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Received February 22, 2010
Revised March 22, 2010
Accepted March 23, 2010

Research Article

HPLC-DAD method for the simultaneous determination of zofenopril and hydrochlorothiazide in oral pharmaceutical formulations

An HPLC method with DAD detection was developed and validated for the simultaneous determination of zofenopril and hydrochlorothiazide in tablets. The separation was carried out through a gradient elution using an Agilent LiChrospher C18 column (250 × 4.0 mm id, 5 μm) and a mobile phase consisting of (A) water–TFA (99.9:0.1 v/v) and (B) acetonitrile–TFA (99.1:0.1 v/v) delivered at a flow-rate of 1.0 mL/min. 8-Chlorotheophylline was used as internal standard. Calibration curves were found to be linear for the two drugs over the concentration ranges of 5.0–40 and 1.0–20 μg/mL for zofenopril and hydrochlorothiazide, respectively. Linearity, precision, accuracy, specificity and robustness were determined in order to validate the proposed method, which was further applied to the analysis of commercial tablets. The proposed method is simple and rapid, and gives accurate and precise results.

Keywords: HPLC / Hydrochlorothiazide / Pharmaceutical formulations / Validation / Zofenopril
DOI 10.1002/jssc.201000123

1 Introduction

Zofenopril is an antihypertensive drug belonging to the family of the angiotensin-converting enzyme (ACE) inhibitors, characterized by high lipophilicity, sustained cardiac ACE inhibition, and antioxidant and tissue protective activities [1–3]. Its chemical name is (4S)-1-[(2S)-3-(benzoylthio)-2-methyl-1-oxopropyl]-4-(phenylthio)-L-proline (Fig. 1A). ACE inhibitors have been developed as a further therapeutic action on the renin-angiotensin-aldosteron system, one of the most important regulators of blood pressure [4–9]. Hydrochlorothiazide, or 6-chloro-3,4-dihydro-2H-1,2,4-benzothiazine-7-sulfonamide (Fig. 1B), is a diuretic of the class of benzothiazines widely used in antihypertensive pharmaceutical formulations, alone or in combination with other drugs, which decreases the active sodium reabsorption and reduces peripheral vascular resistance. The two drugs are successfully used in association in the treatment of hypertension [3, 4]. The fixed combination of zofenopril–hydrochlorothiazide 30–12.5 mg/day is approved for the management of mild-to-moderate hypertension in different European countries. In clinical trials comparing zofenopril–hydrochlorothiazide with each agent administered as monotherapy, combination therapy was clearly more effective in normalizing blood pressure. In addition, combination therapy provided a sustained and consistent blood pressure control over the entire 24 h dosing interval. The efficacy and safety profile of zofenopril–hydrochlorothiazide highlights that this combination is a potentially useful addition to currently available therapy for patients with blood pressure inadequately controlled by monotherapy, as well as for patients who require more rapid and intensive blood pressure control [9]. Reported methods for the determination of hydrochlorothiazide in pharmaceutical formulations include derivative spectrophotometry and HPLC [10]. Quantification of hydrochlorothiazide in biological samples has been developed using liquid chromatography [11–13]. Several methods described the determination of hydrochlorothiazide in combination with other drugs such as valsartan [14], olmesartan [15], bisoprolol [16], captopril [17], losartan [18] and amiloride [19] in pharmaceutical formulations. Quantification of zofenopril and its active metabolite zofenoprilat has been carried out in human plasma by liquid chromatography coupled with tandem mass spectrometry [20]. So far, no method for the simultaneous determination of these drugs in pharmaceutical forms has been described. It was desirable to develop a simple and fast procedure that could be applied in quality control laboratories for the simultaneous determination of the two drugs in the presence of each other. The present methodology shows a simple and rapid method for the simultaneous determination of

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Abbreviation: ACE, angiotensin-converting enzyme

zofenopril and hydrochlorothiazide in pharmaceutical forms. The procedure, based on the use of reversed-phase HPLC, provides accurate and precise results for the quantitation of these two drugs in tablets.

2 Materials and methods

2.1 Chemicals and reagents

Zofenopril was purchased from American Custom Chemicals (San Diego, CA, USA). Hydrochlorothiazide and 8-chlorotheophylline (internal standard, Fig. 1C) were supplied from Sigma-Aldrich (St. Louis, MO, USA). TFA (HPLC grade) was supplied by Fluka Chemika-Biochemika (Buchs, Swizerland). HPLC-grade acetonitrile was provided by Carlo Erba Reagenti (Milan, Italy). All other chemicals were of analytical grade. HPLC grade water was obtained by passage through an Elix 3 and Milli-Q Academic water purification system (Millipore, Bedford, MA, USA).

2.2 Apparatus and chromatographic conditions

HPLC analysis was performed on a Waters (Waters, Milford, MA, USA) system composed of a P600 pump and a W2996 photodiode array detector. A model 7125i sample injector (Rheodyne, Cotati, CA, USA) equipped with a 20-μL loop was used. The separation was achieved using a LiChrospher 100 RP-18 column (250 mm × 4.0 mm id, 5 μm particle size) (Agilent Technologies, Santa Clara, CA, USA). An on-line degassing system model DGU-14A (Shimadzu Corporation, Japan) and a column thermostat oven module Igloo-Cil (Cil Cluzeau Info Labo, France) were used. A Labsonic FALC ultrasonic bath (Falc Instruments, Bergamo, Italy) was used. Chromatograms were recorded on a Fujitsu Siemens Esprimo computer and data were treated with the Empower Pro software (Waters). The mobile phase consisted of water–TFA (99.9:0.1; v/v) (A) and acetonitrile–TFA (99.9:0.1; v/v) (B). The elution was performed using the following gradient: 0–4 min 70:30 (A:B v/v); 4–8 min 30:70 (A:B v/v); 8–15 min 30:70 (A:B v/v). After the run was complete, the column re-equilibration time was 5 min. The mobile phase was prepared daily, filtered through a 0.45-μm, WTP 0.5-μm membrane (Whatmann, Maidstone, UK), sonicated before use and delivered at a flow rate of 1.0 mL/min. Column temperature was kept constant at 25°C. The injection volume was 20 μL. 8-Chlorotheophilline (IS) was used as internal standard. Detector wavelength was set at the maximum absorbance for each analyte, *i.e.* 224 nm for hydrochlorothiazide, 245 nm for zofenopril and 275 nm for IS.

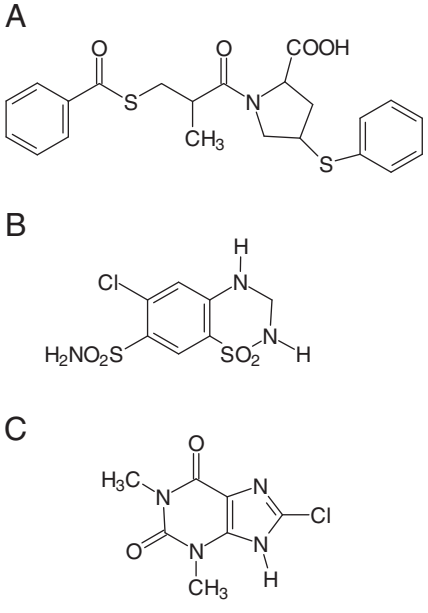

Figure 1. Chemical structures of zofenopril (A), hydrochlorothiazide (B) and 8-chlorotheophylline (internal standard, C).

2.3 Standard solutions

Zofenopril and hydrochlorothiazide stock solutions (1.0 mg/mL of each drug in acetonitrile–water (50:50 v/v) acidified with 0.1% TFA) were freshly prepared. A 1.0 mg/mL of internal standard (8-chlorotheophilline) stock solution in acetonitrile–water (50:50 v/v) acidified with 0.1% TFA was also prepared. Stock solutions were then diluted with the appropriate volume of the same solvent mixture to obtain the desiderate concentration (in the range of 5.0–40 μg/mL for zofenopril and of 1.0–20 μg/mL for hydrochlorothiazide, while the concentration of internal standard was kept constant at 15 μg/mL). The standard solution mixture was prepared by diluting zofenopril stock solution to a concentration of 200 μg/mL and mixing it with hydrochlorothiazide working solution (100 μg/mL) into a volumetric flask to give a solution with a final concentration of 20 μg/mL and 10 μg/mL for zofenopril and hydrochlorothiazide, respectively. The stability of zofenopril, hydrochlorothiazide and internal standard after 2 wk of storage at +4°C was evaluated. The observed %RSD from the initial concentration values were 0.2, 0.3 and 0.2% for zofenopril, hydrochlorothiazide and internal standard, respectively. After 5 wk of storage at room temperature, the observed %RSD from the initial concentration values were 0.2, 0.4 and 0.2% for zofenopril, hydrochlorothiazide and internal standard, respectively. All these percent deviations were within the experimental error of the assays.

2.4 Pharmaceutical forms

Film coated tablets containing zofenopril 30 mg/tablet and hydrochlorothiazide 12.5 mg/tablet, were a commercial product, currently marketed in 25 countries. Inactive ingredients were microcrystalline cellulose, lactose, cornstarch, magnesium stearate, Macrogol 6000.

2.5 Sample preparation

Ten tablets were crushed and combined, and finely powdered. An amount of material was accurately weighed, transferred in a volumetric flask, added with a mixture of acetonitrile–water (50:50 v/v) acidified with 0.1% TFA and sonicated for 10 min and brought to volume with the same solvent. After filtration, the solution, diluted to a concentration within the range of the calibration curve described, was added with internal standard and analyzed by HPLC.

2.6 Method validation

The method was validated according to the United States Pharmacopeia requirements [21]. The following validation characteristics were evaluated: linearity, LOD, LOQ, precision, accuracy, robustness, system suitability, selectivity and specificity.

2.6.1 Linearity, detection and quantitation limits, precision and accuracy

Linearity concentration curves for the assay of zofenopril and hydrochlorothiazide were obtained by injecting eight different concentrations of zofenopril and hydrochlorothiazide standard calibration solutions with concentration ranging from 5.0 to 40 μg/mL and from 1 to 20 μg/mL for zofenopril and hydrochlorothiazide, respectively, while the concentration of internal standard was kept constant at 15 μg/mL. Each solution was injected in triplicate. Peak area ratios (zofenopril/8-chlorotheophylline and hydrochlorothiazide/8-chlorotheophylline) were plotted *versus* the respective compound concentrations.

LOD and LOQ were calculated from the residual standard deviation of the regression line (σ) of the analytical curve and its slope (*S*) in accordance with the equations LOD = 3.3 (σ/*S*) and LOQ = 10 (σ/*S*) [22]. To measure repeatability of the system, 20 consecutive injections were made using a standard solution containing 10 μg/mL of hydrochlorothiazide, 20 μg/mL zofenopril and 15 μg/mL of internal standard. The results were expressed as the percentage RSD (RSD%) for peak area ratio of zofenopril/8-chlorotheophylline and hydrochlorothiazide/8-chlorotheophylline and for the retention time of zofenopril and hydrochlorothiazide, respectively. The intra-day precision was evaluated by injecting sample solutions prepared at low, middle and high concentrations of the analytical curves (5.0–40 μg/mL for zofenopril, 1.0–20 μg/mL for hydrochlorothiazide) containing 15 μg/mL of internal standard, in one day. The inter-day precision was evaluated by injecting the same solutions on three consecutive days. Three determinations for each concentration were performed. Precision was expressed as the standard deviation for peak area ratio for zofenopril/8-chlorotheophylline and hydrochlorothiazide/8-chlorotheophylline, respectively. The accuracy was calculated as the percentage recovery of a known amount of standard added to the sample. 8-Chlorotheophylline standard solution was added to commercial sample solution, which was then analyzed by the proposed method.

2.6.2 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication of its reliability during normal usage [23]. To study the robustness of the proposed method, deliberate modifications in flow rate, wavelength values, and temperature were made.

2.6.3 Specificity

The specificity of the method for zofenopril and hydrochlorothiazide was tested by analyzing a mixture of the inactive ingredients (placebo), the commercial samples containing zofenopril and hydrochlorothiazide and a mixture of standard solutions.

2.6.4 Selectivity

The selectivity of the method was established by studying retention time, separation factor, retention factor, resolution of all peaks and the absorption spectra of each eluted peak.

3 Results and discussion

3.1 Optimization of the HPLC conditions

To effectively and simultaneously separate zofenopril, hydrochlorothiazide and the internal standard under gradient conditions, various chromatographic conditions with different columns (C_8, phenyl, cyano), pHs and mobile phase compositions were investigated. A satisfactory separation was obtained using a LiChrospher C_{18} column and a mobile phase consisting of: (A) water–TFA (99.9:0.1 v/v) and (B) acetonitrile–TFA (99.9:0.1 v/v) delivered at a flow-rate of 1.0 mL/min. The analysis was carried out in the following gradient elution mode: eluent B 30% from 0 to 4 min, then increased to 70% in 4 min and maintained for 7 min at 70%. After the run was complete, the column re-equilibration time was 5 min. The order of elution was 8-chlorotheophylline (t_R = 2.7 min), hydrochlorothiazide (t_R = 3.5 min) and zofenopril (t_R = 14.4 min), respectively, at a flow rate of 1.0 mL/min and at room temperature (Fig. 2). The gradient elution we developed was the one which provides the best results in terms of time, resolution and peak symmetry. Chromatographic parameters are shown in Table 1.

3.2 Method validation

Calibration curves were obtained by plotting peak-area ratios (compound/internal standard) against the respective

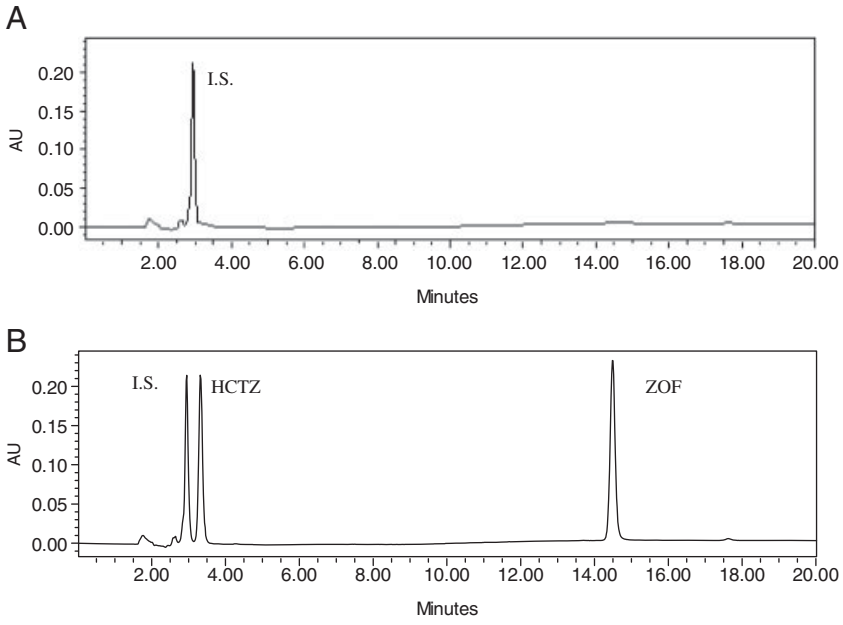

Figure 2. Chromatograms: (A) internal standard 8-chlorotheophylline (15 μg/mL) and (B) mixture of 20 μg/mL zofenopril (ZOF), 10 μg/mL of hydrochlorothiazide (HCTZ) and 15 μg/mL of internal standard 8-chlorotheophylline (IS) in acetonitrile–water (50:50 v/v) acidified with 0.1% TFA. Sample volume is 20 μL.

Table 1. Chromatographic parameters[a)]

Compound	t_R (min)	*k*	rt_R	α	*N*	As
ZOF	14.4	8.1	ZOF/8-CIT = 5.33	4.6	17958	1.4
HCTZ	3.14	1.2	HCTZ/8-CIT = 1.30	1.2	19943	0.8
8-CIT	2.74	0.7	8-CIT/8-CIT = 1.00	1.2	18696	1.0

a) ZOF: zofenopril; HCTZ: hydrochlorothiazide; 8-CIT: 8-chlorotheophylline; t_R: retention time; *k*: retention factor; rt_R: relative retention time; α: separation factor; *N*: theoretical plate number; As: asymmetry factor.

Table 2. The regression analysis data[a)]

	[*c*] (μg/mL)	*a*	*b*	*r*	S_a	S_b	LOD (μg/mL)	LOQ (μg/mL)	*F*
ZOF	5.0–40	0.1653	0.0513	0.9989	0.0256	0.0010	0.026	0.08	2396.06
HCTZ	1.0–20	0.0800	0.0969	0.9989	0.0231	0.0020	0.019	0.06	2259.51

a) ZOF: zofenopril; HCTZ: hydrochlorothiazide; [c] concentration range; *r*: correlation coefficient; LOD: limit of detection; LOQ: limit of quantitation; *a*, *b*: intercept and slope; S_a, S_b: standard deviations of the intercept and slope; *F*-test tabulated (0.05) = 7.71.

Table 3. Intra- and inter-day precision of the proposed RP-HPLC method for ZOF and HCTZ quantitative determination[a)]

Sample	ZOF (μg/mL)				HCTZ (μg/mL)		
	10.0	25.0	35.0		2.50	7.50	15.0
Intra-day ($n = 3$)				Intra-day ($n = 3$)			
SD	0.2	0.1	0.2	SD	0.05	0.04	0.1
Inter-day ($n = 9$)				Inter-day ($n = 9$)			
SD	0.2	0.2	0.3	SD	0.05	0.03	0.2

a) *n*: number of determinations; SD: standard deviation; ZOF: zofenopril; HCTZ: hydrochlorothiazide.

compound concentrations. In both cases, straight regression lines with correlation coefficients above 0.998 were obtained. The *F*-test was applied for both calibration curves and the data provided the conclusive evidence of a linear relationship between concentration and instrumental response [24]. LOD and LOQ were calculated using calibration curves results. Data are summarized in Table 2. System repeatability was determined by injecting a standard solution containing 20 and 10 μg/mL of zofenopril and hydrochlorothiazide, respectively, 20 times in the chromatographic system. For zofenopril and hyrochlorothiazide, %RSD for peak area ratio were 2.44 and 1.24, while RSD% for retention time were 1.33 and 2.44, respectively.

Table 4. Recovery of a standard solution of ZOF and HCTZ added to sample and determined using the proposed HPLC method

Sample[a)]	Zofenopril				Hydrochlorothiazide			
	Nominal content (mg/tablet)	Standard added to commercial sample[a)] (μg/mL)	Standard found (μg/mL)	Recovery[b)]%	Nominal content (mg/tablet)	Standard added to commercial sample[a)] (μg/mL)	Standard found (μg/mL)	Recovery[b)]%
1	30	–	29.9	99.9	12.5	–	12.5	99.8
2	30	2.5	32.2	99.1	12.5	2.5	15.0	100.2
3	30	5	34.9	99.9	12.5	5	17.4	99.7
4	30	7.5	37.8	100.8	12.5	7.5	19.9	99.8
5	30	10	40.1	100.3	12.5	10	22.5	100.1

a) Commercial sample (ZOF and HCTZ tablets).
b) Average of three determinations.

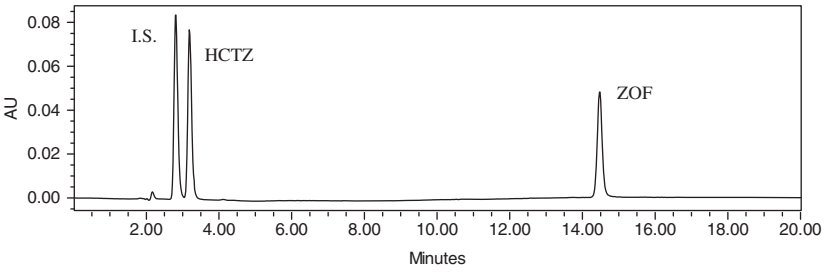

Figure 3. Chromatogram of a commercial sample containing zofenopril (ZOF) and hydrochlorothiazide (HCTZ) and internal standard (IS).

The values obtained demonstrate that the system is reliable for analysis. The precision of the method was evaluated by intra- and inter-day determinations. The one-way ANOVA was used to estimate the total variability within and between days. The results are shown in Table 3. The accuracy of the method was expressed as RSD% of the percentage recovery of a known amount of standard added to the sample. The results are shown in Table 4. The robustness of the method was evaluated by small changes in flow rate, temperature and wavelength values. About 1.6% of difference was observed in the more critical result when the analytical parameters were modified and compared with the original conditions. The specificity of the method was demonstrated by the absence of interferences among zofenopril, hydrochlorothiazide and excipients in the samples, using the criteria defined in the USP 30 assays [21]. A mixture of the inactive ingredients (placebo) added with internal standard, the commercial sample of zofenopril and hydrochlorothiazide and a standard mixture solution were analyzed by the proposed methodology. As it can be observed (Figs. 2 and 3), neither tablet excipients nor impurities interfere in the analysis of zofenopril and hydrochlorothiazide. The absorption spectra of the eluted peaks were achieved using a photodiode array detector and then compared with those of the reference standards. The results showed equivalent spectrophotometric profiles. A commercial sample was analyzed in triplicate and the average recoveries were 101.0% both for zofenopril and hydrochlorothiazide. System suitability test is an important part of liquid chromatographic method. It is used to verify if the chromatographic system is adequate and reliable. Data for five injections of a solution containing 20 μg/mL of zofenopril and 10 μg/mL of hydrochlorothiazide standard solutions were analyzed. The RSD% for peak area ratios were 1.6 and 1.8% for zofenopril and hydrochlorothiazide, respectively. These results agree with those specified in the United States Pharmacopeia [21].

4 Concluding remarks

The validated method is rapid and efficient, and allows the separation of zofenopril and hydrochlorothiazide in the presence of its excipients without using buffers in the mobile phase. Total time of analysis was about 20 min. Good recoveries and good RSD% values confirm that the proposed HPLC method is applicable and reliable for the determination of zofenopril and hydrochlorothiazide in the examined pharmaceutical product. The developed method, because it is sensitive, precise and accurate, is suitable to be used in quality control test of the examined pharmaceutical product.

The authors have declared no conflict of interest.

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