

LIQUID CHROMATOGRAPHIC DETERMINATION OF ALPRAZOLAM WITH ACE INHIBITORS IN BULK, RESPECTIVE PHARMACEUTICAL PRODUCTS AND HUMAN SERUM

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ABSTRACT: Present study describes simple and fast liquid chromatographic method using ultraviolet detector for simultaneous determination of anxiety relief medicine alprazolam with ACE inhibitors i.e; lisinopril, captopril and enalapril employing Prospher Star C₁₈ (25 cm, 0.46 cm, 5 μm). Separation was achieved within 5 min at ambient temperature via methanol:water (8:2 v/v) with pH adjusted to 2.9, monitoring the detector response at 220 nm. Optimum parameters were set up as per ICH (2006) guidelines. Calibration range was found out to be 0.312-10 μg mL⁻¹ for alprazolam and 0.625-20 μg mL⁻¹ for all the ACE inhibitors with correlation coefficients >0.998 and detection limits 85, 37, 68 and 32 ng mL⁻¹ for lisinopril, captopril, enalapril and alprazolam respectively. Intra-day, inter-day precision and accuracy of the assay were in acceptable range of 0.05-1.62% RSD and 98.85-100.76% recovery. Method was determined to be robust and effectively useful for the estimation of studied drugs in dosage formulations and human serum without obstruction of excipients or serum components.

Keywords: Alprazolam, ACE inhibitors, RP HPLC

1. INTRODUCTION

Alprazolam (figure 1), chemically 8-chloro-1-methyl-6-phenyl-4H-s-triazolo [4,3-I] [1,4] benzodiazepine is prescribed to care for anxiety disorders, also given for panic disorder and nausea due to chemotherapy. It binds to specific sites on the gamma-amino-butyric acid (GABA) receptor.

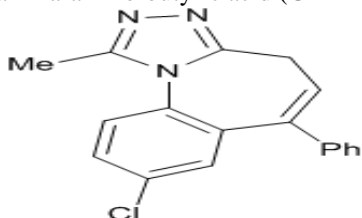


Figure 1: Chemical structures of alprazolam

Literature survey enlightens various articles published on liquid chromatographic methods for its determination either alone or with multiple analytes in dosage formulations and body fluids. It has been determined with fluoxetine hydrochloride [1], sertaline [2] and propranolol hydrochloride [3] in pharmaceutical formulations and with oxazepam, and diazepam in human urine samples [4]. Our previous study describes liquid chromatographic method for simultaneous analysis of ALP with rosuvastatin and diclofenac sodium [5]. Angiotensin-converting-enzyme inhibitor or ACE inhibitor is a drug class used for several decades to cure hypertension [6] and congestive heart failure [7] by dilation of blood vessels, which results in lowering of blood pressure. Several analytical methods like voltammetric [8], kinetic [9], HPLC-UV [10], LC-MS/MS [11] and spectrophotometric method [12] have been reported for the analysis of ACE inhibitors. Our research colleagues reported numerous techniques for ACE inhibitors analysis including LC determination of rosuvastatin with ACE inhibitors [13], lisinopril with statins [14], lisinopril and NSAIDs [15] and captopril and H₂-receptor antagonist [16].

Present study is designed to develop and validate a well-ordered isocratic reverse phase liquid chromatographic method with UV-detection for the simultaneous analysis of alprazolam with ACE inhibitors at isosbestic point. Study

involved easily available and economic chemicals and reagents. ICH guidelines were followed for method validation [17]. The proposed method can fruitfully be applied for the estimation of studied drugs in dosage formulations and human serum without obstruction of excipients or serum components.

2. MATERIALS AND METHODOLOGY

2.1. Materials and reagents

Alprazolam (Genix Pharma Pvt Ltd), lisinopril (Atco Laboratories Ltd.), captopril (BMS Pvt. Ltd.) and enalapril (Merck Sharp and Dome Ltd.) were kindly provided by respective pharmaceuticals. Nerum[®] 0.5 mg, Lisinopril[®] 5 mg, Capoten[®] 25 mg and Renitec[®] 10 mg were bought from pharmacy. Analytical grade solvent including methanol and acetonitrile were obtained from Merck, Germany. Double distilled de-ionized water prepared by using Millipore ultra-pure water system was utilized all through the analysis.

2.2. Instrumentation

Analysis was performed on Shimadzu LC-20 AT VP solvent delivery pump, rheodyne manual injector fitted with a 20 μL loop and a Shimadzu SPD-20A UV visible detector. Separation was accomplished on Prospher Star C₁₈ (25 cm, 0.46 cm, 5 μm) column, data integration was performed on Shimadzu CBM-102 Communication Bus Module and the data was acquired and computed on Shimadzu Class-GC 10 software (version 2). λ_{max} of each analyte was determined on Shimadzu 1800 UV-visible spectrophotometer.

2.3. Calibration curves

50 μg mL⁻¹ stock solutions of alprazolam and studied ACE inhibitors were diluted to 0.312-10 μg mL⁻¹ for ALP and 0.625-20 μg mL⁻¹ for all the ACE inhibitors. These working standard solutions were made ready on the day one and analyzed on each day of analysis for inter-day and intra-day precision of method. Prior to injecting into the system, samples were degassed and filtered (0.45 μm pore size).

2.4. Sample preparation

2.4.1. Pharmaceutical formulations

Twenty tablets of Nerum[®] 0.5 mg, Lisinopril[®] 5 mg, Capoten[®] 25 mg and Renitec[®] were separately granulated in pestle and mortar. Finely triturated content equivalent to 1 mg mL⁻¹ of active pharmaceutical ingredient were separately dissolved in small volume of diluent, mixed well, allowed to stand for 30 min and then sonicated in order to get complete extraction of drugs. The contents of each flask were filtered to remove insoluble inactive ingredients of tablets and finally the volumes were completed with diluent. Into a series of 25 mL volumetric flask, aliquot of each drug was transferred and volume was made up to mark to get the required concentration. The solutions were injected into the system after micro filtration through 0.45 µm millipore filter paper.

2.4.2. Drug serum sample

Blood specimen from human donor was drawn from antecubital area of the arm at Fatmid Foundation Karachi and readily transferred in to an sterile EDTA glass tube. The tube was centrifuged at 1600 x g for 10 min at 4°C. The straw-colored supernatant was treated with 9.0 mL acetonitrile and vortexed for few minute followed by centrifugation for 10 minutes at 10,000 rpm. The transparent serum solution in supernatant was transferred in container. Small amount of serum was spiked with respective concentration of lisinopril, captopril and enalapril and alprazolam and analyzed.

2.5. Chromatographic conditions

Chromatographic separation was achieved at ambient temperature via methanol:water (8:2 v/v) with pH set to 2.9 by means of *o*-phosphoric acid (85%), monitoring the detector response at 220 nm. All the analytes were eluted isocratically adjusting the flow rate 1.0 mL min⁻¹. All the solutions were injected into the system after micro filtration through 0.45 µm millipore filter paper and degassed by sonicator.

2.6. Method development and experimental condition optimization

The most suitable chromatographic conditions were established by assessing preliminary parameter appropriate for analysis. At first, λ_{max} of each analyte was measured on Shimadzu 1800 UV-visible spectrophotometer and the isosbestic point. Diverse ratios of methanol-water and acetonitrile-water with variable pH were examined at ambient temperature for simultaneous investigation of studied drugs to obtain best separation and good resolution. The appropriate flow rate was set by monitoring the eluent in the range of 0.8-1.2 mL min⁻¹.

2.7. Method validation

The developed analytical method was authenticated by validation following the ICH 2006 guidelines [17] for linearity, precision, accuracy, specificity and selectivity, system suitability, robustness, detection and quantitation limits. The system suitability was assessed on every day of validation; specificity studies of method was conducted by injecting the blank, placebo solution, solution of dosage formulation and serum sample spiked with analytes. To establish linearity, six concentration level for API and serum were prepared and chromatographed at isosbestic point, regression characteristics including slope, intercept,

correlation coefficient, standard error and standard error estimates were evaluated. Accuracy and inter-day, intra-day precision were assessed in terms of percent recovery of analytes in pharmaceutical formulation and serum and in terms of percent relative standard deviation for five times determinations at each concentration level for reference standard and three serum samples respectively. The LLoD and LLoQ are the concentration where signal to noise ratio was three and ten times to the baseline noise respectively. For robustness studies, a variety of chromatographic parameters like mobile phase composition, pH and flow rate were deliberately altered and their consequences on analytical results were observed.

3. Results and discussion

Hypertension is one of a chronic medical condition commonly observed in patients suffering from other disease. It is a condition with rare symptoms in which a frequently high pressure greater than 140 over 90 mmHg is applied by the blood to blood vessels. Physicians prescribe ACE inhibitors along with alprazolam to the patient suffering from stress like anxiety. Moreover, to treat panic disorder after chemotherapy, alprazolam is prescribed to hypertensive patient who is taking ACE inhibitors. Attempt has, therefore, been made to develop and validate simultaneous determination of co-prescribed pharmaceutical ingredients in bulk, dosage formulations and in human serum.

3.1. Method development and optimization

Preliminary test were performed to set up the optimized analytical method for determination of alprazolam with ACE inhibitors simultaneously in bulk drug, pharmaceutical formulations and human serum. All the studied analytes showed good solubility in acetonitrile, methanol and in aqueous solutions. To select suitable mobile phase, various ratios of acetonitrile:water and methanol:water with varying pH were employed with continuous monitoring of detector response. It was experienced that retention times of drugs coincide with each other in many of the mobile phases. The best separation and resolution was achieved in 80:20 methanol:water with pH 2.9; while adjusting the detector wavelength at 220 nm for analysis. The UV spectra and chromatograms of alprazolam and studied ACE inhibitors are represented in figures 2 and 3 respectively.

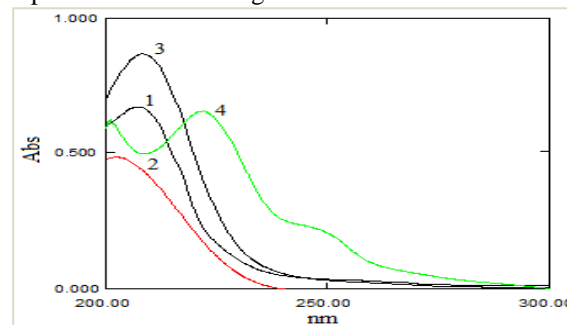


Figure 2 Absorption spectra of lisinopril¹, captopril², enalapril³ and alprazolam⁴

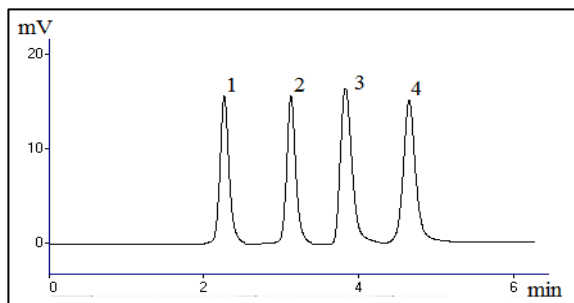


Figure 3: Representative chromatogram of lisinopril¹, captopril², enalapril³ and alprazolam⁴

3.2. Method validation

To ensure the reliability at isosbestic point of 220 nm, ICH guidelines [17] were followed for validation of developed analytical method for simultaneous determination of alprazolam and ACE inhibitors. The studied validation parameters included specificity and robustness of method, system suitability, selectivity, linear range, precision and accuracy, detection and quantitation limits.

3.2.1. System suitability test

While doing system suitability testing, we have come to conclusion that minute variation in chromatographic parameters does not distress the suitability of system. Mobile phase compositions 78:22 to 82:18 with pH range 2.8-3.0 showed no drastic change in analytical results. Similarly, satisfactory outcome was obtained with flow rate 0.9-1.1 mL min⁻¹. System suitability parameters including capacity factor, theoretical plates, resolution and separation factor are presented in Table 1.

Table 1: System suitability parameters

Drug	t _R	k'	N	T	Rs
Lisinopril	2.24	0.18	2845	1.60	0.46
Captopril	3.18	0.45	3354	1.46	1.61
Enalapril	3.82	1.19	3992	1.32	0.92
Alprazolam	4.48	0.24	2904	1.66	-

Retention time (t_R), Capacity factors (k'), Theoretical plates (N), Tailing factor (T), Resolution (Rs)

3.2.2. Specificity

Inevitable fact based on experimental analysis was ascertained by acquiring the chromatograms of serum blank and excipients solution and also the serum solution spiked with samples and solutions of pharmaceutical formulation.

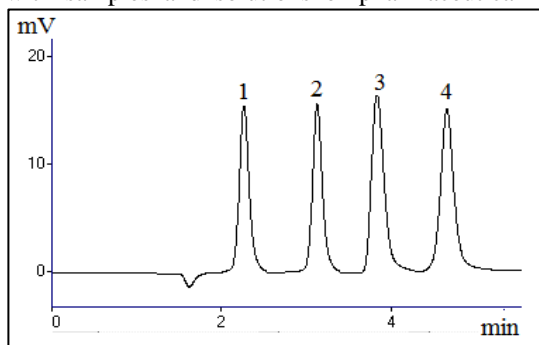


Figure 4 Chromatograms of serum sample spiked with lisinopril¹, captopril², enalapril³ and alprazolam⁴

From figure 4, it is overt that there was no interfering peak of serum or excipients which would interrupt at the same retention time of alprazolam and ACE inhibitors signifying the specificity of method.

3.2.3. Linearity

Calibration curves of alprazolam and ACE inhibitors between concentration and detector response were designed, which were obtained linear within the concentration range of 0.312-10 µg mL⁻¹ for alprazolam and 0.625-20 µg mL⁻¹ for lisinopril, captopril and enalapril respectively with correlation coefficient greater than 0.998 in each case. The regression data is represented in table 2.

3.2.4. Accuracy and precision

Percent recovery values of lisinopril, captopril, enalapril and alprazolam in Lisinopril[®] 5 mg, Capoten[®] 25 mg, Renitec[®] 10 mg and Nerum[®] and precision in terms of percent RSD with respect to repeatability and intermediate precision were examined in the concentration ranges of 0.312-10 µg mL⁻¹ for alprazolam and 0.625-20 µg mL⁻¹ for lisinopril, captopril and enalapril respectively.

Recovery values were found to be in the range of 99.13-100.50% and 98.84-100.76% for dosage formulations and human serum respectively (Table 3) and the RSD were determined be 0.08-1.62% for inter-day and 0.05-1.44% for intra-day precision of method (Table 4).

3.2.5. Detection and quantitation limits

The LLoD and LLoQ values are the concentration levels appearing as detector response three and ten times to the baseline noise. It indicates the sensitivity of method evaluated from the slope of calibration curve and standard deviation. The LLoD was found to be 85, 37, 68 and 32 ng mL⁻¹ and LLoQ was calculated as 257, 111, 206 and 98 ng mL⁻¹ for lisinopril, captopril, enalapril and alprazolam respectively (Table 2).

3.2.6. Robustness

The procedure was repeated many times by altering one chromatographic parameter like mobile phase composition, pH, flow rate and wavelength at a time while keeping the others constant. The subsequent effect was monitored after each analysis. Theoretical plates obtained in between 2000-8000 and tailing factor less than 2 specify the robustness of method. The data is presented in Table 5.

3.3. Application of proposed method

Liquid Chromatographic method for concurrent determination of alprazolam and ACE inhibitors was validated which proved to be reliable with high precision and accuracy. Consistency was confirmed by repeating the method several times with some amendment in proposed chromatographic parameters. Statistical results provided evidences that method can withstand with minor variation in parameters. Moreover, the proposed method is suitable for estimation of alprazolam and studied ACE inhibitors in

Table 2: Regression characteristics and sensitivity of the method

Drug	Linearity $\mu\text{g mL}^{-1}$	Intercept	Slope	R ²	LOD	LOQ	LOD	LOQ
					(ng mL ⁻¹)			
					Bulk drug		Serum	
Lisinopril	0.625-20	39546	13424	0.9987	85	257	47	114
Captopril	0.625-20	28746	18850	0.9982	37	111	15	46
Enalapril	0.625-20	39861	20294	0.9984	68	206	30	100
Alprazolam	0.312-10	75439	42487	0.9986	32	98	21	64

Table 3: Recovery pharmaceutical formulations and in serum

Lisinopril		Captopril		Enalapril		Alprazolam	
Conc	%Rec	Conc	%Rec	Conc	%Rec	Conc	%Rec
<i>Pharmaceutical formulation</i>							
0.625	99.58	0.625	99.82	0.625	100.50	0.315	99.64
1.25	99.13	1.25	100.18	1.25	99.43	0.625	99.86
2.5	100.18	2.5	99.88	2.5	99.52	1.25	100.14
5	99.65	5	99.85	5	99.96	2.5	99.98
10	99.73	10	100.10	10	100.04	5	100.09
20	100.02	20	100.03	20	99.98	10	99.97
<i>Serum</i>							
1.25	100.76	1.25	99.13	1.25	98.85	0.625	99.24
2.5	99.46	2.5	99.52	2.5	99.83	1.25	99.92
5	99.60	5	99.84	5	100.01	2.5	99.49

Table 4: Precision of the proposed method

Lisinopril			Captopril			Enalapril			Alprazolam		
Conc	Interday	Intraday	Conc	Interday	Intraday	Conc	Interday	Intraday	Conc	Interday	Intraday
	%RSD			%RSD			%RSD			%RSD	
0.625	0.83	0.57	0.625	0.17	1.03	0.625	0.15	0.89	0.315	0.24	0.55
1.25	0.90	0.05	1.25	0.53	0.81	1.25	0.79	0.31	0.625	0.57	0.88
2.5	0.43	0.96	2.5	0.19	0.71	2.5	0.63	0.29	1.25	0.56	0.38
5	1.62	0.35	5	0.80	0.13	5	0.08	0.22	2.5	0.68	0.43
10	0.80	0.41	10	0.28	0.29	10	0.48	0.25	5	0.10	0.33
20	0.21	0.36	20	0.77	1.44	20	1.06	0.10	10	1.01	0.42

pharmaceutical formulations and human serum without obstruction of excipients or serum components with high percent recoveries and relative standard deviation (Table 3 and 4) substantiating the applicability of method for everyday analysis with best resolution and separation.

4. CONCLUSION

The presented analytical method describes a safe and least time consuming LC-UV technique for simultaneous measurement of alprazolam and ACE inhibitors in bulk drug, tablet formulation and human serum. Furthermore, the method was validated according to ICH guidelines which confirmed that the proposed method is specific, linear, robust, precise and accurate. The benefits of developed method are its less consumption of reagent, utilization of very small quantity of sample, high sensitivity and less retention times of analytes.

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