Isocratic RP- HPLC Method for Simultaneous Separation and Estimation of Zofenopril and Hydrochlorthiazide in Pharmaceutical Dosage Forms

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Abstract: A simple, rapid, sensitive and accurate isocratic reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed and subsequently validated for the simultaneous determination of Zofenopril and Hydrochlorthiazide in combined dosage form. Chromatographic separation of the two drugs was performed on a Purospher BDS C18 column (250 mm× 4.6 mm id, 5µm particle size). The mobile phase comprising of acetonitrile methanol: 0.02M NaH₂PO₄ buffer (40:20:40) was delivered at a flow rate of 1.0mL/min. The pH of the mobile phase is adjusted to 7.2 with Sodium hydroxide solution. Detection was performed at 245nm. The separation was completed within 10 min and the retention time of hydrochlorthiazide is 4.62 and Zofenopril is 6.86 min respectively. Calibration curves were linear with R² between 0.99-1.0 over a concentration range of 100-600 µg/ml for Zofenopril calcium and 50-300 µg/ml for hydrochlorthiazide. The developed method was successfully applied to determine Zofenopril and hydrochlorthiazide in pharmaceutical formulations.

Key words: Zofenopril , Hydrochlorthiazide; Validation; Pharmaceutical formulations.

Introduction:

Zofenopril is an antihypertensive drug belonging to the family of a sulphydryl angiotensin converting enzyme (ACE) inhibitors, characterized by high lipophilicity, sustained cardiac ACE inhibition, and antioxidant and tissue protective activities¹⁻³. Zofenopril (ZOF) is chemically (4S)-N-[3-(Benzoylsulfanyl)-2(S)-methylpropionyl]-4-(phenylsulfanyl)-L-proline⁴⁻⁵ (Figure 1.). Hydrochlorthiazide (HCT) is chemically 6-chloro-3,4-dihydro-2 H 1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (Figure 1.) is a thiazide diuretic. Thiazides

affect the renal tubular mechanisms of electrolyte reabsorption, directly increasing excretion of sodium and chloride in approximately equivalent amount. it is official in I.P, B.P, USP⁶⁻⁸. Zofenopril (30mg) when combined with low dose hydrochlorthiazide (12.5 mg) showed an additional 4-8mm Hg anti-hypertensive effect with favorable tolerability in line with that of other combinations of an ACE inhibitor and low dose hydrochlorthiazide⁹. A new combination dosage form of Zofenopril and hydrochlorthiazide is being used in treatment of hypertension¹⁰. Literature survey shows that various analytical methods have been reported for estimation of zofenopril and hydrochlorthiazide individually and combination with other drugs⁹⁻¹⁷. Only one Gradient HPLC¹⁸ method was reported for its simultaneous estimation. Isocratic elution technique is preferred for simple samples (i.e., less than two components) and it is more helpful in gradient elution instrumentation is not available in quality control laboratories. Hence, an attempt has been made to develop new isocratic RP-HPLC method for its simultaneous estimation of Zofenopril and Hydrochlorthiazide in pharmaceutical formulation with good accuracy, precision and simplicity. The developed method is validated as per ICH guidelines¹⁹.

Structure of Zofenopril

Structure of Hydrochlorthiazide

Figure 1. Structure of Zofenopril and Hydrochlorthiazide.

Experimental

Equipment

Analysis was performed on Alliance HPLC System (Water's Corporation, USA) consisting of 2695 separation module and 2998 Photo Diode Array (PDA) detector. Chromatographic data was acquired using waters empower software.

Chemicals

Zofenopril (purity $\geq 98\%$) was purchased from American custom chemicals (San diego, CA, U.S.A). Hydrochlorthiazide was kindly supplied by Aurobindo pharma limited, Hyderabad, India. Acetonitrile and methanol of HPLC grade were procured from Rankem lab ltd. Sodium di hydrogen phosphate and sodium hydroxide were A.R grade purchased from Merck chemicals Mumbai, India. Water HPLC grade was obtained from a Milli-Q RO water purification system. Tablets of Zoprazide® was obtained from a commercial source.

HPLC Conditions

A Purospher BDS C₁₈ (250cm*4.6mm, 5 μ) column was used as the stationary phase. The contents of the mobile phase were acetonitrile, methanol and 0.02M NaH₂PO₄ buffer

(adjusted to pH 7.2 with sodium hydroxide solution) in the ratio of 40:20:40 v/v. They were filtered through 0.45 μ m membrane filter, sonicated for 15 min. and pumped from the respective solvent reservoirs to the column at a flow rate of 1 mL/min. The run time was set at 10 min and column temperature was ambient. Prior to the injection of the drug solution, the column was equilibrated for at least 0.5 h with the mobile phase flowing through the system. The analytes were monitored at 245 nm.

Preparation of standard stock solutions and linearity solutions

Standard stock solutions of ZOF and HCT were prepared separately by dissolving 50 mg of each drug in 50 mL volumetric flask with 10 mL of mobile phase and the solutions were sonicated for about 15 min. Then the volume was made up to the mark with mobile phase to get 1 mg/mL standard stock solution. Several aliquots of these standard stock solutions were taken in different 10 mL volumetric flask and diluted up to the mark with mobile phase such that the final linearity concentrations of zofenopril and hydrochlorthiazide were 100-600 μ g/mL and 50-300 μ g/mL, respectively.

Assay standard solution preparation

A working standard solution containing 300 μ g/mL of ZOF and 125 μ g/mL of HCT were prepared from the above standard stock solution.

Preparation of sample solution

Twenty tablets each containing 30 mg of ZOF and 12.5 mg of HCT were weighed and powdered equivalent to dose, transferred to a 100mL volumetric flask, and extracted with mobile phase. The mixture was sonicated for 20 min in an ultrasonic bath. The volume was adjusted to 100mL with mobile phase to get the concentration of 300 μ g/mL of ZOF and 125 μ g/mL of HCT. The solution was filtered through 0.45 μ nylon filter. The filtrate collected after discarding first few milliliters was injected on the above chromatographic system. All the solutions were protected from light.

Method development and optimization

Some important parameters like pH of the mobile phase, concentration of the acid or buffer solution, percentage and type of organic modifier, etc., were tested for a good chromatographic separation. Trails showed that a neutral mobile phase with reverse phase Purospher BDS column gives symmetric and sharp peaks. For this reason 0.02M sodium di hydrogen ortho phosphate buffer solution was preferred and the neutral pH was maintained by using 0.1N sodium hydroxide solution. Acetonitrile and Methanol both were chosen as the organic modifier because both dissolves drug very well. Mobile phase composition of acetonitrile methanol: 0.02M NaH₂PO₄ buffer (40:20:40 v/v) at flow rate of 1 mL/min showed good resolution. When 0.1N sodium hydroxide was used as modifier, resolution between ZOF and HCT was much better at pH 7.0, with a decrease in peak tailing. Retention times of the drugs obtained under these conditions were 4.62 and 6.86 min for HCT and ZOF, respectively. For the quantitative analytical purpose the wavelength was set at 245 nm. The typical chromatogram of the sample is shown in Figure 2.

Figure 2. A Typical chromatogram of Zofenopril and Hydrochlorthiazide.

Validation of the method

System suitability studies

The column efficiency, resolution, and peak asymmetry were calculated for the standard solutions. The values obtained demonstrated the suitability of the system for the analysis of the drug combination was reported in Table 1.

Table 1. System Suitability parameters.

S.No	Parameters	Zofenopril	Hydroclorthiazide	Acceptance criteria
1	Retention time	6.86	4.62	
2	Asymmetry of the peak	0.71	0.62	Not more than 1.5
3	RSD of replicate injections	0.185	0.346	Not more than 2%
4	Theoretical plates	4105	3548	Not less than 2000
5	Resolution	3.8		More than 2

Solution stability studies

In order to demonstrate the stability of both the standard and sample solutions during analysis, both solutions were analyzed over a period of 24 h at room temperature. The results indicated for both the solutions, the retention time and peak area of ZOF and HCT did not show much variation (%RSD less than 2). There was no significant degradation with in the indicated period. Hence it was concluded that both the solutions were stable for 24 h at room temperature.

Specificity

The specificity of the method was checked for the interference of impurities in the analysis of a blank solution (without any sample) and then a drug solution of 20 µg/mL was injected

into the column, under optimized chromatographic conditions, to demonstrate the separation of both ZOF and HCT from any of the impurities, if present. As there was no interference of impurities and also no change in the retention time, the method was found to be specific and also confirmed with the results of analysis of formulation.

Linearity study

The peak areas of ZOF and HCT were linear with respect to the concentration over the range of 100-600 µg/mL and 50-300 µg/mL, respectively. The slope and intercept value for calibration curve $Y=10166x+16891$ ($r = 0.999$) for ZOF and $Y=10498x+39334$ ($r = 0.999$) for HCT.

The results showed that an excellent correlation exists between peak area and concentration of the drugs within the concentration range indicated previously. The data was analyzed by "linear regression least squares fit," and the parameters are listed in Table 2.

Table 2. Linearity Study.

S.NO	Parameters	Zofenopril	Hydrochlorthiazide
1	Linearity range	100-600 µg/mL	50-300 µg/mL
2	Slope	10166	10498
3	Intercept	16891	39334
4	Correlation coefficient(r^2)	0.999	0.998
5	Limit of Detection	0.51 µg/mL	0.22 µg/mL
6	Limit of Quantification	5µg/mL	2µg/mL

Precision

Intraday and Interday precision were evaluated by determining the corresponding responses three times on the same day and on 3 different days for ZOF(200,300,400 µg/mL) and for HCT(100,150,200 µg/mL). The results were reported in terms of RSD and shown in Table 3.

Table 3. Intraday and interday precision data for the quantitative determination of Zofenopril and Hydrochlorthiazide.

Concentration µg/mL	Intraday precision		Interday precision	
	Calculated concentration±SD*	RSD%	Calculated concentration±SD*	RSD%
Zofenopril				
200	199.68±0.422	0.57	200.14±0.091	0.13
300	299.10±0.517	0.21	300.45±0.143	0.14
400	401.01±0.109	0.79	400.96±0.264	0.58
Hydrochlorthiazide				
100	100.28±0.347	0.18	99.24±0.156	0.63
150	149.59±0.169	0.59	150.24±0.106	0.13
200	200.19±0.365	0.47	199.58±0.654	0.34

* Average of six determinations.

Accuracy

To study the reliability, suitability and accuracy of the method recovery studies were carried out. A known quantity of the pure drug was added to the pre-analyzed sample formulation at the level of 50%, 100% and 150% and further dilutions were made and the concentration of the drugs were determined from calibration curve. Recovery studies were carried out six times and the percentage recovery was calculated and presented in Table 4. The lower value of %RSD indicates the method is accurate. The mean recoveries were in the range of 99.15-100.96%, which shows that there is no interference with excipients. The percent recoveries were calculated by using the following equation.

$$\text{Percentage Recovery} = \frac{b - a}{c} \times 100$$

where,

- a- The amount of drug found before the addition of standard drug.
- b- The amount of drug found after the addition of the standard drug.
- c- The amount of standard drug added.

Limit of detection

The limit of detection (LOD) is the smallest concentration that can be detected but not necessarily quantified as an exact value. It was calculated as $3.3 \sigma/S$ as per ICH guidelines. The LOD of ZOF and HCT was found to be 0.51 µg/mL and 0.22 µg/mL respectively.

Limit of quantitation

The limit of quantitation (LOQ) is the lowest amount of analyte in the sample that can be determined with suitable precision and accuracy. It was calculated as $10 \sigma/S$ as per ICH guidelines. The LOQ of ZOF and HCT was found to be 5 µg/mL and 2 µg/mL, respectively.

Table 4. Recovery studies for spiked concentration of Zofenopril and hydrochlorthiazide.

Name of the Drug	Level of % Recovery	Amount of standard		% Recovery
		Added µg/mL*	Recovered µg/mL*	
Zofenopril	50	150	149.96	99.97
	100	300	300.19	100.06
	150	450	449.12	99.80
Hydrochlorthiazide	50	75	75.19	100.25
	100	150	150.34	100.22
	150	225	226.3	100.57

*Average of six determinations.

Ruggedness and Robustness

The ruggedness of the method was determined by carrying out the experiment on different instrument like Waters HPLC and Shimadzu HPLC by different operators using different

columns of similar type like Phenomenex C₁₈, Hypersil C₁₈. Robustness of the method was determined by making slight changes in the experimental conditions such as the composition of the mobile phase, pH of the mobile phase, and flow rate of the mobile phase and the chromatographic characteristics were evaluated.

Tablet studies

The proposed method was successfully applied to the analysis of marketed products (Zoprazide®) and the results obtained are given in Table 5.

Table 5. Analysis of formulation.

Drug	Labeled amount(mg)	Amount of mg/tab found*	%Label claim	%RSD
Zofenopril	30	29.8	99.33	0.474
Hydrochlorthiazide	12.5	12.46	99.68	0.253

* Average of six determinations.

Results and Discussion

The proposed method gave good resolution between ZOF and HCT with in short period of time (≤ 10 min). The different mobile phases at various pH and different flow rates were examined and mobile phase with acetonitrile: methanol:0.02M NaH₂PO₄ buffer (40:20:40v/v) was selected as optimal mobile phase based on system suitability parameters with well resolved peaks of ZOF and HCT resolution of the peaks more than 2. In addition high column efficiency was indicated from the large number of theoretical plates (>3000). The degree of asymmetry was also evaluated using the tailing factor which did not exceed the critical value (1.5) indicating acceptable degree of peak asymmetry. The wavelength for the detection of both compounds was 245 nm with best detector response. Linearity range was observed in concentration of 100-600 $\mu\text{g/mL}$ for ZOF and 50-300 $\mu\text{g/mL}$ for HCT, respectively. Solution stability studies showed that the active pharmaceutical ingredients remain stable for 24 h at room temperature. When a known amount of pure drug solutions were added to the powder sample of tablet dosage form at three different levels and subjected to estimation of the drugs by the proposed method, there was a high recovery of ZOF and HCT, indicating that the proposed method is highly accurate. The intra-day and inter-day variations of the method was studied on the same day and on three different days and observed low %RSD less than 1. This shows that the present HPLC method is highly precise. The tablets were found to contain 99.92 to 100.07% of ZOF and 99.92 to 99.98% of HCT. The limit of detection and limit of quantification for ZOF was found to be 0.51 and 5 $\mu\text{g/mL}$ respectively and for HCT, 0.22 and 2 $\mu\text{g/mL}$ respectively, which suggests that nanogram quantity of drugs, can be estimated accurately. The changes in flow rate, pH of mobile phase, composition of mobile phase did not affect the percentage assay of drug confirming the robustness of the method. Ruggedness of the method was confirmed as no significant changes were observed on analysis using different instrument and different analyst. The proposed method was found to

be Specific as there is no interference of impurities and Peak purity values for peaks of both ZOF and HCT confirmed the specificity.

Conclusion

The RP-HPLC method developed for simultaneous analysis of ZOF and HCT can be used for routine quality control of their bulk drug mixture and their combined dosage form.

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