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Short communication

Development and validation of a reversed-phase HPLC method for simultaneous estimation of ambroxol hydrochloride and azithromycin in tablet dosage form

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ABSTRACT

A simple, precise and accurate reversed-phase liquid chromatographic method has been developed for the simultaneous estimation of ambroxol hydrochloride and azithromycin in tablet formulations. The chromatographic separation was achieved on a Xterra RP18 (250 mm × 4.6 mm, 5 μm) analytical column. A Mixture of acetonitrile–dipotassium phosphate (30 mM) (50:50, v/v) (pH 9.0) was used as the mobile phase, at a flow rate of 1.7 ml/min and detector wavelength at 215 nm. The retention time of ambroxol and azithromycin was found to be 5.0 and 11.5 min, respectively. The validation of the proposed method was carried out for specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness. The linear dynamic ranges were from 30–180 to 250–1500 μg/ml for ambroxol hydrochloride and azithromycin, respectively. The percentage recovery obtained for ambroxol hydrochloride and azithromycin were 99.40 and 99.90%, respectively. Limit of detection and quantification for azithromycin were 0.8 and 2.3 μg/ml, for ambroxol hydrochloride 0.004 and 0.01 μg/ml, respectively. The developed method can be used for routine quality control analysis of titled drugs in combination in tablet formulation.

1. Introduction

Azithromycin [9-de-oxy-9a-aza-9a-methyl-9a-homoerythromycin A dihydrate] is an azalide, a subclass of macrolide antibiotics. It is used to treat certain bacterial infections, most often bacteria causing middle ear infections, tonsillitis, throat infections, laryngitis, bronchitis, pneumonia and sinusitis [1,2]. Ambroxol hydrochloride [trans-4-(2-Amino-3,5-dibrombenzylamino)-cyclohexanol hydrochloride] is a mucolytic agent used in the treatment of respiratory disorders associated with viscid or excessive mucus. Ambroxol hydrochloride reduces bronchial hyper-reactivity, stimulates cellular surfactant production, increases the amount of antibiotic penetration and thus reduces daily dose of them and exhibits anti-inflammatory properties as well [3].

A new tablet formulation commercially available in combination of ambroxol hydrochloride 60 mg and azithromycin 500 mg. Literature survey revealed that some analytical methods have been used for individual estimation of azithromycin and ambroxol hydrochloride. HPLC methods with an UV detector [4], or with an amperometric electrochemical detector [5], pre-column derivatization method [6] and a spectrophotometric determination method [7] for azithromycin has been described. A spectrofluorimetric methods have been described [8,9] for the analysis of several macrolides including azithromycin. A FIA-amperometric method for azithromycin determination is also described [10]. Several spectrophotometric methods have been used for the qualitative and quantitative determination of ambroxol. These are simple UV spectrophotometry [11–13] and flow injection spectrophotometry [14]. In another study, the spectrophotometric determination of ambroxol was carried out by liquid–liquid extraction using bromothymol blue with a flow injection system [15]. Capillary electrophoresis applied for the determination of ambroxol in body fluids [16]. Ambroxol determination in human plasma, urine and pharmaceutical formulations using gas chromatography [17,18], high-performance liquid chromatographic methods [19–24] and HPLC–MS methods [25]. However no references have been found for simultaneous determination of azithromycin and ambroxol hydrochloride in pharmaceutical preparations. The present manuscript describes a simple, rapid, precise and accurate isocratic reversed-phase HPLC method for the simultaneous deter-

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mination of azithromycin and ambroxol hydrochloride in the same tablet dosage form.

2. Experimental

2.1. Chemicals

Azithromycin (94.12%) and ambroxol hydrochloride (99.67%) were obtained from Yeshwant Mahavidyalaya, Nanded, (MS), India. Dipotassium hydrogen phosphate (AR Grade), acetonitrile (HPLC Grade) were purchased from E. Merck (India) Ltd. Worli, Mumbai, India. The 0.45-μm nylon filters were purchased from Advanced Micro Devices Pvt. Ltd. Chandigad, India. Double distilled water was used throughout the experiment. Tablets were purchased from Indian market, containing ambroxol hydrochloride 60 mg and azithromycin 500 mg per tablet.

2.2. Equipments

Analysis was performed on a chromatographic system of Waters 2695 separation module (USA) equipped with an auto injector, Waters 2487 Dual λ absorbance detector. A chromatographic separation was achieved on Xterra RP18 (250 mm × 4.6 mm, 5 μm) analytical column. Data acquisition was made with Empower software. The peak purity was checked with the photodiode array detector.

2.3. Standard solutions and calibration graphs

Standard stock solution of ambroxol hydrochloride (2.4 mg/ml) was prepared in diluent which was a mixture of acetonitrile and water (40:60, v/v). To study the linearity range of each component, serial dilutions were made by adding this standard stock solution in the different weights of azithromycin in the range of 250–1500 μg/ml of azithromycin and 30–180 μg/ml of ambroxol hydrochloride. A graph was plotted as concentration of drugs versus peak area response. It was found to be linear for both the analytes. From the standard stock solution, a mixed standard solution was prepared containing 1000 μg/ml of azithromycin and 120 μg/ml of ambroxol hydrochloride. The system suitability test was performed from five replicate injections of mixed standard solution.

2.4. Sample preparation

Twenty tablets were weighed and finely powdered. The average weight of tablets is determined with the help of weight of 20 tablets. A portion of powder equivalent to the weight of one tablet was accurately weighed into 100 ml A-grade volumetric flasks and 70 ml diluent was added. The volumetric flasks were sonicated for 20 min to effect complete dissolution of the azithromycin and ambroxol hydrochloride, the solutions were then made up to volume with diluent. The solution was filtered through 0.45 μm nylon filter. The aliquot portion of the filtrate was further diluted to get final concentration of 1000 μg/ml of azithromycin and 120 μg/ml of ambroxol hydrochloride. Fifty microlitres of the test solution was injected and chromatogram was recorded for the same, and the amounts of the drugs were calculated.

2.5. Method validation

The HPLC method was validated in terms of precision, accuracy and linearity according to ICH guidelines [26]. Assay method precision was determined using nine-independent test solutions. The intermediate precision of the assay method was also evaluated using different analyst on three different days. The accuracy of the assay method was evaluated with the recovery of the standards from excipients. Three different quantities (low, medium and high) of the authentic standards were added to the placebo. The mixtures were extracted as described in Section 2.4, and were analyzed using the developed HPLC method. Linearity test solutions were prepared as described in Section 2.3. The LOD and LOQ for analytes were estimated by injecting a series of dilute solutions with known concentration. To determine the robustness of the method, the final experimental conditions were purposely altered and the results were examined. The flow rate was varied by (±) 0.1 ml/min. The percentage of organic modifier was varied by (±) 5%. Column temperature was varied by (±) 5 °C and pH of mobile phase was varied by (±) 0.1.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

During the analysis of basic drugs like azithromycin and ambroxol, one of the well known problem in pharmaceutical industry is peak tailing. Since these compounds strongly interact with polar ends of HPLC column packing materials, causing severe peak asymmetry and low separation efficiencies. High purity silica backbone and advances in bonding technology have alleviated the tailing problem of polar compounds in HPLC to a significant extent. During the optimization of the method, different columns (Inertsil C8, 250 mm × 4.6 mm, 5 μm; Zorbax C18 250 mm × 4.6 mm, 5 μm; Symmetry C18 250 mm × 4.6 mm, 5 μm) and two organic solvents (acetonitrile and methanol) were tested. The chromatographic conditions were also optimized by using different buffers like phosphate, acetate and citrate for mobile phase preparation. After a series of screening experiments, it was concluded that phosphate buffers gave better peak shapes than their acetate and citrate counterparts. With methanol as solvent both the peaks shows less theoretical plates and more retention time compared to acetonitrile. The chromatographic separation was achieved on a Waters Xterra RP18, 250 mm × 4.6 mm, 5 μm column, by using a mixture of acetonitrile–dipotassium phosphate (30 mM) (50:50, v/v) as mobile phase. Xterra packing materials are synthesized using Waters Hybrid particle technology. Hybrid particles contain both inorganic (silica) and organic (organosiloxane) components sharing the advantages of both. In separate steps, these particles are surface bonded to attach C18 groups, then endcapped to further reduce the concentration of residual silanols. These residual silanols are the main cause of peak tailing for basic compounds. Xterra columns are having high pH (2–12) and temperature (20–60 °C) stability. About mobile phase, due to the lack of other chromophore than the ester group in azithromycin and, therefore, the need to work at a low wavelength (215 nm), acetonitrile was considered as organic solvent instead of methanol. On the other hand, pH was adjusted in the highest value (pH 9) permitted to avoid problems with silica dissolution but to obtain the lower ionization degree in the amino groups to increase retention. As pH was reduced below 9.0, azithromycin was eluting earlier and at pH 8.0 it is eluting at retention time of 4.7 min and merging with ambroxol peak. The pH of mobile phase was adjusted with diluted orthophosphoric acid. Moreover, the stability of azithromycin and ambroxol is lower in acidic medium. Temperature was increased to facilitate mass exchange with the corresponding decrease of peak broadening and increase in sensibility. Fifty degree celsius was a compromise, because at 70° the peaks were narrower, but column life was rather short.

At 50 °C column temperature and pH 9.0 of mobile phase, the peak shape of azithromycin and ambroxol was found symmetrical.

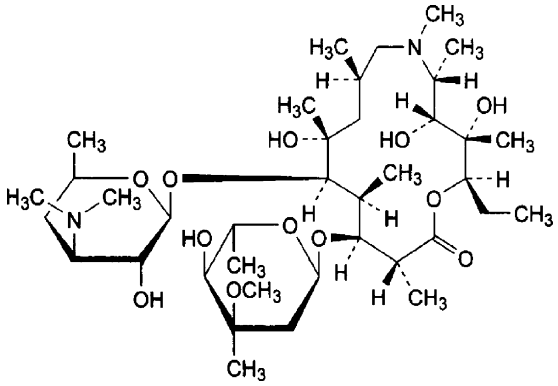

Fig. 1. Structure of azithromycin.

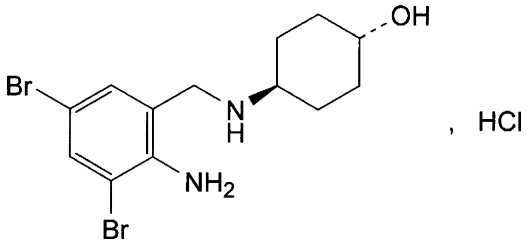

Fig. 2. Structure of ambroxol hydrochloride.

The flow rate kept was 1.7 ml/min to achieve adequate retention time of two peaks (Figs. 1 and 2).

3.2. Validation of method

3.2.1. Specificity

The specificity of the HPLC method is illustrated in Fig. 3 where complete separation of ambroxol and azithromycin was noticed in presence of tablet excipients. In addition there was no any interference at the retention time of ambroxol and azithromycin in the chromatogram of placebo solution. In peak purity analysis with photo diode detector, purity angle was less than purity threshold for both the analytes. This shows that the peak of analytes was pure and excipients in the formulation did not interfere the analytes.

3.2.2. Accuracy

Accuracy of the method was calculated by recovery studies at three levels by standard addition method (Table 1). The mean percentage recoveries obtained for ambroxol hydrochloride and azithromycin were 99.40 and 99.90%, respectively.

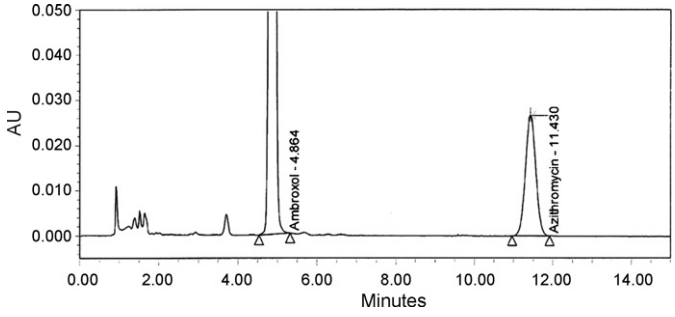

Fig. 3. A typical chromatogram of a tablet sample solution containing 120 μg/ml of ambroxol hydrochloride and 1000 μg/ml of azithromycin.

Table 1
Results of the recovery analysis of azithromycin and ambroxol hydrochloride.

Compound	Wt spiked (mg)	Wt recovered (mg)	Recovery (%)	R.S.D. (%) $n=3$
Azithromycin	125.80	124.88	99.27	0.51
	531.23	531.32	100.02	0.34
	797.11	800.44	100.42	0.35
Ambroxol HCl	14.14	14.12	99.9	0.43
	59.12	58.71	99.3	0.58
	89.14	88.29	99.1	0.42

R.S.D.: relative standard deviation. Wt: weight.

3.2.3. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The system precision is a measure of the method variability that can be expected for a given analyst performing the analysis and was determined by performing five replicate analyses of the same working solution. The relative standard deviation (R.S.D.) obtained for azithromycin and ambroxol hydrochloride was 1.51 and 0.29%, respectively (Table 2).

The intra- and inter-day variability or precision data are summarized in Table 3. The intra-day precision of the developed LC method was determined by preparing the tablet samples of the same batch in nine determinations with three concentrations and three replicate each. The R.S.D. of the assay results, expressed as a percentage of the label claim, was used to evaluate the method precision. The inter-day precision was also determined by assaying the tablets in triplicate per day for consecutive 3 days. The results indicated the good precision of the developed method (Table 3).

3.2.4. Linearity

Linearity was determined for azithromycin in the range of 250–1500 μg/ml; and for ambroxol hydrochloride, 30–180 μg/ml. The correlation coefficient ('r') values for both the drugs were >0.999. Typically, the regression equation for the calibration curve was found to be $y = 35107.11x - 16468.18$ for ambroxol hydrochloride and $y = 504.51x - 5017.3$ for azithromycin.

Table 2
System suitability parameters.

Parameters	Ambroxol hydrochloride	Azithromycin
Theoretical plates[a]	9913	8986
USP resolution[a]	–	19.12
Peak symmetry[a]	1.02	1.04
% R.S.D.	0.29	1.51

[a] USP-NF 29 section 621, pp. 2135.

Table 3
Intra- and inter-day assay precision data ($n=9$).

Actual concentration	Measured concentration (μg/ml), R.S.D. (%)	
	Intra-day	Inter-day
Ambroxol hydrochloride (μg/ml)		
30	30.12, 0.23	30.03, 1.25
120	120.36, 0.52	120.42, 0.89
180	180.24, 0.48	180.37, 1.38
Azithromycin (μg/ml)		
250	250.48, 0.68	250.11, 1.02
1000	1000.84, 0.27	999.38, 1.54
1500	1500.41, 0.78	1501.17, 0.97

Data expressed as mean for "measured concentration" values.

Table 4
Results of robustness study.

Factor	Level	Mean % assay ($n = 3$)		% R.S.D. of results	
		Ambroxol hydrochloride	Azithromycin	Ambroxol hydrochloride	Azithromycin
pH of mobile phase	8.9	100.6	99.8	0.42	1.17
	9.1	101.3	100.1	0.98	0.72
Flow rate (ml/min)	1.6	99.6	101.5	1.05	0.85
	1.8	100.3	101.4	0.52	0.78
Column oven temperature (°C)	45	98.9	100.7	0.74	1.07
	55	100.4	101.8	0.96	0.63
% of acetonitrile	45	100.8	99.9	1.24	0.81
	55	101.3	100.5	0.73	0.68

3.2.5. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of azithromycin and ambroxol hydrochloride were determined by calibration curve method [21]. Solutions of both azithromycin and ambroxol hydrochloride were prepared in the range of 0.4–10 and 0.005–0.125 μg/ml respectively and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were calculated by using following equations.

$$\text{LOD} = \frac{3.3 \times \text{Syx}}{b}; \quad \text{LOQ} = \frac{10.0 \times \text{Syx}}{b}$$

where Syx is residual variance due to regression; b is slope.

LOD and LOQ for azithromycin were 0.8 and 2.3 μg/ml respectively and for ambroxol hydrochloride were 0.004 and 0.01 μg/ml, respectively.

3.2.6. 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.

Robustness of the method was investigated under a variety of conditions including changes of pH of the mobile phase, flow rate, percentage of acetonitrile in the mobile phase and column oven temperature. The mixed standard solution is injected in five replicates and sample solution of 100% concentration is prepared and injected in triplicate for every condition and % R.S.D. of assay was calculated for each condition. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters has proven that the method is robust (Table 4).

4. Conclusion

A simple, specific, linear, precise, and accurate RP-HPLC method has been developed and validated for quantitative determination of azithromycin and ambroxol hydrochloride in new tablet formulation. The method is very simple and specific as both peaks are well separated from its impurities and excipient peaks with total runtime of 15 min, which makes it especially suitable for routine quality control analysis work.

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