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Contributed Paper

Development and Validation of HPLC with UV Detection Method: Analysis of Cilazparil in Pharmaceutical Dosage Forms

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ABSTRACT

A simple and rapid liquid chromatographic method was developed and validated for the determination of cilazapril (CZP) in bulk drug and pharmaceutical dosage form. Chromatographic separation has been achieved within 10 minutes by using an Cyano column (150 mm × 4.6 mm I.D., 5 µm particle size) as the stationary phase with a mobile phase consisted of formate buffer pH 3.75 and methanol (50:50 *v*/*v*) at a flow rate of 1.2 mL/min. Detection was performed at 227 nm using an ultraviolet detector. The method was validated in accordance with International Conference on Harmonization guidelines with respect to linearity, sensitivity, selectivity, accuracy, precision and robustness. Regression analysis showed good correlations ($\mathbf{R}^2$=0.9995) for CZP in the concentration range of 2-200 µg/mL. The percentage recovery was in the range of 99.20%-99.96% for CZP from the pharmaceutical dosage form. The proposed method is suitable for determination of CZP in pharmaceutical dosage form and bulk drug.

Keywords: cilazapril, cyano column, HPLC analysis, pharmaceutical dosage forms

1. INTRODUCTION

Cilazapril [1-6] (CZP), chemically known as 9(s)-[1(s)-(ethoxycarbonyl)-3-phenylpropylamino]-octahydro-10-oxo-6H-pyridazo [1,2-a] [1,2] diazepine-1(s)-carboxylic acid monohydrate, is a long acting pyridazine angiotensin-converting enzyme inhibitor used in the treatment of high blood pressure (hypertension) and congestive heart failure (reduction in the heart pumping action). In treating hypertension, CZP may be used alone or in combination with thiazide diuretics (e.g., hydrochlorothiazide). After absorption, CZP is hydrolyzed to the active metabolite, cilazaprilat. The cilazaprilat prevents the conversion of angiotensin I to angiotensin II by inhibition of angiotensin-converting enzyme. Angiotensin II is a vasoconstrictor and a negative feedback mediator for rennin activity. Rennin is an enzyme released by the kidneys that stimulate the production of angiotensin I. Due to negative feedback effect on rennin activity

the production of angiotensin I is decreased. Therefore, the production of angiotensin II is also decreased. The lower angiotensin II level decreases sodium & water reabsorption, vasoconstriction and vascular that finally results in reduced blood pressure

The CZP is official in European Pharmacopoeia [7], which describes potentiometric titration with 0.1M sodium hydroxide for its assay. Various techniques have been reported for the quantification of CZP. Cilazapril in pharmaceutical dosage forms or biological fluids either in combination with its active metabolite cilazaprilat or other drug (hydrochlorothiazide) was quantitated by many methods, such as derivative spectrophotometry [8, 9], chemometry [10], HPLC with UV detection [11-14], HPLC/MS/MS [15], capillary zone electrophoresis [16], HPLC with amperometric detection [17] and Vierordt's [9] methods.

Several techniques have been reported for the assay of CZP alone in biological fluids/pharmaceuticals formulations and include enzyme immune assay (EIA) [18], GC-MS [19], derivative spectrophotometry [20], voltammetry [21, 22] and amperometric biosensor [23]. EIA [18], GC-MS [19], voltammetry [21, 22] and amperometric biosensor [23] methods may have the highest sensitivity, but the determination process is complicated, costly and time consuming. In addition EIA [18], GC-MS [19] methods were not applied to pharmaceutical formulations. Although the derivative spectrophotometric [20] method is simple and easy to perform, it suffers from decreased selectivity due to measurement in ultraviolet region, narrow range of linearity and less precise with %RSD values >2.0. As a result, the applications of these methods [18-23] to quantify CZP in pharmaceutical formulations are limited.

For reasons of robustness and knowledge of analysts with the HPLC with UV detection technique, presently most of the separations are performed by HPLC method. To the best of our knowledge two HPLC with UV detection [20, 24] methods are reported for the determination of CZP in pharmaceutical formulations. The disadvantages of the reported HPLC methods [20, 24] are low sensitivity, lack of accuracy, lesser precision, use of internal standard, use of triple solvent system and long retention time (>5 minutes) that leads to a longer runtime for a single sample. Besides, the method reported by Gumieniczek and Przyborowski [24] was not fully validated.

The aim of the present work to develop and validate a rapid, sensitive, precise and accurate HPLC method, without the use of internal standard, for determination of CZP in bulk and in pharmaceutical dosage forms.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals and solvents were analytical and HPLC grade, respectively and used as received. All solutions were prepared in Milli-Q water from Merck Specialties Private Ltd, Hyderabad, India. Pharmaceutical grade cilazapril was obtained as gift sample from the Hetero Drugs Limited, Hyderabad, India. Inhibace tablets (claimed to contain 2.5 mg and 5.0 mg CZP per tablet, manufactured by Roche Scientific Company India Pvt.Ltd, Mumbai, India) were purchased from local pharmacy store. Potassium formate and methanol were obtained from Sd fine-chem Ltd, Mumbai, India. Formic acid was obtained Nice Chemicals (P) Ltd, Kochi, India.

2.2 Equipment and Chromatographic Conditions

Samples were analyzed on an Shimadzu HPLC class VP series isocratic HPLC system (Shimadzu Corporation, Kyoto, Japan), attached with a LC-10 AT pump, a G1312A vacuum degasser, and an variable wavelength programmable UV/Visible detector SPD-10A. The detector wavelength was fixed at 227 nm

and peak areas were integrated automatically using the Shimadzu class VP series version 5.03 computer software program. Other apparatus included a Shimazdu (Tokyo, Japan) electronic weighing balance model BL 220 H for weighing the samples and an Elico pH meter (Hyderabad, India) LI 120 model.

A Cyano Column (150 mm × 4.6 mm I.D., 5 μm particle size) was maintained at ambient temperature. The mobile phase was composed of a mixture of formate buffer (pH 3.75, adjusted with formic acid) and methanol in the ratio of 50:50 *v/v*. The formate buffer was prepared by mixing 50 mL of 4 M formic acid and 2 gm of potassium formate in a total volume of 1.0 L. Prior to use, the mobile phase was filtered and degassed via 0.45 μm membrane filter. The flow rate of the mobile phase was set at 1.2 mL/min. Measurements were made with 20 μL of injection volume. The total run time was about 10 minutes.

2.3 Preparation of Standard Solutions

Standard stock solution of CZP (1 mg/mL) was prepared in mobile phase. Series of working standard solutions were diluted with the same solvent to the desired concentration for linearity (2, 5, 10, 20, 40, 60, 80, 100, 150 and 200 μg/mL), system suitability studies (100 μg/mL), sensitivity (2 μg/mL), selectivity (100 μg/mL), accuracy (2, 100, 200 μg/mL), precision (2, 100, 200 μg/mL) and robustness (2, 200 μg/mL).

2.4 Preparation of Tablet Sample Solutions

To determine the concentration of CZP in tablet dosage forms (label claim: 2.5 and 5 mg per tablet), 50 tablets were weighed, their mean weight was determined and they are finely powdered. A precisely weighed powder sample equivalent to 50 mg of CZP was transferred into a 50 mL volumetric flask containing 25 mL mobile phase. The content of the flask was sonicated for 15 min and the resulting solution was filtered through 0.45 μm membrane filter. The volume was completed with mobile phase and the solution reached 1 mg/mL (stock solution). An appropriate aliquot of the stock solution was transferred into a volumetric flask and diluted with the mobile phase to obtain concentration equal to 100 μg/mL of CZP. The solution was filtered through 0.45 μm membrane filter before analysis.

3. Results and Discussion

3.1 Method Development

To develop an efficient and simple HPLC method for the assay of CZP, preliminary tests were conducted to select the suitable and optimum conditions. HPLC parameters, such as detection wavelength, ideal mobile phase and their proportions, flow rate and column temperature were carefully studied. The HPLC parameters were finally chosen based on the criteria of peak properties like height, area, retention time and peak symmetry.

The ultraviolet spectra of CZP dissolved in mobile phase showed the maximum absorption wavelength at 227 nm. Therefore, 227 nm was selected as detection wavelength. Different combinations of toluene, methanol, propanol, dichloromethane, orthophosphate buffer & formate buffer were tested. The optimum condition at formate buffer-methanol (50:50, *v/v*), was reached. The mobile phase with different pH (3-7) was tried. The best peak shape and tailing factor with reasonable analysis time for CZP was accomplished at pH 3.75. Therefore, mobile phase with pH 3.75 was chosen. Two different stationary phases were investigated and the peak properties were compared. The best peak properties were obtained by using Cyano Column (150 mm x 4.6 mm, 5 μm particle size). The mobile phase with flow rates in the range 0.8-1.6 mL/min was investigated. At the flow rate 1.2 mL/min, symmetric and well retained peak was obtained. Therefore, the flow rate 1.2 mL/min was selected. The effect

of temperature on the column efficiency was studied. Different temperatures of 15°C-35°C with 5°C increments were evaluated. The chromatograms were recorded. It was found that the temperature had a negligible influence on peak shape, therefore room temperature was chosen. Under the described chromatographic conditions, the retention time was 2.67 min.

3.2 HPLC Method Validation

After the successful optimization, the optimized HPLC method was validated in accordance to the International Conference on Harmonization guidelines [25]. Parameters such as system suitability, selectivity (components of mobile phase and excipients of tablet interferences), sensitivity (LOD and LOQ), linearity range, accuracy (recovery), precision (repeatability and intermediate precision) and robustness were all validated.

3.2.1 System suitability

The system suitability was determined by injecting six replicates of the CZP standard solutions (100 μg/mL) and analyzing for its retention time, peak area, theoretical plates, plates per meter, height equivalent to theoretical plate, and peak asymmetry. The system suitability results revealed %RSD of less than 1.1% for all the parameters. As shown in Table 1, the proposed method meets the accepted requirements.

Table 1. System suitability parameters of the proposed method.

Parameter	Mean value*	RSD (%)
Retention Time (t) (Min)	2.669	1.080
Peak area	2825215	0.420
Theoretical Plates (n)	3436	0.922
Plates per Meter (N)	13744	0.906
Height equivalent to theoretical plate (HETP) (mm)	$9.0x10^{-7}$	1.026
Peak asymmetry	1.084	0.886

* Average of five determinations

3.2.2 Selectivity (components of mobile phase and excipients of tablet interference)

Blank mobile phase (without drug), CZP standard and tablet sample solutions were all injected into the HPLC column to assure the selectivity of the optimized method. A comparison of the retention time of CZP in tablet sample solution and in the standard solution was exactly the same. Figures 1 and 2 showed that there were no interferences at the retention time for CZP due to the excipients in tablet dosage forms. There were no peaks in mobile phase blank (Figure 3). Hence, the proposed method was found to be selective and is suitable for the quantification of the CZP in tablet dosage forms.

3.2.3 Sensitivity

The sensitivity of the proposed method was investigated via measurement of the limit of detection (LOD) and limit of quantitation (LOQ) at a signal-to-noise ratio of 3 and 10, respectively. It was achieved by injecting working standard solution of CZP with known concentration (2 μg/mL) into the HPLC column five times. The LOD and LOQ were found to be 0.025 and 0.083 μg/mL, respectively. These values suggest that the developed method is sensitive to quantify CZP.

3.2.4 Linearity

A ten-point (2, 5, 10, 20, 40, 60, 80, 100, 150 and 200 μg/mL) calibration curve was prepared. The peak area for each concentration was obtained by injecting 20 μl of the CZP working standard solution into the column. Calibration curves were plotted by taking the mean peak area on the Y-axis and the concentration of CZP (μg/mL) on the X-axis. The linearity was assessed by the least square regression method.

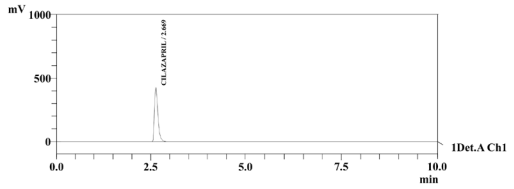

Figure 1. Chromatogram of CZP in standard solution.

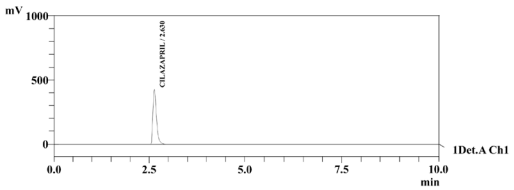

Figure 2. Chromatogram of CZP in tablet sample solution.

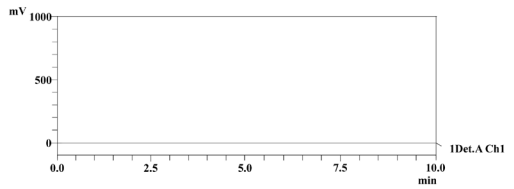

Figure 3. Chromatogram of mobile phase blank (with out drug).

The regression line confirmed linearity in the tested range, 2-200 µg/mL (Figure 4). The regression line was linear with R^2 of 0.9995. The method parameters and regression data are shown in Table 2.

3.2.5 Precision and accuracy

The precision and accuracy of the method was determined by intra-day (repeatability) & inter-day (intermediate) assay and was expressed as relative standard deviation and recovery percentage, respectively. Five replicate injections of the standard solutions of CZP at concentrations 2, 100 and 200 µg/mL prepared. The intra-day variation was assessed over one

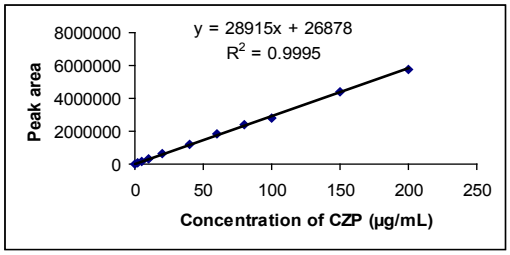

Figure 4. Linearity of the proposed method.

Table 2. Regression data of the proposed method.

Parameter	Value
Linearity (µg/mL)	2-200
Regression equation: y = mx+c	y = 28915x + 26878
Intercept (c)	26878
Slope (m)	28915
Regression coefficient (R^2)	0.9995

y = peak area, x = concentration of CZP in µg/mL

day, while inter-day assay was carried out over 3 days. Table 3 provides data obtained from the intra-day & inter-day experiments. The relative standard deviation values for intra-day and inter-day precision were in the range of 0.595-0.889% and 0.685-0.900% (acceptance criteria proposed: RSD-not more 2.0%), respectively. The percent recovery values for intra-day and inter-day accuracy were in the range of 98.00-99.98% and 99.01-100.50% (acceptance criteria proposed: %Recovery range- 80 to 120%), respectively. The results (Table 3) indicating that the method has sufficient precision and accuracy.

3.2.6 Recovery

Accuracy was further determined by the recovery study of known concentration of CZP standard added to a preanalysed tablet sample solution. The recovery study was performed five times. The average recovery data (Table 4) of CZP showed results between 99.60% and

Table 3. Intra and inter-day assay of the proposed method.

Concentration of CZP (μg/mL)		RSD (%)	Recovery (%)
Taken	Found* ± SD		
Intra-day assay			
2	1.96± 0.013	0.663	98.00
100	99.98± 0.595	0.595	99.98
200	199.96 ± 1.778	0.889	99.98
Inter-day assay			
2	2.01 ± 0.016	0.796	100.50
100	100.02 ± 0.686	0.685	100.02
200	199.94± 1.800	0.900	99.97

* Average of five determinations

Table 4. Recovery data of the proposed method.

Labelled claim (mg)	Concentration of CZP (mg)		RSD (%)	Recovery (%)
	Added	Found* ± SD		
2.5	1.25	3.74 ± 0.026	0.695	99.73
5.0	2.5	7.47± 0.061	0.815	99.60

* Average of five determinations

99.73% with relative standard deviation between 0.695% and 0.815%.

3.2.7 Robustness

The robustness of the method was illustrated by assaying the CZP standard solutions (2 and 200 μg/mL), when mobile phase flow rate (±0.2 mL/min), mobile phase composition ratio (±2%), mobile phase pH (±0.2) and detection wavelength (±1nm) were deliberately varied. The results (Table 5) obtained from the assay of the CZP standard solutions were not affected by the varying conditions; recovery values were 99.00-100.50% with relative standard deviation 0.700-0.953%. The proposed method thus remained unaffected by slight but deliberate changes in the analytical conditions (Table 5).

Table 5. Robustness of the proposed method.

Variable	Concentration of CZP (μg/mL)		Recovery (%)	RSD (%)
	Taken	Found* ± SD		
Mobile phase (50:50 ± 2%)**	2	2.01 ± 0.016	100.50	0.796
	200	200.02± 1.402	100.01	0.700
pH of the mobile phase (3.75±0.2)	2	1.95 ± 0.015	99.50	0.769
	200	199.92 ± 1.600	99.96	0.800
Flow rate (1.2 ± 0.1 mL/min)	2	1.93 ± 0.015	99.00	0.777
	200	197.79± 1.885	99.89	0.953
Detection wavelength (227 ± 1 nm)	2	1.93 ± 0.018	99.00	0.932
	200	198.94± 1.789	99.87	0.895

* Average of three determinations

**mobile phase composition: formate buffer and methanol

3.3 Application of the Method to Assay of CZP Content in Pharmaceutical Dosage Forms

The above-mentioned validation results indicated that the proposed method gave satisfactory results with CZP in bulk. Therefore its pharmaceutical dosage forms (Inhibace tablets) were subjected to the assay of their CZP contents by the proposed method. For this purpose, 20 μL of tablet sample solution prepared in the section 2.4, "Preparation of Tablet Sample Solutions", was injected into the HPLC system. The chromatograms were recorded and the peak area was calculated. The percent recovery values are determined. The label claim percentages were in the range of 99.20 ± 0.562% and 99.96 ± 0.760% (Table 6). Good recoveries with low relative standard deviation values indicate the non interference of excipients commonly present in the pharmaceutical dosage form.

3.4 Comparison with the Official Method

The results obtained above were compared with that obtained from the official potentiometric

titration method [7] by statistical analysis with respect to the accuracy (by student *t*-test) and precision (by variance *F*-test). At 95% confidence level, no significant differences were found between the calculated and theoretical values of *t*- and *F*-tests confirming similar accuracy and precision in the determination of CZP by both methods (Table 6).

4. Conclusion

A simple and rapid HPLC analytical method equipped with UV detection at 227 nm has been developed for the quantification of CZP. The results of validation undertaken according to the International Conference on Harmonization guidelines reveal that the method is linear, sensitive, selective, accurate, precise and robust. The method is appropriate for the routine analysis of CZP in either bulk or in pharmaceutical dosage forms.

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Table 6. Comparison between proposed and official methods.

Method	Labelled claim (mg)	Found* (mg) ± SD	Recovery (%)	RSD (%)
Official	2.5	2.490 ± 0.019	99.60	0.763
	5.0	4.990 ± 0.026	99.80	0.521
Proposed	2.5	2.499 ± 0.019	99.96	0.760
		t* value*= 1.28, ***F* value*****= 3.59		
	5.0	4.980 ± 0.028	99.20	0.562
		t* value*= 1.46, ***F* value*****= 4.16		

* Average of five determinations

**Tabulated *t*-value at 95% confidence level is 2.306

***Tabulated *F*- value at 95 % confidence level is 6.390

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