International Journal of Chemistry and Pharmaceutical Sciences

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Research Article **Open Access**

Analytical Method Development and Validation for the Simultaneous Estimation of Enalapril and Hydrochlorothiazide by RP-HPLC Method in Bulk and Pharmaceutical Dosage Form

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A B S T R A C T

A Reverse Phase High Performance Liquid Chromatography method was developed for the determination of Enalapril and Hydrochlorothiazide in bulk and Pharmaceutical dosage form. The separation was achieved on an Inertsil ODS (250x4.6mm),5µm column by using a mixture of Phosphate buffer and acetonitrile in a ratio of55:45 v/v at a flow rate of 1ml/min. The detection was made at 215 nm. Calibration curve was linear over the concentration range of 10-60 µ g/ml of Enalapril and Hydrochlorothiazide. The proposed method was validated as per the ICH guidelines. The method was rapid, accurate, precise, specific and found to be suitable for the quantitative analysis of the drug in Bulk and Pharmaceutical dosage form

Keywords: Enalapril, Hydrochlorothiazide, RP-HPLC

A R T I C L E I N F O

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Article History: Received 10 September 2016, **Accepted** 12 October 2016, **Available Online** 27 November 2016

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Manuscript ID: IJCPS3228

PAPER-QR CODE

Citation: *Jyothi Keerthi et al.* Analytical Method Development and Validation for the Simultaneous Estimation of Raltegravir and Lamivudine by RP-HPLC Method in Bulk and Pharmaceutical Dosage Form. *Int. J. Chem, Pharm, Sci.*, 2016, 4(11): 592-603.

1. Introduction

Analytical methods: The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias[1]. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors.

Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs[2].

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B). [3,4]

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behaviour[5]. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge.

The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines. Scouting experiments are frequently performed during method development to establish the performance limits of the method, prior to formal validation experiments[6-9]. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients[10].

Enalapril is a prodrug that belongs to the angiotensin-converting enzyme (ACE) inhibitor class of medications. It is rapidly metabolized in the liver to Enalapril at following oral administration. Enalapril is a potent, competitive inhibitor of ACE, the enzyme responsible for the conversion of angiotensin I (ATI) to angiotensin II (ATII). ATII regulates blood pressure and is a key component of the renin-angiotensin-aldosterone system (RAAS). Enalapril may be used to treat essential or Reno vascular hypertension and symptomatic congestive heart failure[11].

Hydrochlorothiazide is a thiazide diuretic often considered the prototypical member of this class. It reduces the reabsorption of electrolytes from the renal tubules. This results in increased excretion of water and electrolytes, including sodium, potassium, chloride, and magnesium. It has been used in the treatment of several disorders including edema, hypertension, diabetes insipidus and hypoparathyroidism. Hydrochlorothiazide, a thiazide diuretic, inhibits water reabsorption in the nephron by inhibiting the sodium-chloride symporter (SLC12A3) in the distal convoluted tubule, which is responsible for 5% of total sodium reabsorption. Normally, the sodium-chloride symporter transports sodium and chloride from the lumen into the epithelial cell lining the distal convoluted tubule. The energy for this is provided by a sodium gradient established by sodium-potassium ATPase on the basolateral membrane. Once sodium has entered the cell, it is transported out into the basolateral interstitium via the sodium-potassium ATPase, causing an increase in the osmolarity of the interstitium, thereby establishing an osmotic gradient for water reabsorption[12].

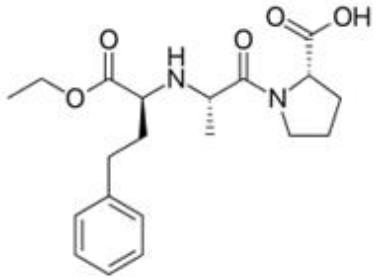


Figure 1: Enalapril

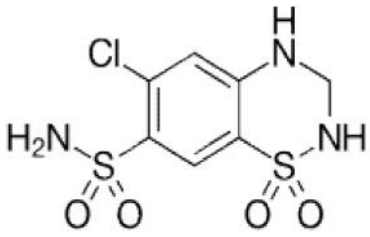


Figure 2: Hydrochlorothiazide

2. Materials and Methods

Apparatus

The instrument used for the study was Shimadzu (LC20ATVP) HPLC, Separation module 2690, UV detector with Spin chrome software version 2.

Reagents and Materials

The solvents used were Methanol, Acetonitrile, Potassium dihydrogen ortho phosphate, Dipotassium hydrogen phosphate, Tri Ethyl Amine of HPLC Grade and HPLC Water.

Selection of detection wavelength:

The sensitivity of method that uses UV- Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected. Standard solutions of Enalapril and Hydrochlorothiazide were scanned in the UV range (200-400nm) and the spectrums obtained were overlaid and the overlain spectrum was recorded. From the overlain spectrum, 215 nm was selected as the detection wavelength for the present study.

Selection of mobile phase

Initially the mobile phase tried was Methanol and water, Methanol, Buffer and water in various proportions. Finally, the mobile phase was optimized to a mixture of 55 volumes of mixed phosphate buffer and 45 volumes of acetonitrile were prepared. The mobile phase was sonicated for 10min to remove gases and filtered through 0.45µ membrane filter

Chromatographic trials for Simultaneous Estimation of Enalapril and Hydrochlorothiazide by RP- HPLC.

Trial – 1 Chromatographic condition

Mobile phase : Methanol: ACN: Water
Column : Analytical (Hyperchrom) ODS
pH : 5.0
Ratio : 50:10:40
Column : Inertsil ODS 3V (250×4.6× 5µ)
Wavelength : 215 nm
Flow rate : 1ml/min

Preparation of mixed standard solution

Weigh accurately 10 mg of Enalapril and 10mg of Hydrochlorothiazide in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Enalapril and of Hydrochlorothiazide is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

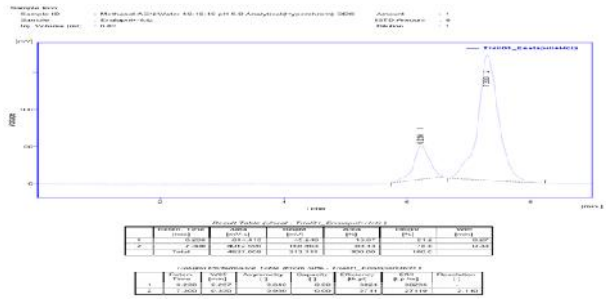


Figure 3: Trail 1 Chromatogram

Observation: The Efficiency was not satisfactory for Hydrochlorothiazide.The peak response of Enalapril was very less. Hence it was not taken for optimization.

Trial- 2Chromatographic conditions

Mobile phase : Methanol: ACN: Phosphate buffer
pH : 4.5
Ratio : 50:30:20
Column : Inertsil ODS 3V (250×4.6 ×5µ)
Wavelength : 215nm
Flow rate : 1ml/min

Preparation of mixed standard solution: Weigh accurately 10mg of Enalapril and Hydrochlorothiazide in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From the above stock solution 10µg/ml of Enalapril and Hydrochlorothiazide is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

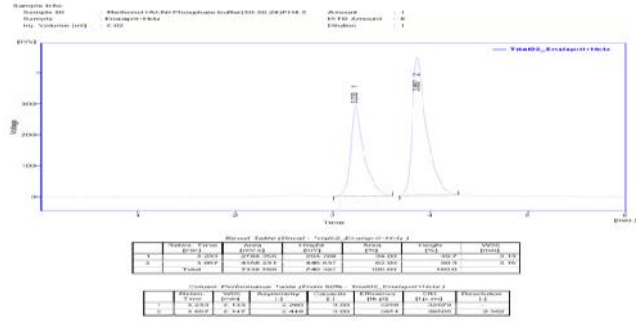


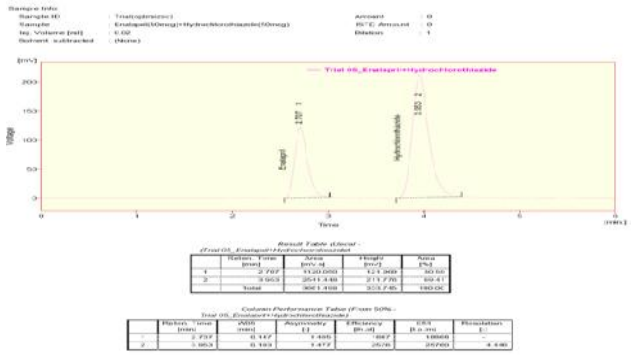
Figure 4: Trail 2 Chromatogram

Observation: Efficiency of both the drugs was good.The run time is very more and the peaks of Enalapril and Hydrochlorothiazide showed tailing. Hence it was not taken for optimization.

Optimized Chromatographic conditions

Mobile phase : Mixed Phosphate buffer: ACN
Ratio : 55:45
Column : Inertsil ODS (250×4.6× 5µ)
Wavelength : 215 nm
Flow rate : 1ml/min

Preparation of mixed standard solution: Weigh accurately 10 mg of Enalapril and Hydrochlorothiazide in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10µg/ml of Enalapril and Hydrochlorothiazide is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.



Trail 5: Chromatogram (Optimized Method)

Observation:

All the system suitability requirements were met and the peak Asymmetry factor was less than 2 for both Hydrochlorothiazide and Enalapril.The efficiency was more than 2000 Hydrochlorothiazide and Enalapril and hence this method was selected as optimized method.

Procedure

Mobile Phase:

A mixture of 55 volumes of mixed phosphate buffer and 45 volumes of acetonitrile were prepared. The mobile phase was sonicated for 10min to remove gases and filtered through 0.45µ membrane filter for degassing of mobile phase.

Preparation of buffer:

Weigh accurately about 1.625 gm of potassium dihydrogen phosphate and 0.3 gm of di potassium hydrogen phosphate were dissolved in 1000ml of water. Sonicate it for 10minutes to remove gases.

Preparation of samples for Assay

Preparation of mixed standard solution

Weigh accurately 10mg of Enalapril and 10 mg of Hydrochlorothiazide in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Enalapril and Hydrochlorothiazide is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Tablet sample

5 tablets were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of Hydrochlorothiazide and Enalapril (10µg/ml) were prepared by dissolving weight equivalent to 10 mg of Hydrochlorothiazide and Enalapril and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 10ml with mobile phase. Further dilutions are prepared in 5 replicates of 10µg/ml of Hydrochlorothiazide and Enalapril was made by adding 1 ml of stock solution to 10 ml of mobile phase.

Calculation

The amount of Hydrochlorothiazide and Enalapril present in the formulation by using the formula given below, and results shown in above table:

$$\%\ \text{Assay} = \frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{DS}} \times \frac{\text{DT}}{\text{WT}} \times \frac{\text{P}}{100} \times \frac{\text{AW}}{\text{LC}} \times 100$$

Where,

AS: Average peak area due to standard preparation
AT: Peak area due to assay preparation
WS: Weight of Hydrochlorothiazide and Enalapril in mg
WT: Weight of sample in assay preparation
DT: Dilution of assay preparation

3. Results and Discussion

Method Validation Parameters

1. Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by injecting blank and sample

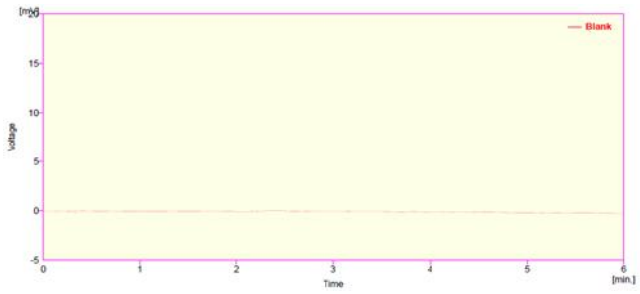


Figure 5: Chromatogram of Blank

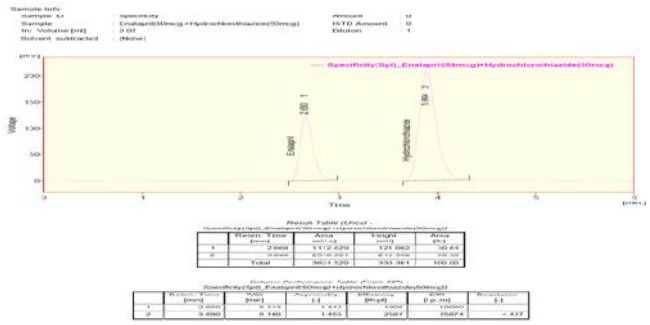


Figure 6: Chromatogram of Sample

2. Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.Standard stock solutions of Enalapril and Hydrochlorothiazide (microgram/ml) were prepared by dissolving 10 mg of Enalapril and 10 mg Hydrochlorothiazide dissolved in sufficient mobile phase and dilute to 100 ml with mobile phase. This solution contains 30-70 µg/ml of Enalapril and Hydrochlorothiazide

Acceptance criteria: Correlation coefficient should be not less than 0.99.

3. Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 30-70 ppm for Enalapril and Hydrochlorothiazide respectively

4. Accuracy

Accuracy of the method was determined by recovery experiments. There are mainly 2types of recovery studies are there.

a) Standard addition method: To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration.
b) Percentage method: For these assay method samples are prepared in three concentrations of 50%, 100%, and 150% respectively.

Acceptance criteria: The mean % recovery of the Enalapril and Hydrochlorothiazideat each level should be not less than 95.0% and not more than 105.0%.

Assay procedure

10µL of the standard and sample solutions of Enalapril and Hydrochlorothiazidewere injected into the HPLC system and the chromatograms were recorded. Amount of drug present in the Tablets were calculated using the peak areas.

5. Precision

Method precision also called as repeatability/Intra-day precision indicates whether a method gives consistent results for a single batch. Method precision was demonstrated by preparing six test solutions at 100% concentration as per the test procedure & recording the chromatograms of six test solutions. The % RSD of peak areas of six samples was calculated. The method precision was performed on Enalapril and Hydrochlorothiazide formulation.

Acceptance criteria

The % RSD for the area of sample injections results should not be more than 2.

Selection of solvent

Solutions of Enalapril and Hydrochlorothiazidewere prepared by dissolving in mobile phase and UV spectrum of each was recorded by scanning between 200-400 nm.

Validation of the Method

Linearity

Enalapril and Hydrochlorothiazide:

Serial dilutions of Enalapril and Hydrochlorothiazide (30-70 ppm) were injected into the column and detected at a wavelength set at 215 nm. The calibration curve was obtained by plotting the concentration vs. peak area and the correlation coefficient was found to be 0.9962 and 0.9963 respectively.

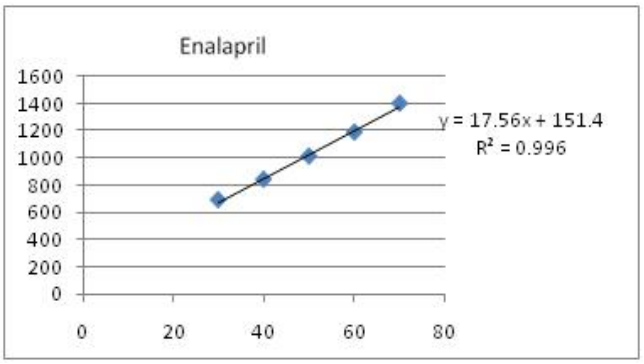


Figure 6: Linearity Graph of Enalapril

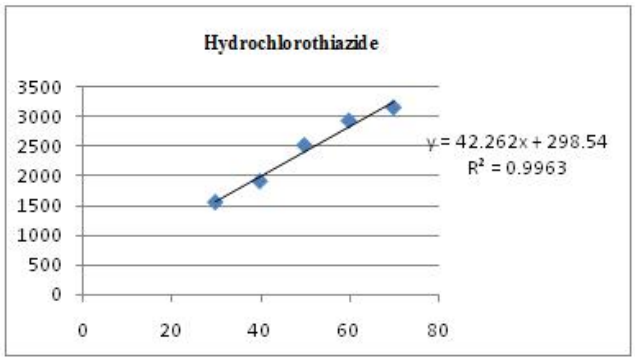


Figure 6: Linearity graph of Hydrochlorothiazide

Table 1: Linearity of Enalapril

S.No.	Conc.(µg/ml)	Area
1	30	695.227
2	40	845.541
3	50	1115.117
4	60	1290.460
5	70	1370.799

Table 2: Linearity of Hydrochlorothiazide

S.No.	Conc.(µg/ml)	Area
1	30	1552.124
2	40	1907.304
3	50	2515.072
4	60	2929.514
5	70	3154.098

Table 3: Recovery results for Enalapril

Recovery level	Accuracy Enalapril				Average % Recovery
	Amount taken (mcg/ml)	Area	Average area	% Recovery	
50%	50	1147.472	1142.193	101.985	101.54
	50	1147.472			
	50	1131.636			
100%	60	1282.181	1287.862	103.48	
	60	1290.460			
	60	1290.945			
150%	70	1391.221	1388.523	99.18	
	70	1373.610			
	70	1400.738			

Table 4: Recovery results for Hydrochlorothiazide

Recovery level	Accuracy Hydrochlorothiazide				Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	%Recovery	
50%	50	2581.774	2573.486	102.06	102.81
	50	2581.774			
	50	2556.911			
100%	60	2933.859	2948.693	105.45	
	60	2936.438			
	60	2975.781			
150%	70	3186.091	3175.224	100.94	
	70	3146.856			
	70	3192.726			

Table 5: Result of Robustness study

Parameter	Enalapril		Hydrochlorothiazide	
	Retention time (min)	**Tailing factor**	**Retention time(min)**	**Tailing factor**
Flow Rate				
0.8 ml/min	3.727	1.558	5.457	1.589
1.2 ml/min	2.127	1.464	3.113	1.421
Wavelength				
213nm	2.707	1.412	3.960	1.500
217nm	2.657	1.382	3.903	1.477

Table 6: Results for Method precision of Enalapril and Hydrochlorothiazide

Enalapril		
S.No.	**Rt**	**Area**
1	2.660	1109.066
2	2.667	1110.202
3	2.680	1113.271
4	2.683	1112.450
5	2.680	1108.599
6	2.690	1109.570
Avg	2.676667	1110.526
Stdev	0.011057	-
%RSD	0.412278	-

Hydrochlorothiazide		
S.No.	**Rt**	**Area**
1	3.890	2518.891
2	3.900	2515.559
3	3.917	2514.373
4	3.903	2512.866
5	3.913	2517.609
6	3.923	2519.468
Avg	3.907667	2516.461
stdev	0.012193	-
%RSD	0.311401	-

Table 7: Results for Ruggedness

Enalapril	%Assay	Hydrochlorothiazide	%Assay
Analyst 01	99.516	**Analyst 01**	100.144
Anaylst 02	99.38	**Anaylst 02**	100.461

Table 8: Results for LOD & LOQ

Drug name	LOD (µg)	LOQ (µg)
Enalapril	0.79	0.98
Hydrochlorothiazide	2.95	3.79

4. Conclusion

A new method was established for simultaneous estimation of Enalapril and Hydrochlorothiazide by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Enalapril and Hydrochlorothiazide by using C18 column (4.6×250mm) 5µ, flow rate was 1ml/min, mobile phase ratio was (55:45 v/v) mixed phosphate buffer: Methanol, detection wavelength was 215nm. Precision and recovery studies were also found to be with the range. The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Enalapril and Hydrochlorothiazidein pharmaceutical dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims. Hence the suggested RP-HPLC method can be used for routine analysis of Enalapril and Hydrochlorothiazide in API and Pharmaceutical dosage form

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