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# Development and validation of HPLC method to quantify zofenopril in tablet

**Wafa Bouaissi[1, 2,*] and Najib Ben Hamida[1]**

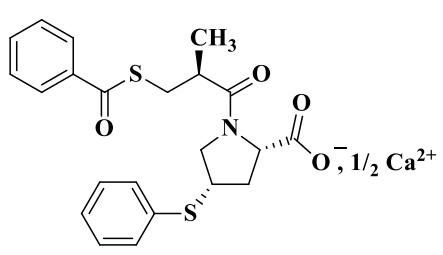
[1] Laboratory of Analytical Chemistry and Electrochemistry, Department of Chemistry, Faculty of science of Tunis, Campus university of El Manar, 2092 Tunis, Tunisia.
[2] Laboratoire National de Contrôle des Médicaments et de Dépistage du Dopage, 11 bis Rue Djebel Lakhdar, 1006 Bab Saâdoun, Tunis-Tunisie

**Abstract**: An accurate, simple, reproducible and sensitive liquid chromatography method was developed and validated for the zofenopril calcium determination in tablet according to the International Conference on Harmonization (ICH) guidelines. The analyses were performed at ambient temperature on a reversed-phase Prontosil LC18 column (250 mm × 4.6 mm I.D. grain size of 5 µm). The detection of the dosage form was carried out at 205 nm. The mobile phase was composed of acetonitrile: phosphate buffer (pH 2.5, 0.02 M) (80/20, v/v), and it was eluted isocratically at a 2.0 mL $min^{-1}$ flow rate. The retention time for zofenopril was found to be 4.27 min. The method was validated in terms of specificity, linearity, quantification limit, detection limit, accuracy and precision. The response was linear in the range of 90 - 210 µg $mL^{-1}$. The correlation coefficients ($R^2$) regression are greater than 0.995. The relative standard deviation values for inter-and intra-day precision are less than 1%. Recoveries ranged between 99.34 and 100.21 %. The stressed samples were analyzed and this proposed method was found to be specific and stable since no interfering peaks of degradation compounds and excipients were noticed. The method was successfully applied for the determination of zofenopril calcium in the pharmaceutical formulation.

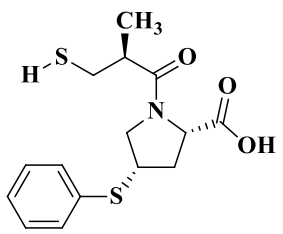
**Keywords**: Liquid chromatography; zofenopril calcium; ICH; zofenil.

## Introduction

Zofenopril Calcium, [1(S), 4(S)]-1(3-mercapto-2 methyl-1-oxopropyl)4-phenyl-thio-Lproline-S-benzoylester , is a pro-drug designed to undergo metabolic hydrolysis and yield the active free sulfhydryl compound zofenoprilat (Fig. 1), an angiotensin converting enzyme (ACE) inhibitor[1,2].It is a highly lipophilic ACE inhibitor [3], characterized by long-lasting tissue penetration and sustained cardiac ACE inhibition[4]. This characteristic confers this drug ancillary antioxidant and cardioprotective properties, including the ability to improve endothelial function in animals and humans, making it a potentially useful tool for the treatment of both hypertension and myocardial infarction[5–7]. Zofenopril has been successful and safely used in the treatment of acute myocardial infarction[8–10], heart failure[11,12], and essential hypertension[13–19]. Zofenopril calcium is a chemically stable, white crystalline powder, with a melting point higher than 250 °C and a molecular weight of 448.59 [3]. The water solubility of zofenopril is 0.3 mg $mL^{-1}$ and the pH of the saturated solution is 6.7. It is slightly soluble in dimethyl formamide and methanol and practically insoluble in isopropanol, butanol, acetone, acetonitrile, and ethyl acetate.




Zofenopril calcium




Zofenoprilat

**Figure 1.** Chemical structure of zofenopril calcium and zofenoprilat

**Corresponding author: Wafa Bouaissi*
*E-mail address*: bouaissiwafa@gmail.com
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The literature survey reveals that several workers developed different methods to determine the zofenopril alone or in combination with some diuretics, in human plasma, in bulk and in pharmaceutical dosage forms, which include liquid chromatography (LC) [20], UV–visible spectrophotometry[21], gas chromatography–mass spectrometry (GC–MS) [22], and liquid chromatography–mass spectrometry (LC–MS) [23–26]. Various analytical methods have been reported for estimation of zofenopril and hydrochlorthiazie in a combined dosage form. A method was carried out on a C18 column using methanol/water (pH 2.5 with $H_3PO_4$) as the mobile phase delivered in a gradient mode [27] and isocratic method was carried out on a C18 column using the mobile phase comprising acetonitrile/methanol/ $NaH_2PO_4$ buffer (0.02 M pH 7.2) (40:20:40) [28]. The aim of the present work was to develop and validate a simple stability-indicating LC method for the determination of zofenopril calcium in pure form and present in formulation according to ICH guidelines [29]. The existing methods are costly, but the developed method is detailed and the time for the analysis is minimized. The forced degradation study of zofenopril calcium in acid, base and peroxide oxidation indicated the specificity of the developed method in the presence of degradation products. Hence, the method is well suitable for the estimation of the commercial formulation of zofenopril calcium.

## Experimental Section

### Instrumentation

The LC analysis was performed on an LC G1600 (Varian) equipped with Prostar 240 pump, a photodiode array detector (ProStar 330) and a Prostar 410 Auto sampler. The sample injection was performed via a Rheodyne 7725i valve with a 20 µL loop. The detector was set at 205 nm and peak areas were integrated automatically by HP computer using a galaxy demo Workstation software. The experiments were carried out on a reversed-phase Prontosil LC 18 column (250 mm × 4.6 mm I.D., with a particle size of 5 µm). For the constant chromatographic column temperature control, a column oven (Prostar 410, Varian) was incorporated into the system. The pH of mobile phase buffers was adjusted by means of a model Metrohm 744 pH meter. Solutions and mobile phase were freshly prepared at the time of use, filtered through 0.45 µm membrane filter and degassed using a sonicator.

### Reagents and chemicals

Zofenopril (purity > 99.5 %, HPLC) and pharmacopoeia grades of the excipients were purchased from the National Laboratory for Drug Control and Screening for Doping (Tunisia). Zofenil 30 mg was procured from France pharmacy. Water and acetonitrile were of HPLC grade, from Labscan. Potassium dihydrogen phosphate (Fluka), sodium hydroxide (Acros Organics) and orthophosphoric acid (85 %) (Panreac) were used in the preparation of phosphate buffer. Stock solutions of zofenopril and sample solution are prepared in the mobile phase. Fresh working solutions are prepared daily. All solutions are filtered through 0.45 µm membrane filter and degassed using a sonicator.

### Preparation of solutions:

*Preparation of phosphate buffer 20 mM (pH 2.5)*

About 272.18 mg of potassium dihydrogen phosphate $KH_2PO_4$ was accurately weighed, transferred into 200 mL volumetric flask and dissolved in HPLC grade water and adjusting the pH =2.5 by adding a few milliliters of orthophosphoric acid, filtered through 0.45µm filter and degassed by sonication.

*Preparation of mobile phase*

Mobile phase was prepared by mixing accurately measured volumes of 800 mL acetonitrile and 200 mL of phosphate buffer in a 1000 mL bottle, filtered, sonicated and used for the analysis.

*Preparation of standard drug solution*

Stock standard solution of zofenopril was prepared by dissolving appropriate amounts in water to obtain final drug concentrations of 300 µg $mL^{-1}$. For the calibration standards, five Calibrators of each drug were prepared by diluting the stock solution with the same mobile phase to obtain a range from 60 % to 140 % of the test concentration.

*Placebo preparation*

The placebo solution was prepared by mixing the excipients: lactose, hypromellose, cellulose microcrystalline, starch, macrogol 400, macrogol 6000, colloidal silica and magnesium stearate in the mobile phase.

*Preparation of the reconstituted form solution*

The pharmaceutical form reconstituted of concentration 150 µg $mL^{-1}$ was prepared by mixing the active compound and the placebo in the mobile phase.

*Preparation of test solution*

Twenty tablets of zofenopril are powdered and mixed thoroughly. An amount of the powder equivalent to 30 mg of the drug is dissolved in water by sonication, and filtered through 0.45 µm filter. The filtrate is diluted to 100 mL with the mobile phase. The resulting solution is again sonicated and filtered through 0.45 µm filter and used for the analysis.

### Forced degradation studies

#### *Hydrolysis acidic and alkaline*

A solution of zofenopril 200 µg $mL^{-1}$ was prepared in water and diluted with 0.1 N HCl and 0.1 N NaOH to volume. The samples were kept on a hot plate at 80 ºC for basic hydrolysis for 5 min, and at 80 ºC for acid hydrolysis for 1 h.

***Oxidative degradation study***

Sample solution was treated with a solution of 3% $H_2O_2$ at 80 °C for 4 min**.**

**Results and Discussion**

**Study of the Optimum Conditions**

The various parameters affecting the retention time of zofenopril have been studied and optimum conditions have been selected. The effect of the flow-rate, column temperature, pH, organic modifier, and counter-ion on the retention time of zofenopril were investigated by HPLC.

***Detection wavelength***

The UV spectrum of zofenopril in water (200 µg $mL^{-1}$) was registered in the wavelength range from 200 to 400 nm as shown in Fig. 2.

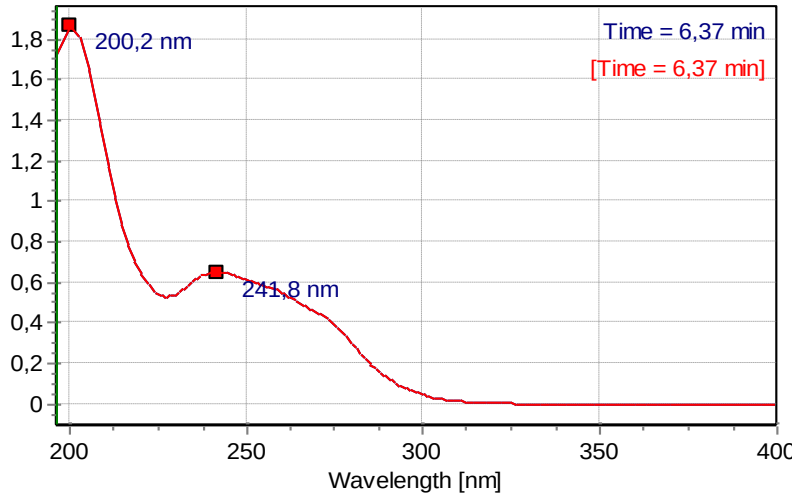


**Figure2.** UV Spectrum of zofenopril 200 µg $mL^{-1}$

***Effect of mobile phase composition***

Chromatograms of zofenopril obtained at five mobile phase compositions (40/60; 50/50; 60/40; 70/30 and 80/20, v/v) consisting of a mixture of acetonitrile and phosphate buffer (pH 2.5; 20 mM) at 25°C with a flow rate of 2 mL $min^{-1}$ are shown in Fig.3.

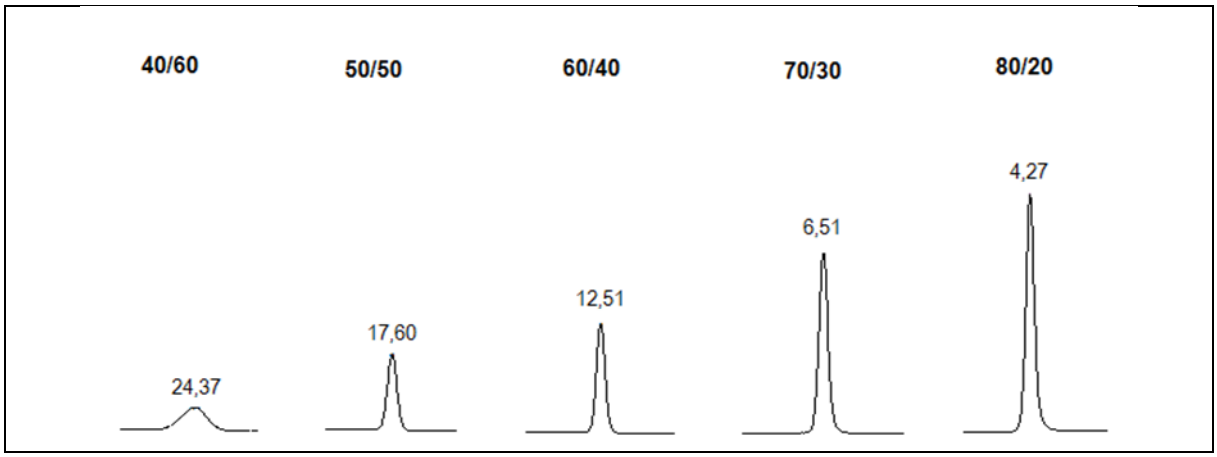


**Figure 3.** Effect of mobile phase composition on the retention time of zofenopril; mobile phase: acetonitrile-buffer (pH 2.5; 20 mM); stationary phase: Prontosil LC 18, 5 µm (250 x 4.6 mm ID); flow rate: 2 mL $min^{-1}$; T=25 °C and λ=205 nm

To investigate the effect of the amount of the organic modifier, various concentrations of acetonitrile modifier in aqueous phosphate buffer pH 2.5 were tested at ambient temperature and a flow rate of 2 mL $min^{-1}$. The amounts of the organic modifier were adjusted in order to obtain a comparable retention time. Their influence on the retention time of zofenopril can be seen in Fig. 3.

At lower concentration (40 %) of acetonitrile, the elution of zofenopril was obtained at higher retention time 24.37 min with a lower intensity of the peak. On the other hand, as could be expected in reverse-phase systems, an increase in the organic modifier concentration resulted in a decrease in retention time. The decrease in polarity of the mobile phase (as the acetonitrile content increases), results in the

preferential partitioning of the drug in the mobile phase as compared to a mobile phase with lower amounts of organic modifier, favoring the rapid elution of the molecule. Moreover, the good separation of zofenopril was obtained in 80 % of acetonitrile with at short retention time of 4.27 min. Thus, a mobile phase consisting of 20 mM phosphate buffer, pH 2.5–acetonitrile (20/80, v/v) at a flow-rate of 2 mL $min^{-1}$ and at ambient temperature was applied for the elution of zofenopril.

***Flow rate***

At flow rates of 0.5, 1, 1.5 and 2 mL $min^{-1}$, the effect on the time scale of HPLC elution in the column at the retention time of zofenopril was investigated at 25 °C (Fig. 4). The mobile phase was a mixture of 20 mM phosphate buffer pH 2.5- acetonitrile (20/80, v/v). Fig. 4 shows that higher flow rate yielded a rapid elution of zofenopril ($t_R$ = 4.27 min) but a lower flow rate of 0.5 mL $min^{-1}$ and the retention time was 11.73 min.

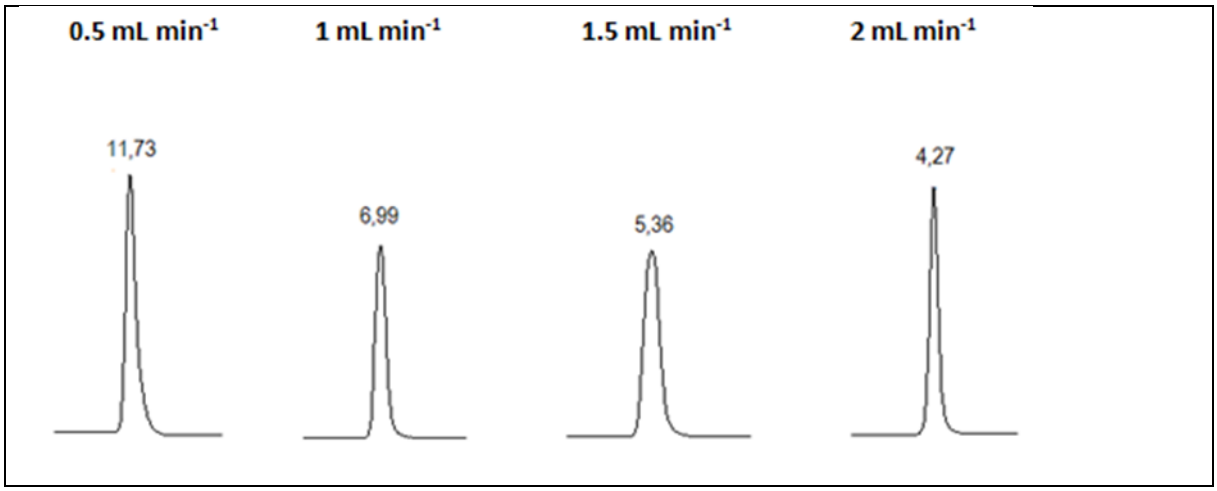


**Figure 4.** Effect of flow rate on the retention of zofenopril; mobile phase: phosphate buffer, pH 2.5-/acetontrile (20/80, v/v); T= 25 °C; stationary phase: Prontosil LC 18, 5 µm (250×4.6 mm I.D.) and λ=205 nm

***Effect of pH***

As was mentioned earlier, the pH of the mobile phase influences both peak shape and retention time of prolinecontaining substances. The chromatograms were obtained with a mobile phase mixture of 20 mM phosphate buffer pH in the range of 2.5-/6 and acetonitrile (20/80, v/v) at 25 °C and a flow rate of 2 mL $min^{-1}$.

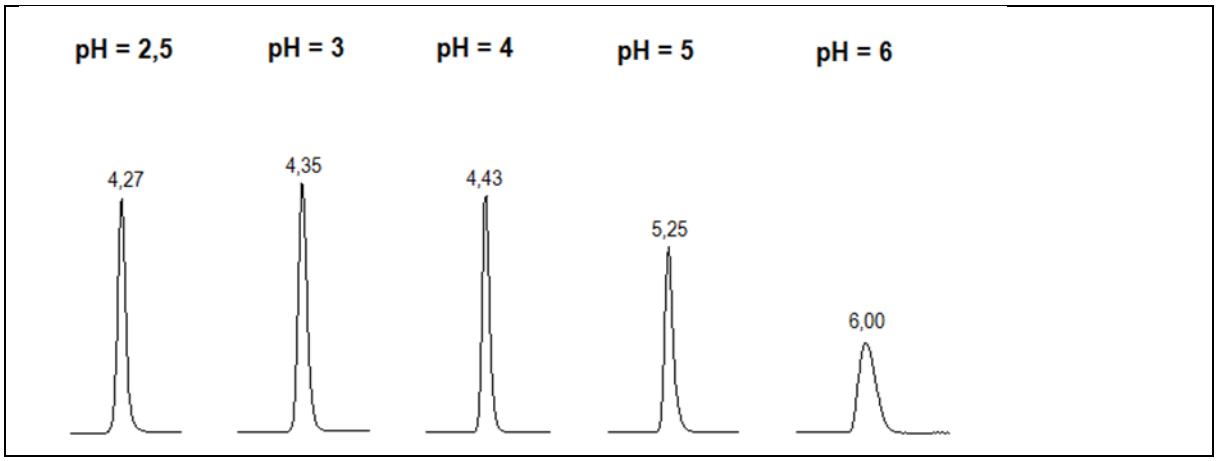


**Figure 5.** Effect of pH on the peak shape and retention time of zofenopril; mobile phase: phosphate buffer-acetontrile (20/80, v/v); flow rate: 2.0 mL $min^{-1}$; T= 25 °C; stationary phase: Prontosil LC 18, 5 µm (250 × 4.6 mm I.D.) and λ=205 nm

At low pH, a rapid elution of zofenopril was observed. On the other hand, it clearly appears that an increase in the pH of the mobile phase increases the retention of zofenopril.

The curve $t_R$ = f (pH) in Fig.6 shows that the change in pH affects the retention time of zofenopril. The variation of retention, according to pH is linear.

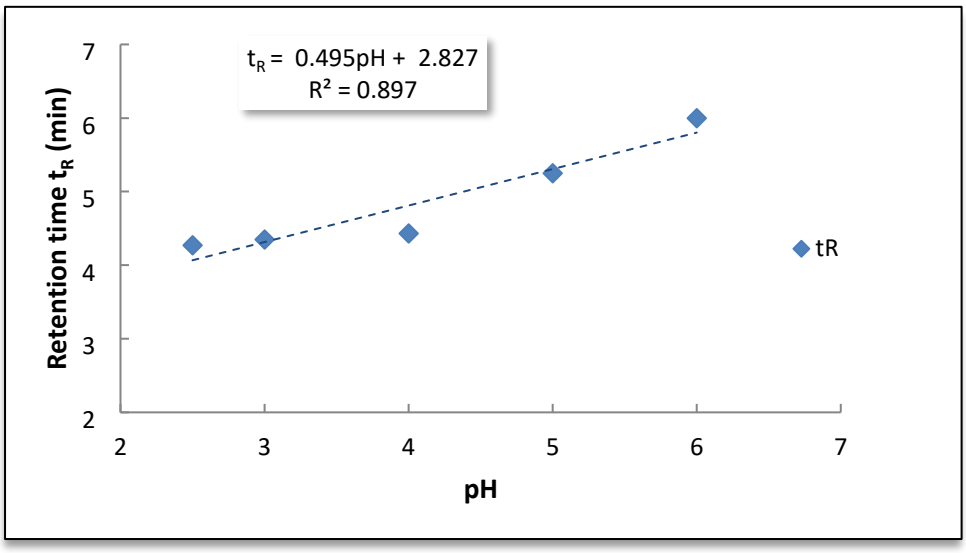


**Figure 6.** Influence of pH on the retention time of zofenopril

***Column temperature***

The chromatographic behavior of zofenopril obtained at several column temperatures with a mobile phase consisting of a mixture of 20 mM phosphate buffer pH 2.5-acetonitrile (20/80, v/v) at a flow rate of 2 mL $min^{-1}$ is shown in Fig.7. Elevated temperature led to decrease the retention time and produced a rapid elution of zofenopril.

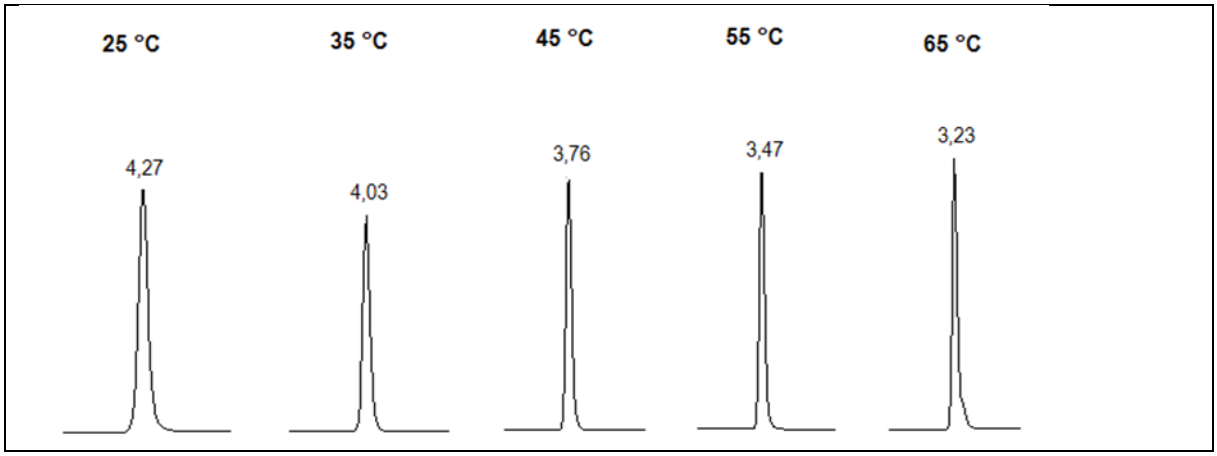


**Figure 7.** Effect of column temperature on the retention time of zofenopril; mobile phase: phosphate buffer, pH 2.5-acetontrile (20/80, v/v); flow rate: 2.0 mL $min^{-1}$; stationary phase: Prontosil LC 18, 5 µm (250 × 4.6 mm I.D.) and λ=205 nm

**Chromatographic Conditions**

The chromatographic analysis was performed at room temperature with isocratic elution. The mobile phase consisted of phosphate buffer (20 mM, pH 2.5) and acetontrile (20/80, v/v). The pump was set at a flow rate of 2.0 mL $min^{-1}$, sample volume of 20 µL was injected onto the HPLC column and elute was monitored at 205 nm.

**Method validation**

The developed method was validated according to International Conference on Harmonization (ICH) guidelines for validation of analytical procedures[29, 30]. Different standard solutions and the reconstituted form solutions were prepared by diluting standard stock solution with mobile phase in the concentration range 90-210 µg $mL^{-1}$. Diluted samples were injected and chromatograms were taken under standard chromatographic conditions. The peak area was plotted against corresponding concentrations to obtain the calibration graph. The specificity of an analytical method may be defined as the ability to determine the analyte in the presence of additional components such as impurities, degradation products, and matrix compounds. The interference of the excipients of the pharmaceutical formulation was determined by the injection of a sample containing only placebo (a mixture of all the tablet excipients) and a sample

containing placebo with zofenopril at the concentration of 0.2 mg $mL^{-1}$. The stability-indicating capability of the method was determined by subjecting the reference standard solution (0.2 mg $mL^{-1}$ of zofenopril calcium) to accelerated degradation conditions such as: acidic, basic and oxidative to evaluate the interference in the quantification of zofenopril. Precision of the analytical method was expressed in the relative standard deviation (RSD) of a series of measurements. The intra-day and inter-day precisions of the proposed method were determined by estimating the corresponding responses (three concentrations/three replicates each) of the sample solution on the same day and on three different days respectively. The signal-to-noise ratio (*S/N*) method was adopted for the determination of limits of detection and limit of quantification. The limit of detection was estimated at three times the *S/N* ratio and the limit of quantification was estimated as ten times the *S/N* ratio. To evaluate the accuracy of the method, recovery test was performed by adding known amounts of standard of zofenopril in the level of 60, 100 and 140 % of zofenopril in the tablets (three replicates of each level) to common tablet excipients (lactose, hypromellose, cellulose microcrystalline, starch, macrogol 400, macrogol 6000, colloidal silica and magnesium stearate). The accuracy of the assay was determined by comparing the found concentration with the injected concentration.

**Linearity**

Five concentrations of the standard solution and five concentrations of the reconstituted form solution in 90 to 210 µg $mL^{-1}$ ranges were analyzed by HPLC. Calibration curves were constructed by plotting average peak areas versus concentrations. Linearity was determined by the regression equations for the method. This experiment was repeated five times for both solutions. The detector responses are found to be linear (Fig.8).

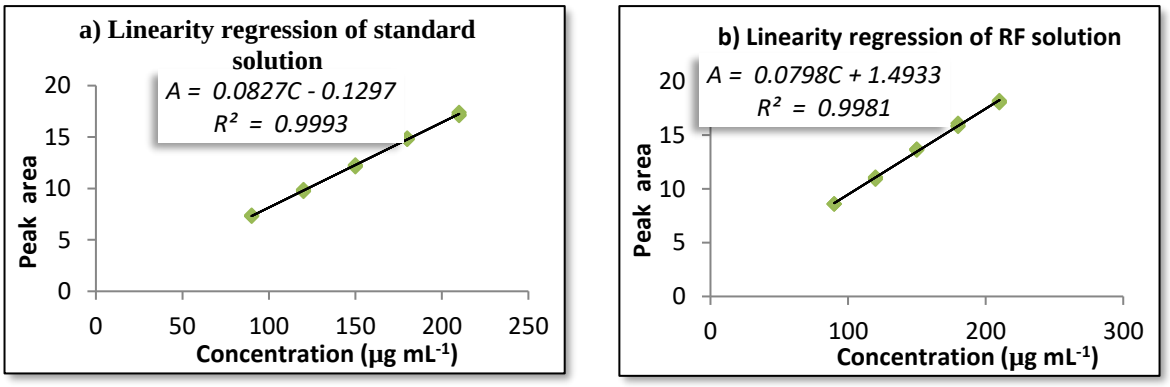


**Figure 8**. Calibration Curve of zofenopril in standard solution (a) and in reconstituted form solution (b)

The slope, intercept of the straight line and regression equations are summarized in Table.1. The correlation coefficients between the concentration of the drug and detector response are found to be higher than 0.995.

**Table 1**. Linearity parameters of zofenopril by HPLC.

| | Zofenopril in standard solution | Zofenopril in reconstituted form solution |
|---|---|---|
| Slopes | 0.0827 | 0.0798 |
| Intercepts | -0.1297 | 1.4933 |
| Regression equations | A = 0.0827C-0.1297 | A = 0.0798C+1.4933 |
| Correlation coefficients | 0.9993 | 0.9981 |

This linearity should be verified by the following statistical tests: Cochran test and Fisher test[31]. The Cochran test estimates the variance in the group divided by the sum of variances of the entire group. If $C_{calculated} < C_{theoretical}$ the test is true, the variance is homogeneous. The Fisher test is mainly used for variance comparison. The ratio of two variances is compared with the F theoretical value. If $F_{Calculated} < F_{Theoretical}$ the difference is not significant, the two variances are coherent. This test is performed to control the least square regression (linearity): the slope must be significantly different from 0 ($F_{Calculated} > F_{theoretical}$) and the linearity adjustments must be non-significant ($F_{Calculated} < F_{Theoretical}$).

The statistical evaluation of the linearity study is presented in table.2. The obtained statistical parameters demonstrated that the method had a good linearity over the considered concentration range.

**Table 2**. Statistical parameters of the linearity of zofenopril.

| **Statistical tests (p = 0.05%)** | **Zofenopril in standard solution** | **Zofenopril in reconstituted form solution** | **Theoretical values** |
|---|---|---|---|
| **Cochran Test –** Homogeneity of variance ($C_{Calc}$) | 0.463 | 0.462 | $C_{th}$ (0.05; 5; 2) = 0.684 |
| **Fisher Test -** Significant slope ($F_{1Calc}$) | 17582.897 | 1828.019 | $F_{1th}$ (0.05; 1; 13) = 4.67 |
| **Fisher Test -** Validity of regression ($F_{2\,Calc}$) | 0.107 | 0.041 | $F_{2th}$ (0.05; 3; 10) = 3.71 |

**Specificity**

The selectivity of the method was confirmed by observing potential interferences caused by excipients of tablet formulations and degradation products under stress conditions as indicated by ICH[29].

The comparison of chromatograms obtained from the standard solution, the sample solution (the reconstituted dosage form of tablets) and a placebo solution (prepared from the excipients) (Fig.9) shows that there was no interference of peaks to the determination of zofenopril. The retention time of zofenopril was confirmed by comparing the retention time with that of the standard.

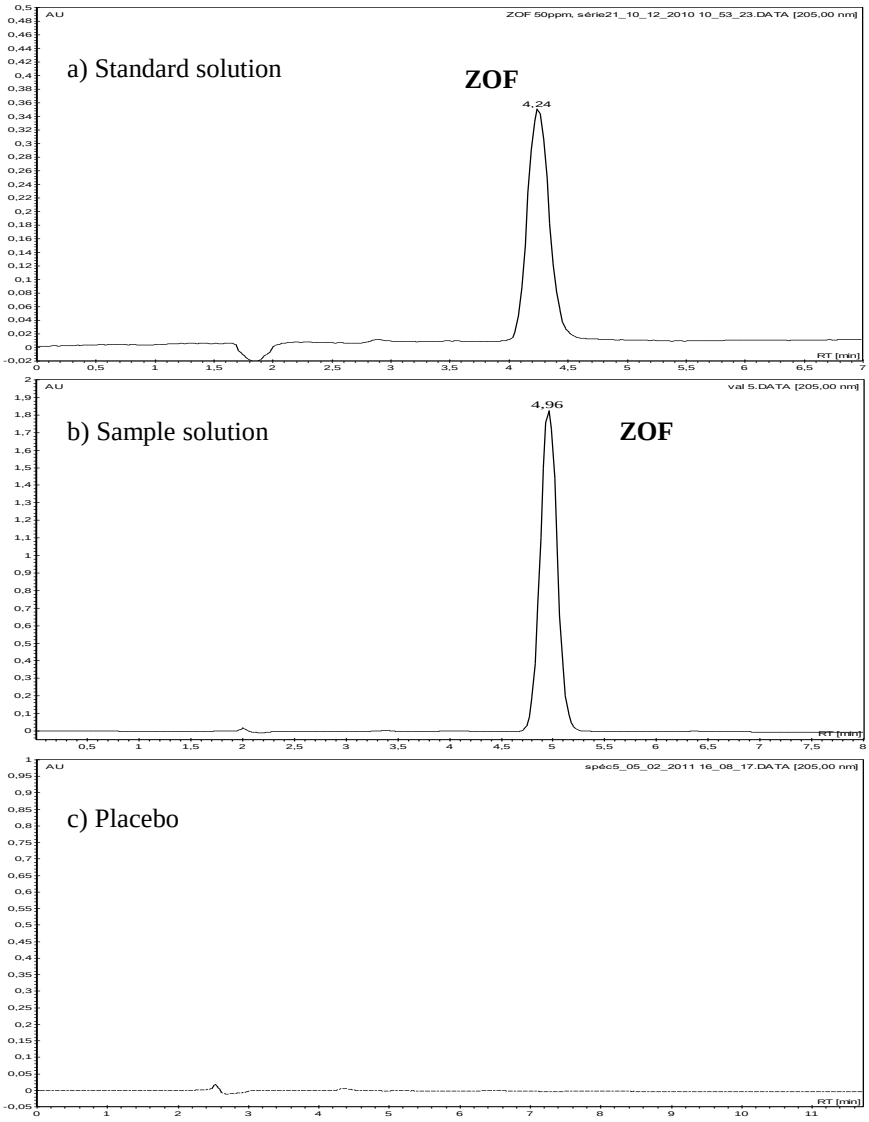


**Figure 9**. Typical HPLC chromatograms of (a) standard solution; b) sample solution; c) placebo. Chromatographic conditions: mobile phase: acetonitrile/buffer (20 mM; pH 2.5) (80/20; v/v); stationary phase Prontosil LC 18, 5 µm (250 x 4.6 mm I.D.); flow rate: 2 mL $min^{-1}$; T=25 °C and λ=205 nm

Specificity was also studied by performing the forced degradation study using acid and alkaline hydrolysis and chemical oxidation at 80 °C (Fig.10).

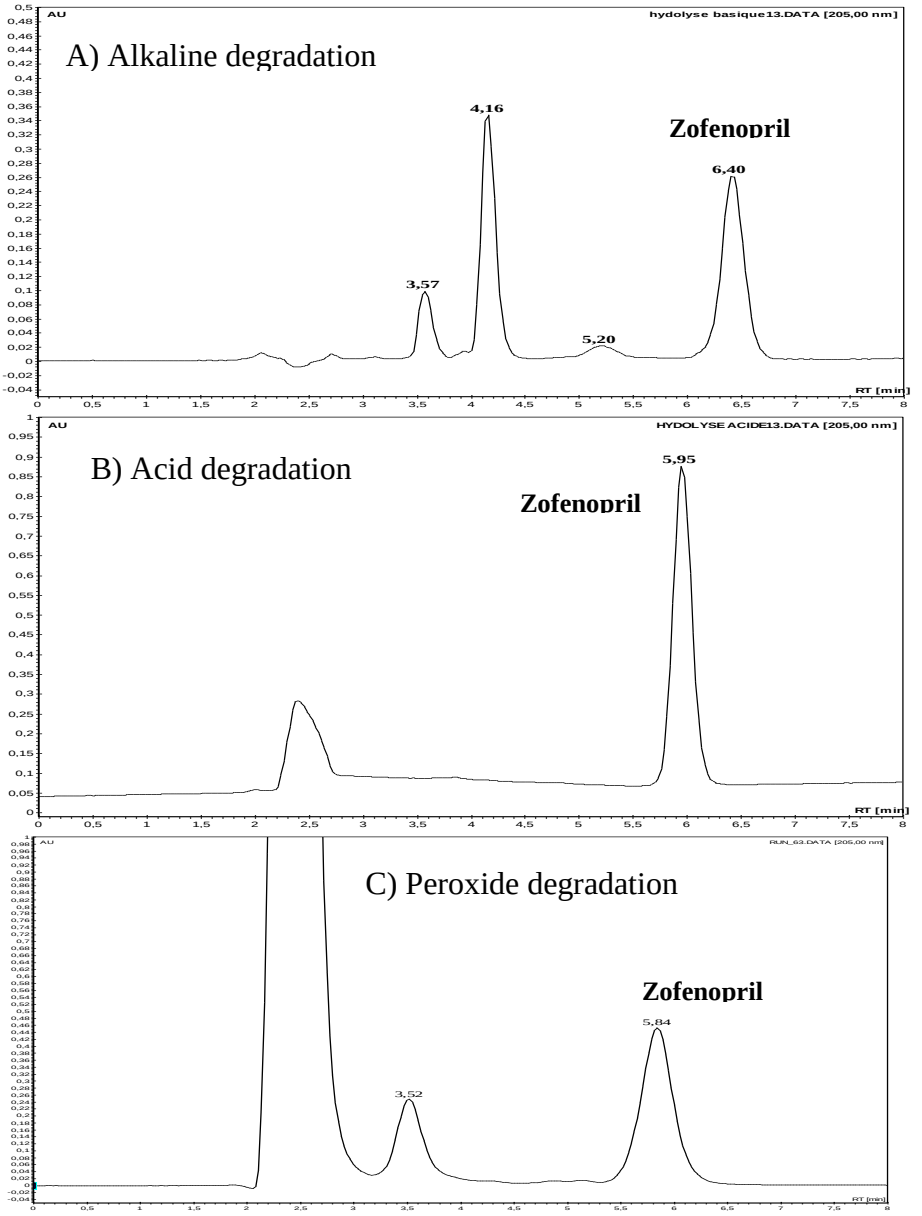


**Figure 10**. Chromatograms obtained from zofenopril (A) after alkaline degradation (0.1N NaOH for 5 min at 80 °C); (B) after acid degradation (0.1N HCl for 1h at 80 °C) and (C) after peroxide degradation (3 % hydrogen peroxide for 4 min at 80 °C). Chromatographic conditions: mobile phase: acetonitrile/buffer (20 mM; pH 2.5) (80/20; v/v); stationary phase: Prontosil LC 18, 5 µm (250 x 4.6 mm I.D.); flow rate: 2 mL $min^{-1}$; T=25 °C and λ=205 nm

During the study it was observed that upon treatment of zofenopril with acid (0.1 N HCl), base (0.1 N NaOH), and hydrogen peroxide (3 %) the degradation was observed in the base and with hydrogen peroxide, whereas no degradation was observed in acid. Further, it is important to note that from the degradation chromatograms (Fig.10) that no peak interfered with zofenopril. Hence, these results proved the good selectivity of the proposed method.

**Accuracy**

To evaluate the accuracy of the method, recovery test was performed by adding known amounts of standard of zofenopril in the level of 60, 100 and 140 % of zofenopril levels in the tablets (three replicates of each level) to common tablet excipients. The accuracy of the assay was determined by comparing the found amount with the added amount. The results obtained are shown in Table.3. The obtained values confirm the accuracy of the proposed method.

**Table 3**. Recovery percentage of zofenopril determined during method validation.

| Level (%) | Injected Conc. **(**µg mL$^{-1}$**)** | Found Conc. (µg mL$^{-1}$) [a)] | (%) Recovery [b)] |
|---|---|---|---|
| 60 | 90 | 90.19 ± 0.50 | 100.21 |
| 100 | 150 | 149.01 ± 1.06 | 99.34 |
| 140 | 210 | 209.83 ± 0.55 | 99.92 |

a) Mean ± SD (n= 3).
b) (Found concentration/ Injected concentration) x 100.

**Precision**

The intra-day precision was determined by analysis of three different preparations in concentrations of 90.0, 150.0 and 210.0 µg mL$^{-1}$ on the same day. The inter-day precision was studied by comparing the assay on three different days. The results are shown in Table.4. The obtained R.S.D. (%) values, lower than 1.0 %, attested the precision of the method.

**Table 4**. Precision determined during method validation.

| Concentration (µg mL$^{-1}$) | | Relative standard deviation (%) | |
|---|---|---|---|
| | | **Intra-day [a)]** | **Inter-day [b)]** |
| 90 | | 0.82 | 0.96 |
| 150 | | 0.47 | 0.59 |
| 210 | | 0.35 | 0.53 |

a) Analyzed on the same day (n=3).
b) Analyzed on three different days (n=9).

**Detection and quantification limits**

The detection limit DL and quantification limit QL were calculated by using the equations: DL = 3.3 x s/S and QL = 10 x s/S, where s is the standard deviation of the response and S is the slope of the calibration curve. DL was 0.38 µg mL$^{-1}$ and QL was 1.28 µg mL$^{-1}$. These values are adequate for the determination of zofenopril in pharmaceutical samples.

**Method application**

The proposed, developed and validated method was successfully applied to the analysis of zofenopril in their marketed formulation (zofenil 30 mg) (Fig.11). There was no interference of excipients commonly found in tablets as described in specificity studies. The percentage of zofenopril was found to be 104.3 ± 0.27 % (Mean ± SD, n = 3) with the RSD = 0.25 % < 2 %.

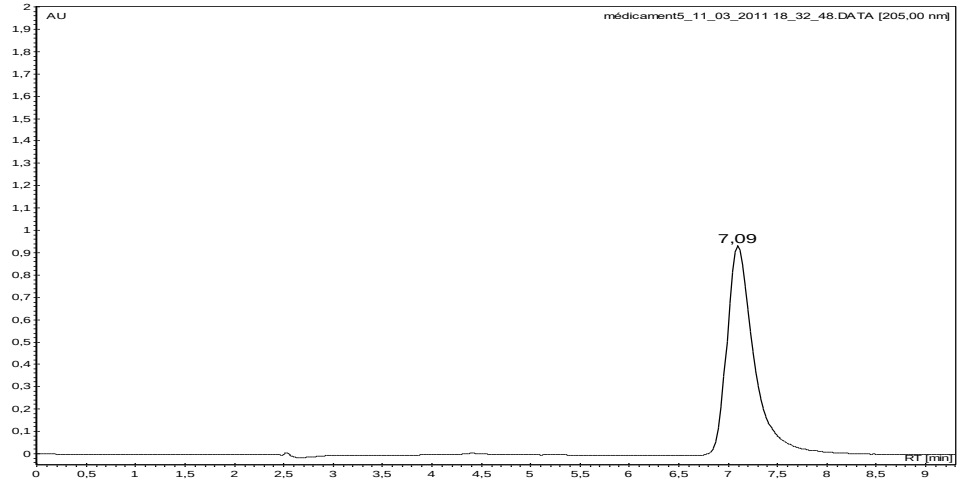


**Figure 11**. Chromatogram registered from the marketed zofenopril formulation

**Conclusion**

The RP-HPLC method developed and validated allows a simple and fast quantitative determination of zofenopril calcium from their formulation. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was found to be specific for the drug of interest irrespective of the excipients present and the method was found to be simple, accurate, precise, and stable under forced degradation stress conditions. So

the established method can be employed in the routine analysis of the marketed formulation

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