Validated RP-LC Method for Simultaneous Determination of Zofenopril and Hydrochlorothiazide in Pharmaceutical Preparations

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Abstract

A simple, selective, and precise reversed-phase column liquid chromatographic method has been developed and validated for the simultaneous determination of zofenopril (ZOF) and hydrochlorothiazide (HCT) in pharmaceutical preparations. Analyses were 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. Detection was performed using an UV–vis detector monitored at 270 nm. Quantitation was achieved using an external calibration curve. The linearity for ZOF concentrations ranging between 1.0–70.0 µg/mL, and between 2.5–35.0 µg/mL for HCT (r > 0.999) was established. The recovery (R%) of the active ingredients from the samples ranged between 99.42% and 100.67%. Intra- and inter-day precisions were less than 1.5%. The limits of detection (LOD) and quantitation (LOQ) were 0.129 and 0.292 µg/mL for ZOF, and 0.183 and 0.556 µg/mL for HCT, respectively. There was no background interference for either of the two active ingredients during chromatographic analysis. The proposed method could be used for quality control of these two componenets in combination.

Introduction

Zofenopril (ZOF); (4S)–*N*-[(2S)-3-benzoylthio-2-methylpropionyl]-4-phenylthio–L-proline (Figure 1A) is a new oral pro-drug, which acts an ACE inhibitor (1). ZOF has been used in the treatment of acute myocardial infarction (2), heart failure (3), and essential hypertension (4–5). Hydrochlorothiazide (HCT); 16-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (Figure 1B) is a thiazide-class diuretic used individually as a anti-hypertensive agent, or in combination with other anti-hypertensive drugs (6). In hypertensive patients, treatment using the combination of ZOF and HCT has been shown to produce a significantly greater blood pressure decrease compared to ZOF alone (7). A new combination dosage form of ZOF and HCT is being to be used for hypertension treatment (8–9).

Quantitative retention-activity relationships of angiotensin converting enzyme inhibitors including ZOF were studied by mixed micellar liquid chromatography (LC) (10). The pro-drug ZOF and its active metabolite zofenprilate were determined in human plasma by gas chromatography-mass spectrometry (GC–MS) (11,12) and LC–MS–MS (13,14). Several analytical methods have been developed for the determination of HCT in pharmaceutical preparations and biological fluids using spectrometry (15), voltammetry (16), GC (17), capillary electrophoresis (18), and LC (15, 19–22). The main objective of this study was to develope and validate a simple, rapid and precise reversed-phase (RP-)LC method for simultaneous determination of ZOF and HCT in combinated pharmaceutical preparations especially. Validation was in accordance with the ICH (20,21) guidelines Q2A and Q2B. For this purpose, system suitability, specificity, linearity, precision, accuracy, limit of detection, limit of quantitation, robustness, and stability were determined. An RP-LC device could be found in every laboratory, and it is also popular in pharmaceutical industry comparing to the other techniques (10–14). This method is proposed for use in routine analysis in quality control laboratories.

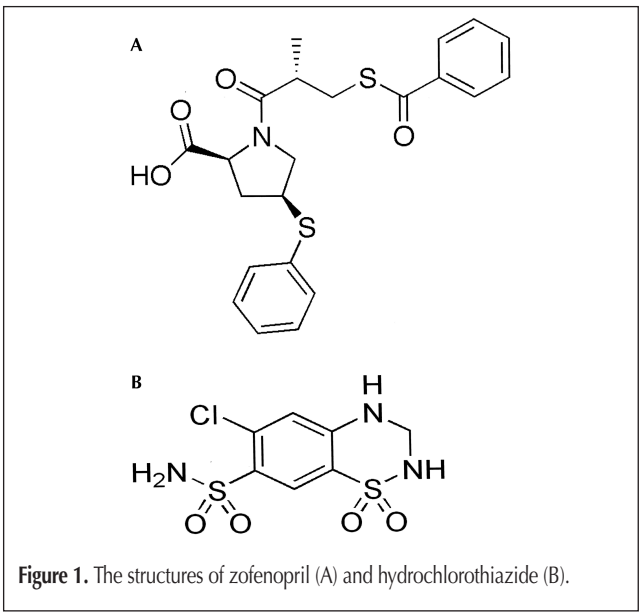

Figure 1. The structures of zofenopril (A) and hydrochlorothiazide (B).

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Experimental

Chemicals and Reagents

The ZOF calcium reference standard was purchased from Chengdu Sino-Strong Pharmaceutical Co., Ltd. (Chengdu, P.R. China). The HCT reference standard and tablet excipients were kindly supplied by Deva Holding A.S. (Istanbul, Turkey). The combined pharmaceutical preparation (Zofenil Duo, Menarini, France) containing 30 mg of ZOF calcium and 12.5 mg HCT per tablet was purchased from a public pharmacy. Methanol (HPLC grade) and *o*-phosphoric acid (analytical grade) were obtained from Merck (Darmstadt, Germany). A 0.45-µm Millipore nylon membrane (Millipore, Saint-Quentin, Yvelines, France) was used for the filtration of the final extract before injection. HPLC-grade water was prepared using the AquaMAX-ultra water purification system from Young Lin Inst (Anyang, Korea).

Instrumentation and chromatographic conditions

The analyses were performed on a Thermo Separation Products Liquid Chromatograph (TX) which consisted of a P4000 solvent delivery Spectra system equipped with a Rheodyne injection valve with a 20 µL loop, a UV3000 detector, an SN4000 digital interface module and a TSP (Thermo Separation Products) PC1000 Ver 3.5.1 (OS/2 warp 4.0) software system. Separation was achieved using a Gemini C18 column (150 × 4.6 mm i.d., 5 µm; Phenomenex, Texas,) operating at 25°C, with a flow rate of 1.0 mL/min. Elution of the compounds of interest was achieved using the mobile phase solvent mixtures of methanol (solvent A) and water (solvent B) adjusted to pH 2.5 with H_3PO_4 as follows: Linear gradient elution from 30% A and 70% B to 80% A and 20% B, 0–5 min; isocratic elution 80% A and 20% B at 5–12 min; and 10 min post-time (30% A and 70% B) prior to the next injection. UV detection was achieved at a wavelength of 270 nm, and the injection volume was 20 µL. Each determination was carried out six times. Quantitation was achieved by the peak-area ratio method using the external standard calibration curve.

Preparation of Reference Standard Solutions

Stock standard solutions of 0.5 mg/mL of ZOF and HCT were freshly prepared weekly in 60% methanol and kept at +4°C. The standard working solutions of ZOF and HCT were prepared daily by diluting stock solutions with the same solvent to obtain a range of 1.00–70.0 µg/mL for ZOF and 2.5–35.0 µg/mL for HCT.

Method Validation

System suitability test

The system suitability tests were performed in accordance with USP 24/NF 19 to confirm the adequacy of the reproducibility of the equipment for the analysis to be performed. System suitability was determined by six replicate analyses of both compounds at concentrations of 30.0 and 12.5 µg/mL for ZOF and HCT, respectively, and RSD values of the retention times were calculated. The tailing factor, capacity factor, and resolution were also determined.

Specificity

To determine the specificity, a placebo solution containing microcrystalline cellulose, lactose monohydrate, corn starch, hypromellose, colloidal silica, and magnesium stearate, was prepared and injected. It was also determined by checking the spectra of ZOF and HCT with a PDA detector.

Linearity

Linearity was evaluated by the analysis of standard working solutions containing mixtures of ZOF and HCT at nine different concentrations. ZOF solutions within the concentration range of 1.0–70.0 µg/mL and HCT solutions within the concentration range of 2.5–35.0 µg/mL were prepared. Six replicate injections of each concentration were performed. The analyte peak area values were plotted against the corresponding concentrations of the analytes.

Assay precision and accuracy

To determine inter-day and intra-day precisions, three different concentrations of the standard stock solutions (3.0, 30.0, and 60.0 µg/mL for ZOF and 5.0, 15.0, and 30.0 µg/mL for HCT) were injected into the LC system on the same day and on different days. Accuracy and recovery studies of ZOF and HCT were performed using the method of standard addition by adding known amounts of the reference compounds to the sample solution. The amounts of the analytes added corresponded to 80% (24.0 and 10.0 µg/mL for ZOF and HCT), 100% (30.0 and 12.5 µg/mL for ZOF and HCT), and 120% (36.0 and 15.0 µg/mL for ZOF and HCT) of analyte concentrations in the samples. The peak areas were calculated and obtained values were fitted to the straight-line equation of the calibration curves. The recovery percentages were calculated.

Limits of detection and limits of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to 3SD/m and 10SD/m criteria, respectively (23,24), where SD is the standard deviation of the intercept, and m is the slope of calibration curve.

Robustness

Assessment of the influence of the variations of pH of the mobile phase (2.5 ± 0.1), flow rate (1.0 ± 0.1), and column temperature (25°C ± 1) was carried out.

Stability

Stability trials for the analytes of interest were performed in the standard working solutions as a validation parameter of the proposed method. The short time stability was tested and determined at room temperature for a period of 24 h and the long term stability was tested and determined at 4°C for a period of 1 month.

Assay procedure for tablets

Ten tablets were separately weighed and then powdered. Quantities of 30.0 mg ZOF and 12.5 mg HCT were accurately weighed and transferred into a 100 mL volumetric flask. Sixty milliliters of MeOH was then added and the mixture was sonicated for 30 min in an ultrasonic bath. The volume was diluted to the mark with MQ water, and then the solution was sonicated for 15 min and filtered. Appropriate dilutions were prepared with 60% MeOH, so that the final concentrations of ZOF and HCT were 30.0 and 12.5 µg/mL , respectively.

Results and Discussion

Method development

Testing of different analytical columns (C8, C18, and CN), different mobile phases containing buffers (phosphate, ammonium acetate and trifluoroacetic acid) with different pHs (2.5–5), and organic modifiers (acetonitrile, methanol) was performed for RP-LC analysis. The C18 column showed a better performance when compared to the other columns and it was selected because it is commonly available in the laboratory. When a Gemini C18 column (150 mm) was used, retention times were shortened and sharper peaks were obtained for each of the compounds. Mobile-phase composition was also optimized by the use of several trials. Methanol was chosen as the organic modifier, because it resolved the drugs well and produced symmetrical and sharp peaks with the acidic mobile phase. When acetonitrile was used, retention times were shortened; however, the conditions were not suitable for HCT. Apart from H_3PO_4, when the other buffer solutions were used, tailing occurred in the peaks. The sensitivity decreased or broad peaks occurred at pH values above or below 2.5. When using isocratic elution with different solvent mixtures, it was observed that ZOF and HCT had eluted as broad peaks with tailing. A methanol–water ratio of 80:20 was the best for ZOF, and a 30:70 ratio was found to be the most appropriate for HCT. This problem was solved with linear gradient elution.

The UV absorption spectra of ZOF and HCT are presented in Figure 2. In spite of the fact that 215 nm seemed to be more appropriate for all the drugs with high absorption values, it was not used due to increased interferences. The preferred wavelength for quantitative purposes was 270 nm.

Good separation was achieved and the retention times of ZOF and HCT were determined as 11.16 and 2.86 min, respectively (Figure 3).

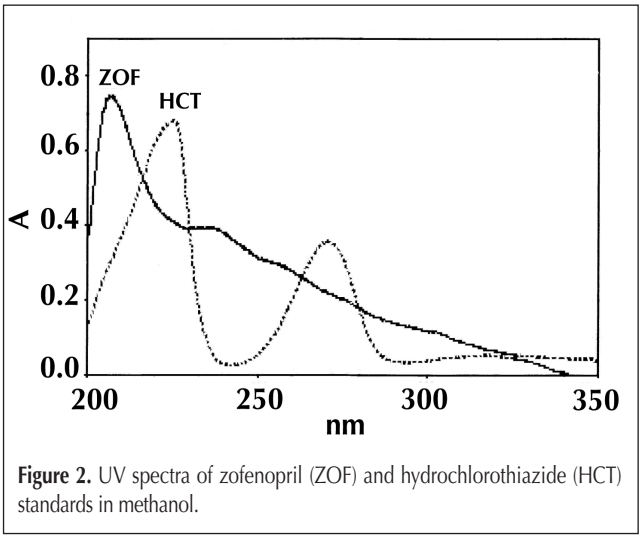

Figure 2. UV spectra of zofenopril (ZOF) and hydrochlorothiazide (HCT) standards in methanol.

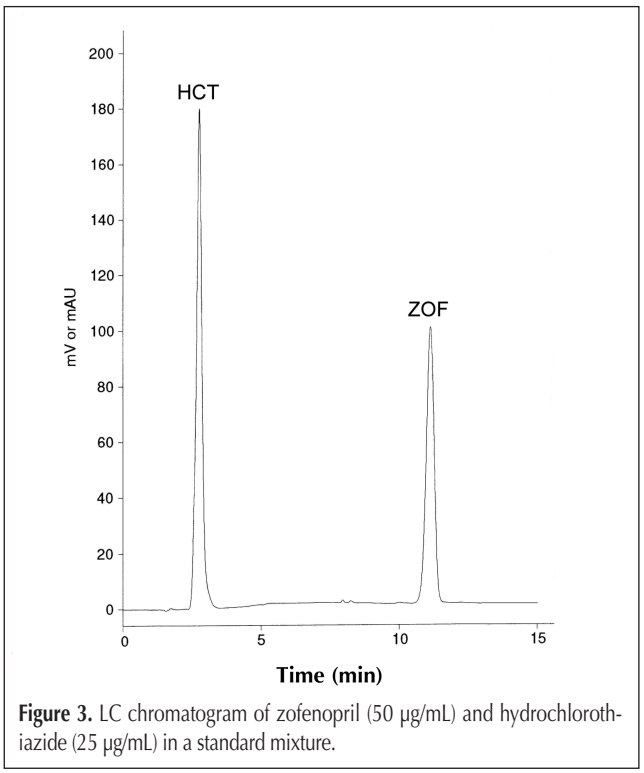

Figure 3. LC chromatogram of zofenopril (50 µg/mL) and hydrochlorothiazide (25 µg/mL) in a standard mixture.

Method validation

System suitability test

The retention times of ZOF and HCT were 11.16 and 2.86 min with RSDs (%) of 1.07 and 1.12, respectively. The tailing factors were 1.0 and 0.9, and capacity factors were 21.3 and 4.6 for ZOF and HCT, respectively. Resolution was 20.8.

Specificity

A relevant chromatogram of a placebo sample can be seen in Figure 4 where no interfering peaks were observed at 270 nm.

Linearity and calibration

The linear concentration ranges were 1.0–70.0 µg/mL for ZOF, and 2.5–35.0 µg/mL for HCT. The obtained equations of the cali-

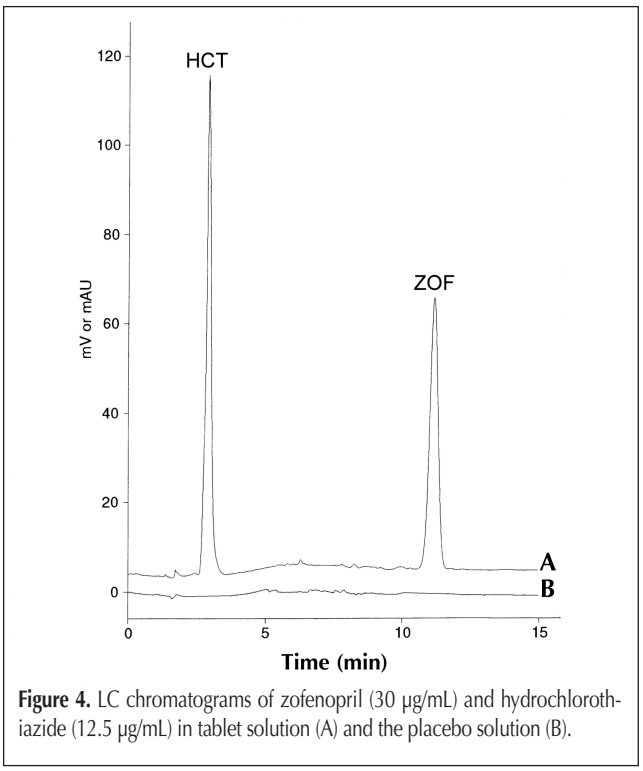

Figure 4. LC chromatograms of zofenopril (30 µg/mL) and hydrochlorothiazide (12.5 µg/mL) in tablet solution (A) and the placebo solution (B).

bration curves for ZOF and HCT were y = 38741x +14143 (r = 0.9998), and y = 106801x – 72112 (r = 0.9999), respectively, where y is the peak area at 270 nm and x is the analyte concentration in µg/mL.

Table IA. Intra-day Variability of Zofenopril and Hydrochlorothiazide (*n* = 6)

Conc. added (µg/mL)	Conc. found (mean ± SD) (µg/mL)	Precision (%RSD)
Zofenopril		
3.0	2.99 ± 0.03	1.0
30.0	30.23 ± 0.3	1.0
60.0	60.52 ± 0.6	1.0
Hydrochlorothiazide		
5.0	5.04 ± 0.05	1.0
15.0	14.94 ± 0.2	1.3
30.0	30.18 ± 0.4	1.3

Table IB. Inter-day variability of Zofenopril and Hydrochlorothiazide (*n* = 6)

Conc. added (µg/mL)	Conc. found (mean ± SD) (µg/mL)	Precision (%RSD)
Zofenopril		
3.0	3.02 ± 0.03	1.0
30.0	29.92 ± 0.4	1.3
60.0	60.27 ± 0.9	1.5
Hydrochlorothiazide		
5.0	5.02 ± 0.04	0.8
15.0	15.11 ± 0.2	1.3
30.0	29.99 ± 0.3	1.0

Table II. Results from Recovery Studies by Standard Addition Method (*n* = 6)

Conc. in tablet solution	Conc. added (µg/mL)	Total conc. found (mean ± SD) (µg/mL)	Precision (%RSD)	Recovery of added drug (%)
Zofenopril				
30.0	24.0	54.14 ± 0.2	0.4	100.58
30.0	30.0	60.08 ± 0.3	0.5	100.27
30.0	36.0	65.79 ± 0.2	0.3	99.42
Hydrochlorothiazide				
12.5	10.0	22.48 ± 0.1	0.4	99.80
12.5	12.5	25.06 ± 0.1	0.4	100.48
12.5	15.0	27.60 ± 0.2	0.7	100.67

Table III. Robustness Results of LC Method*

	Condition (±RSD%)			
	Optimum		Upper/Lower	
Factor	ZOF	HCT	ZOF	HCT
Variations of pH	2.5 ± 0.2	2.5 ± 0.3	2.6/2.4 ± 0.3	2.6/2.4 ± 0.5
Flow rate	1.0 ± 0.3	1.0 ± 0.4	1.1/0.9 ± 0.2	1.1/0.9 ± 0.4
Column temp.	25°C ± 0.1	25°C ± 0.2	26°C/24°C ± 0.2	26°C/24°C ± 0.3

*ZOF; 30 µg/mL and HCT; 15 µg/mL

Assay precision and accuracy

Intra- and inter-day results showed that the RSD values for concentrations of ZOF and HCT ranged from 0.8 to 1.3% (Table I). The proposed method is reproducible, because the precision values were < 1.5%. The percent recovery of the added standards to the assay samples was calculated within the range of 99.42–100.67% (Table II).

LOD and LOQ

In this study, the LOD and LOQ values were found to be 0.129 and 0.392 µg/mL for ZOF, and 0.183 and 0.556 µg/mL for HCT.

Robustness

Evaluation of the robustness of method revealed that the analytical procedure was valid under slightly different conditions (Table III).

Stability

Stability studies showed that both compounds were stable, no decomposition products (peaks) were observed during chromatography, and no changes in peak areas were observed during the analytical procedure (Table IV).

Tablet studies

The validated RP-LC method was applied for the simultaneous determination of ZOF and HCT in a combined dosage form. Characteristic chromatograms of a tablet and a placebo extract can be seen in Figure 4. The mean assay results, expressed as a percentage of the label claim, are listed in Table V.

Conclusions

A validated RP-LC method has been developed for the simultaneous determination of ZOF and HCT in a combined dosage form. The proposed method complies with ICH guidelines in accuracy, precision and stability of both standard and tablet samples. There were no matrix interferences caused by the excipients. RP-LC is the most popular device in pharmaceutical industry when comparing to GC–MS (11,12) and micellar liquid chromatograph (10). It could be found in every laboratories easily considering to LC–MS–MS (13,14). We propose this method for routine analysis and quality control of pharmaceutical preparations containing the combination of ZOF and HCT.

Table IV. Results from Stability Experiments (*n* = 6)

Conc. (µg/mL)	Room temperature		+ 4°C	
	Mean	RSD (%)	Mean	RSD (%)
Zofenopril				
3.0	3.01	0.9	3.02	0.9
30.0	30.01	1.1	30.16	1.2
60.0	60.33	1.2	60.26	1.4
Hydrochlorothiazide				
5.0	4.99	0.9	5.01	1.0
15.0	15.09	1.2	14.96	1.4
30.0	30.05	1.2	30.12	1.2

Table V. Results from Assay Experiment (n = 6)*

	Zofenopril	Hydrochlorothiazide
Mean amount of drug found (mg/tablet)	30.24	12.58
Mean recovery (%)	100.80	100.64
RSD	0.67	0.45

*The label claimed was 30 mg zofenopril calcium and 12.5 mg hydrochlorothiazide.

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