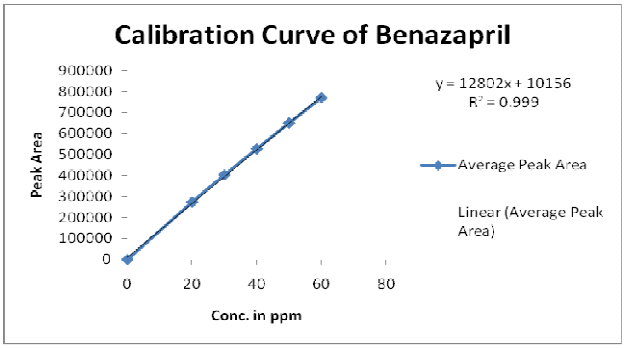

Fig-: Calibration Curve of Benazepril

Table-: Chromatographic Data for Linearity Study of Hydrochlorothiazide

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

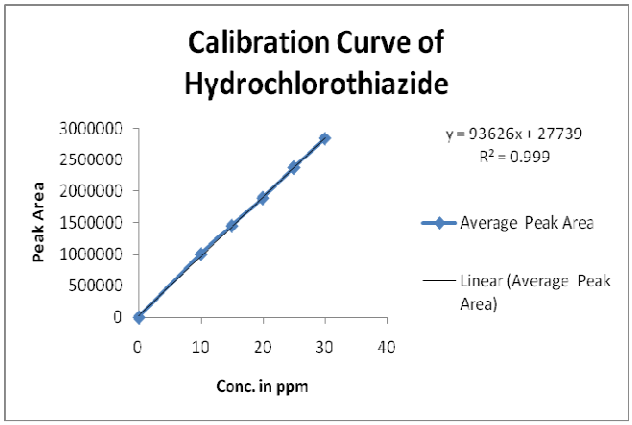

Fig-: Calibration Curve of Hydrochlorothiazide

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

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

Table-: The accuracy results for Benazepril

%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%	

Table-: The accuracy results for Hydrochlorothiazide

%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.

Precision:

Repeatability:

Table-: Results of repeatability for Benazepril:

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

Table-: Results of Repeatability for Hydrochlorothiazide:

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Hydrochlorothiazide	3.603	1687589	367859	8659	1.79
2	Hydrochlorothiazide	3.608	1685987	368547	8679	1.80
3	Hydrochlorothiazide	3.600	1685987	367985	8645	1.80
4	Hydrochlorothiazide	3.696	1685754	365874	8695	1.79
5	Hydrochlorothiazide	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 Benazepril

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

Table: Results of Intermediate precision for Hydrochlorothiazide

S.No.	Peak Name	Rt	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	Resolution	% Assay
1	Hydrochlorothiazide	3.623	1698587	385482	8789	1.81	9.8	98%
2	Hydrochlorothiazide	3.611	1698574	385698	8759	1.80	9.8	98.2%
3	Hydrochlorothiazide	3.696	1698532	385748	8754	1.81	9.9	98.7%

4	Hydrochlorothiazide	3.696	1698574	386958	8754	1.81	10.01	99.7%
5	Hydrochlorothiazide	3.696	1698532	385755	5798	1.80	9.98	98.5%
6	Hydrochlorothiazide	3.642	1698547	386558	8762	1.80	10.02	98.2%
Mean			1698558					
Std. Dev.			23.77113					
% RSD			0.001399					

Day 2:

Table-: Results of Intermediate precision Day 2 for Benazepril

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Benazepril	2.198	536854	8758	5789	1.58
2	Benazepril	2.196	536985	8795	5726	1.59
3	Benazepril	2.178	536587	8746	5742	1.58
4	Benazepril	2.142	532546	8754	5746	1.59
5	Benazepril	2.177	534587	8725	5798	1.58
6	Benazepril	2.177	538598	8726	5785	1.59
Mean			536026.2			
Std. Dev.			2131.492			
% RSD			0.397647			

Table-: Results of Intermediate precision Day 2 for Hydrochlorothiazide

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	Resolution
1	Hydrochlorothiazide	3.611	1678598	356875	8875	1.82	9.9
2	Hydrochlorothiazide	3.623	1678985	358985	8856	1.83	10.01

3	Hydrochlorothiazide	3.684	1678984	358754	8862	1.82	9.9
4	Hydrochlorothiazide	3.697	1678985	352412	8849	1.83	10.01
5	Hydrochlorothiazide	3.684	1678549	358987	8873	1.82	9.9
6	Hydrochlorothiazide	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.

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

Benazepril

Result: = 1.04µg/ml

Hydrochlorothiazide

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

Benazepril

Result: =2.1µg/ml

Hydrochlorothiazide

Result: =6.3µg/ml

Robustness:

Variation in flow rate:

Table-: Results for Robustness

Benazepril

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

Hydrochlorothiazide

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.

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Benazepril and Hydrochlorothiazide 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.
Benazepril was found to be freely soluble in water; soluble in alcohol, in methanol, ethanol and in glacial acetic acid and also soluble in Acetonitrile. Hydrochlorothiazide was found to be is slightly soluble in water, freely soluble in sodium hydroxide solution, in n-butyl amine, and in dimethyl formamide; sparingly soluble in methanol; insoluble in ether, in chloroform, and in dilute mineral acids. Methanol: Phosphate Buffer (pH-4.2) (37:63 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 Benazepril and Hydrochlorothiazide in bulk drug and in Pharmaceutical dosage forms.

ACKNOWLEDGEMENT

The Authors are thankful to Sura Labs, Dilshukhnagar, Hyderabad for providing the necessary facilities for the research work.

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