



High Performance Liquid Chromatographic Method for Simultaneous Determination of Alprazolam with Antihistamines in Bulk Drug, Pharmaceutical Formulation and Human Serum

S. AKRAM, S. N. ALI⁺⁺, A. QAYOOM, S. IQBAL*, N. NAZ, I. MEMON**

Department of Chemistry, NED University of Engineering and Technology, Karachi.

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Abstract A high performance liquid chromatographic method with UV detection for simultaneous quantification of alprazolam with antihistamine was developed and validated following the ICH guidelines. Separation was achieved on Shimadzu Shim-pack CLC-ODS (M) 25 M column employing mobile phase 80:20 methanol-water with pH 3.5 at 230 nm adjusting flow rate 1.0 mL min⁻¹. The linear concentration ranges were 0.2-12.5 µg mL⁻¹ for alprazolam and 0.4-25 µg mL⁻¹ for antihistamines with correlation coefficient greater than 0.998 and detection limits 2.85, 6.92, 10.56, 4.9 ng mL⁻¹ for promethazine, levocetirizine, alprazolam, and loratadine respectively. Validation showed RSD < 2% and 98.05-101.89% recovery. Proposed method was successfully applied for quantification of studied drugs in pharmaceutical formulations without excipients interference and also in human serum.

Keywords: Alprazolam, Antihistamine, Method Validation, HPLC, Pharmaceutical Formulation

1. INTRODUCTION

Analytical method development and validation is a major step in quality assessment of chemical analysis in material science (Zoonen *et. al* 1999). Numerous methods have been developed and validated in different domain of science according to the application e.g. toxicological, environmental, food science and forensic etc. (Tavernier *et. al* 2004). Statistical data analysis is also an important prerequisite to evaluate the credibility and reliability of analytical measurement. Accuracy and precision of measurement together provide the picture of an analytical method whether it is fit for purpose to meet the requirement (González *et. al* 2007). There are a number of internationally recognized organizations which provide guidelines regarding method development and validation including United States Food and Drug Administration (FDA) (US Food and Drug Administration 1994, US Food and Drug Administration (2001), International organization for standardization (International organization for standardization 1994, International organization for standardization 1995), United states pharmacopeia (United States Pharmacopoeia 2007, United States Pharmacopoeia 1994), International Conference on Harmonization (International organization for standardization 1994), Cooperation on International Traceability in Analytical Chemistry (Chemistry C-ooITiA 2005) and Association of Official Analytical Chemists (Official Methods of Analysis 2005, Official Methods of Analysis 1990). Importance of method

development in pharmaceuticals to establish the identity, physical characteristics, purity and potency of analyte (Araujo 2009).

Present study is about development and validation of a method for simultaneous liquid chromatographic determination of alprazolam (Fig.) with three antihistamines of wide use promethazine, levocetirizine and loratadine (Fig.1). Validation has been performed following ICH guidelines (International organization for standardization 1994). Applicability of method has been established by analyzing the studied analytes in pharmaceutical formulation and human serum without any observable response of interfering species.

2. EXPERIMENTAL

2.1. Reagents and Chemicals

Standard active pharmaceutical ingredient alprazolam and pharmaceutical formulation of Xanax[®] 0.5 mg were obtained from Pfizer Pakistan Ltd. Promethazine, levocetirizine and loratadine were obtained from Eros Pharmaceuticals Pvt. Ltd. Pakistan and commercial formulations Theoclate Avomine[®] 25 mg (sanofi-aventis Pakistan Limited), T-Day[™] 5 mg (Novartis) and Jardin 10 mg (High-Q) were purchased from a local pharmacy located in Karachi. Analytical grade methanol and orthophosphoric acid were purchased from Merck (Darmstadt, Germany). Double distilled deionized water was used throughout.

⁺⁺Corresponding author: Email: saeeda_khowaja@hotmail.com

*Department of Chemistry, Jinnah Govt. College Nazimabad Karachi, Karachi.

**Department of Chemical Engineering, NED University of Engineering and Technology, Karachi

2.2. Instrumentation

Separation and analysis of studied analytes were achieved by using Shimadzu Corporation, Japan liquid chromatograph along with LC-20AT binary gradient solvent delivery pump AOC-20, auto-sampler SIL-20AHT/20ACHT UFLC with 1 μ L sample volume auto injector AOC-20i, UV-visible detecting device SPD-20A/20AV coupled with Shimadzu CBM-20A Communication Bus Module and LC solution GPC software (version 1.25, Shimadzu Corporation, Japan) for data acquirement and interpretation. Shimadzu-1800 UV/Vis spectrophotometer was used to determine isosbestic point for analysis.

2.3. Chromatographic parameters

Liquid chromatographic analysis were performed by using Shimadzu Shim-pack CLC-ODS (M) 25M column (4.6mm i.d. $\times$ 25cm) with optimized parameters including 80:20 v/v methanol: water mobile phase composition, pH 3.5 adjusted by o-phosphoric acid with detection wavelength 230 nm at 1.0 mL min⁻¹ elution rate. Mobile phase was filtered and degassed by using 0.45 μ m pore size filter with millipore vacuum filter system and an ultrasonic bath (Elma LC-30H model Singen, Germany).

2.4. Standard solution preparation

10 mg standard of each analyte including alprazolam, promethazine, levocetirizine and loratadine were weighed accurately, dissolved in small volume of diluent and finally made up to mark having final concentration 100 μ g mL⁻¹. All solutions were accompanied with filtration and sonication before analysis.

2.5. Calibration standards preparation

Calibration standards were prepared by serial dilution of standard solutions of alprazolam, promethazine, levocetirizine and loratadine in 10 mL volumetric flask by transferring required aliquot of standard solutions 0.2-12.5 μ g mL⁻¹ for alprazolam and 0.4-25 μ g mL⁻¹ for promethazine, levocetirizine and loratadine. Finally each solution was filtered in 1.5 mL vial through 0.45 μ m pore size membrane and placed into AOC-20i auto injector for analysis.

2.6. Pharmaceutical formulations

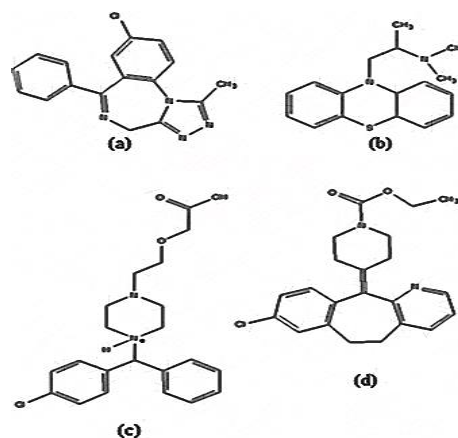
Pharmaceutical formulations with brand name Xanax[®] (0.5 mg), TheoclaveAvomine[®] (25 mg), T-Day[™] (5 mg) and Jardin (10 mg) were finely triturated in pestle and mortar. Accurately weighed analyteequivalent to 10 mg active pharmaceutical ingredient was dissolved in small volume of diluent and sonicated for complete dissolution. Solutions with final concentration 100 μ g mL⁻¹ were filtered with 0.45 μ m membrane and further analyzed.

2.7. Drug Serum

Blood of a healthy person was collected at Fatmid Foundation Karachi, with pre-treatment of plasma separation by centrifugation at 1600 $\times$ g for 10 min at 4°C. For final clear solution of serum, 9.0 mL acetonitrile with 1.0 mL of plasma was mixed and again centrifuged at speed of 10,000 rpm for 10 min. Alprazolam, promethazine, levocetirizine and loratadine were spiked with appropriate concentration in clear supernatant solution for the chromatographic study of drugs in serum solution at isosbestic point.

2.8. Method validation

ICH Q2 (R1) guidelines were followed to validate developed method by the study of system suitability, linearity, precision, accuracy, limit of detection and quantification, robustness, selectivity and specificity.



Alprazolam^a, promethazine^b, levocetirizine^c, loratadine^d.

System suitability of newly developed method was measured by six repeated analysis of single sample. Specificity was analyzed by determining the interference of unwanted species by injecting standard solutions, spiked excipients sample, solution of commercial formulations and spiked serum solution. Linearity of method was assessed in the concentration range 0.2-12.5 μ g mL⁻¹ for alprazolam and 0.4-25 μ g mL⁻¹ for promethazine, levocetirizine and loratadine. To evaluate inter and intra-day precision, solution in the same concentration ranges were analyzed by injecting six replicates. Accuracy was assessed by determining the recovery of active pharmaceutical ingredient in Xanax[®], TheoclaveAvomine[®], T-Day[™] and Jardin. Sensitivity of method was established with the relation of standard deviation and slope of calibration curve as LOD = 3.3 SD/ α and LOQ = 10 SD/ α respectively. Robustness studies were carried out by making deliberate changes in chromatographic parameters including mobile phase composition 80:20 $\pm$ 2mL, pH 2.6-3.6 and flow rate in range of 0.7-1.2 mL min⁻¹.

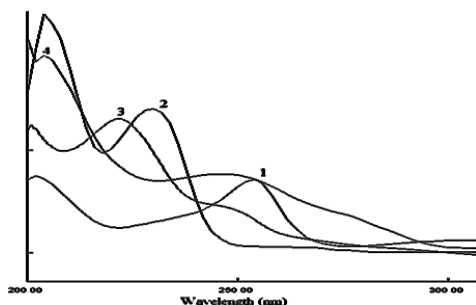


Fig. 2: UV spectra of alprazolam¹, promethazine², levocetirizine³, loratadine⁴

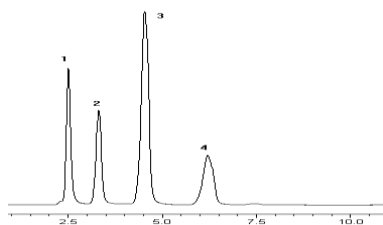


Fig.3: Chromatograms representing alprazolam¹, promethazine², levocetirizine³, loratadine⁴

Table 1: System Suitability				
Drugs	tr ^{a)}	N ^{b)}	T ^{c)}	Res ^{d)}
Promethazine	2.4	2160	1.22	--
Levocetirizine	3.1	2472	1.07	3.11
Alprazolam	4.4	2701	1.03	4.30
Loratadine	6.1	2551	1.00	4.04

^{a)}retention time, ^{b)}theoretical plates, ^{c)}tailing factor, ^{d)}resolution

3. RESULT AND DISCUSSION

3.1. Method development and optimization

Benzodiazepines are wide class of drugs with clinical uses and abuses (Chouinard 2004., He *et al.*, 1998). Alprazolam is short acting anxiolytic benzodiazepines, well recognized for its anti-anxiety and sedative-hypnotic action (Rainville *et al.*, 2012) along with various side effects including cognitive functioning, dizziness, drowsiness and slow response which were diminished by extended release (XR) formulation of alprazolam (Leufkens *et al.*, 2007). It has already been analyzed by HPLC (Rainville *et al.*, 2012). Simultaneous recommendation of alprazolam and studied antihistamine reveals the significance of method for their quantitation and identification. This study is about to propose a new rapid, efficient, economical and valid HPLC-UV method for simultaneous determination of alprazolam with promethazine, levocetirizine and loratadine in raw material, pharmaceutical formulation and human serum.

Chromatographic parameters for newly developed analytical method were optimized to achieve best separation with high resolution. Initially, UV spectra of each drug were obtained to determine the isosbestic point (**Fig. 2**) which was found to be 230 nm.

Various composition of methanol: water and acetonitrile: water (60:40, 70:30, 80:20, 90:10) were tried with variable pH ranging from 2.6-3.6. Separation was observed by varying flow rate in the range 0.7-1.2 mL min⁻¹. Significant resolution with symmetrical peaks and retention time less than seven minutes were obtained by establishing mobile phase ratio 80:20 v/v methanol: water with pH 3.5 and 1.0 mL min⁻¹ elution rate. (**Fig. 3**) represents the separation of alprazolam with promethazine, levocetirizine and loratadine.

3.2. Method validation

ICH guidelines were followed to validate newly developed method by performing system suitability test, linearity, accuracy, precision, specificity and selectivity, detection, and quantitation limits and robustness of the method.

3.3. System suitability test

System suitability is an important step of method validation. It was evaluated by injecting solution of each standard six times on each day of analysis and instrumental response was reported as retention time, theoretical plates (N), tailing factor (T) and resolution factor (Res) (**Table. 1**). Obtained data have theoretical plates greater than 2000 and resolution greater than 1.5 showing system suitability for the method.

Table 2: Regression characteristics				
Parameters	Promethazine	Levocetirizine	Alprazolam	Loratadine
Slope	20231	17458	45037	17031
Linearity (µg mL ⁻¹)	0.4-25	0.4-25	0.2-12.5	0.4-25
Intercept	19248	18809	84412	14502
Correlation coefficient	0.9983	0.9998	0.9985	0.998
Standard error	0.548	0.596	0.565	0.890
Standard error estimate	1.06	1.034	0.944	1.299
LOD (ng mL ⁻¹)	6.92	10.56	2.85	4.9
LOQ (µg mL ⁻¹)	0.02097	0.03200	0.00864	0.0149

3.4. Linearity and range

Linearity and range were extracted by plotting calibration curves at seven different concentration levels as 0.2-12.5 $\mu\text{g mL}^{-1}$ for alprazolam and 0.4-25 $\mu\text{g mL}^{-1}$ for promethazine, levocetirizine and loratadine with minimum correlation coefficient 0.998 for each analyte. Regression characteristics describing intercept and slope along with the data of standard error and standard error estimate are reported in (Table. 2).

3.5. Precision and accuracy

Inter-day and intra-day precision were estimated in the concentration range 0.4-25 $\mu\text{g mL}^{-1}$ for each

antihistamine and 0.2-12.5 $\mu\text{g mL}^{-1}$ for alprazolam by analyzing sample solution six times within a day and two repeated days of method validation. RSD for each analyte was found to be 1.20, 1.69, 1.29 and 1.77% respectively (Table. 3).

Accuracy of method was reported by determining the recovery of alprazolam, promethazine, levocetirizine and loratadine in pharmaceutical formulation and human serum as 98.05-101.83, 98.07-100.15, 99.23-100.08, and 98.06-101.62 and 99.94-101.10, 99.93-99.98, 98.05-101.89 and 98.12-99.96 respectively (Table 4).

Table 3: Precision of method

Promethazine			Levocetirizine			Alprazolam			Loratadine		
Conc $\mu\text{g mL}^{-1}$	%RSD ^a	%RSD ^b	Conc $\mu\text{g mL}^{-1}$	%RSD ^a	%RSD ^b	Conc $\mu\text{g mL}^{-1}$	%RSD ^a	%RSD ^b	Conc $\mu\text{g mL}^{-1}$	%RSD ^a	%RSD ^b
25	0.64	0.59	25	0.62	0.47	12.5	0.76	0.44	25	0.84	0.51
12.5	0.14	0.28	12.5	0.56	0.39	6.25	0.38	0.53	12.5	1.44	1.06
6.25	0.632	1.20	6.25	0.01	0.34	3.12	0.43	1.29	6.25	0.39	1.77
3.12	0.36	0.21	3.12	0.45	0.69	1.6	0.36	0.05	3.12	0.27	0.10
1.6	0.33	0.14	1.6	1.67	0.52	0.8	0.05	0.36	1.6	0.02	0.05
0.8	0.11	0.03	0.8	0.34	0.37	0.4	0.23	0.33	0.8	0.07	0.43
0.4	0.27	0.30	0.4	0.37	0.04	0.2	0.45	0.17	0.4	0.36	0.76
Human Serum											
Promethazine			Levocetirizine			Alprazolam			Loratadine		
Conc $\mu\text{g mL}^{-1}$	%RSD		Conc $\mu\text{g mL}^{-1}$	%RSD		Conc $\mu\text{g mL}^{-1}$	%RSD		Conc $\mu\text{g mL}^{-1}$	%RSD	
12.5	0.027		12.5	0.32		6.25	0.20		12.5	0.49	
6.25	0.045		6.25	0.22		3.12	0.11		6.25	0.05	
3.12	0.004		3.12	0.28		1.6	0.03		3.12	0.09	

^aInter-day precision and ^b intra-day precision

Table 4: Recovery of method

Pharmaceutical Formulation											
Theoclave Avomine [®]			T-Day TM			Xanax [®]			Jardin		
Conc $\mu\text{g mL}^{-1}$	%Rec	%Error	Conc $\mu\text{g mL}^{-1}$	%Rec	%Error	Conc $\mu\text{g mL}^{-1}$	%Rec	%Error	Conc $\mu\text{g mL}^{-1}$	%Rec	%Error
12.5	98.91	-1.10	12.5	100.08	0.08	6.25	98.05	-1.99	12.5	100.06	0.06
6.25	98.07	-1.97	6.25	99.23	-0.78	3.12	98.29	-1.74	6.25	101.62	1.60
3.12	100.15	0.15	3.12	100.02	0.02	1.6	101.83	1.80	3.12	98.06	-1.98
Human Serum											
Promethazine			Levocetirizine			Alprazolam			Loratadine		
12.5	99.93	-0.073	12.5	100.10	0.10	6.25	101.10	1.08	12.5	98.12	-1.921
6.25	100.00	0.001	6.25	101.89	1.85	3.12	99.94	-0.06	6.25	99.96	-0.044
3.12	99.98	-0.022	3.12	101.13	1.12	1.6	99.98	-0.02	3.12	99.94	-0.063

Table 5:Robustness									
		Promethazine		Levocetirizine		Alprazolam		Loratadine	
Parameters		T	N	T	N	T	N	T	N
Flow rate (mL/min)	0.9	0.891	1655	1.037	1985	0.996	2248	0.944	2148
	1.0	1.227	2144	1.082	2468	1.036	2692	1.005	2538
	1.1	1.201	2108	1.047	2236	1.011	2466	0.974	2359
Wave length(nm)	228	1.328	2172	1.133	2432	1.071	2630	1.037	2469
	230	1.210	2086	1.047	2384	1.001	2590	0.982	2459
	232	1.190	2047	1.020	2372	0.985	2606	0.953	2499
pH	3.4	1.312	2464	1.115	3725	1.025	4763	0.965	4572
	3.5	1.223	2154	1.079	2475	1.032	2691	1.006	2549
	3.6	1.293	2326	1.148	3235	1.014	4376	0.912	4568
Mobile phase	78:22	1.032	1304	1.594	1972	1.420	2583	1.351	2759
	80:20	1.224	2160	1.079	2472	1.032	2701	1.007	2551
	82:18	1.169	1259	1.575	1965	1.455	2627	1.366	2545

3.6. Detection and quantification limits

Slope of calibration curve of lowest concentration and instrumental precision represents the overall sensitivity of method. Three times and ten times of baseline signal termed as LOD and LOQ was found 2.85, 6.92, 10.56, 4.9 ngmL⁻¹ and 0.00864, 0.02097, 0.03200, 0.0149 µgmL⁻¹ alprazolam, promethazine, levocetirizine and loratadine respectively.

3.7. Robustness

Deliberate changes in chromatographic parameters i.e. methanol:water ratio up to ± 2 mL, pH (± 0.1) and flow rate (± 0.1 mLmin⁻¹) were made to assess robustness of developed method. Theoretical plates and tailing factor represented in (Table 5) confirms that method is compatible even after slight changes in parameters.

3.8. Specificity

Specificity studies were established at optimum condition by injecting blank, spiked excipients solution, pharmaceutical formulation and spiked serum solution followed by filtration through 0.45µm millipore filter paper to examine possible interference of excipients and endogenous components of serum (Fig. 5).

3.9. Application in pharmaceutical formulation

Analytical method development and validation is a continuous process for drug discovery. It is an essential step of any pharmaceutical development program. In the present study the newly developed analytical method was employed for simultaneous determination of alprazolam with antihistamine in bulk drug commercial formulations, Xanax® 0.5 mg, Avomine® 25 mg, T-Day™ 5 mg and 10 mg Jardin. Chromatogram represented in (Fig. 3) shows high resolution and symmetrical peaks for all the studied analytes with high accuracy and precision less than 1.98%. It is therefore concluded that developed method is suitable for simultaneous determination of alprazolam with

antihistamine in bulk drug and pharmaceutical formulation without significant interferences of unwanted species.

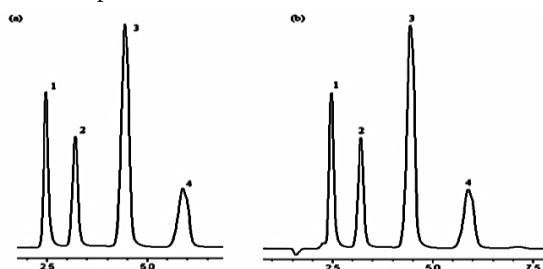


Fig.4: Chromatograms representing alprazolam¹, promethazine², levocetirizine³ and loratadine⁴ in pharmaceutical formulation*and human serum*Fig 1:

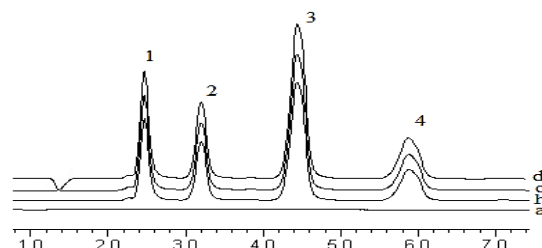


Fig. 5: Chromatograms of (a) placebo (b) spiked excipients solution, (c) pharmaceutical formulation and (d) spiked serum solution in alprazolam¹, promethazine², levocetirizine³ and loratadine⁴

3.10. Application in human serum

All the optimized parameters of developed method were successfully applied for simultaneous determination of studied drugs in human serum. This study was conducted in clear serum solution spiked with analyte obtained by centrifugation of blood sample with acetonitrile. Results showed good percent recovery in the range 99.94-101.10, 99.93-99.98, 98.05-101.89 and 98.12-99.96 for alprazolam, promethazine, levocetirizine and loratadine respectively.

4. **CONCLUSION**

A rapid, efficient, sensitive, economical, RP-HPLC method was reported in our study for simultaneous determination of alprazolam with three antihistamines. ICH guidelines were followed in order to conduct its validation process for suitability test, linearity, precision, accuracy, limit of detection and quantitation, robustness, selectivity and specificity. Over all precision was found good with high percent recovery. This method is successfully applicable for simultaneous determination of alprazolam with selected antihistamine in pharmaceutical formulation and human serum without any interference.

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