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Short communication

Validated LC method for in-vitro analysis of azithromycin using electrochemical detection

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1. Introduction

The azalide antibacterial azithromycin is a semisynthetic acid stable erythromycin derivative with an expanded spectrum of activity and improved tissue pharmacokinetic characteristics relative to erythromycin. Azithromycin is absorbed rapidly and distributes widely throughout the body except cerebrospinal fluid. Its unique pharmacokinetic properties include extensive tissue distribution and high drug concentrations within cells (including phagocytes). These attributes result in much greater tissue or secretion drug concentrations compared to simultaneous serum concentrations. The terminal elimination half life was reported to be 68 h and is prolonged because of extensive tissue sequestration and binding [1–3]. It is very effective against upper and lower respiratory tract infections, sexually transmitted diseases, and skin and soft tissue infections. A 3 day oral regimen of once-daily 500 mg azithromycin has been shown to be effective in patients with acute bronchitis, pneumonia, sinusitis, pharyngitis, tonsillitis and otitis media. The commonly observed side effects include diarrhea, nausea, abdominal pain, headache and dizziness [3].

Azithromycin has been measured in biological fluids by microbiological method [4] and high performance liquid chromatography using electrochemical [5–7], fluorescence [8] and mass spectrometry [9] for detection. However, there is noticeable shortage of methods described in the literature for its determination in pharmaceutical dosage forms and in in-vitro dissolution studies except the USP method [10] which describes the use of high pH mobile phase (pH 11.0) and thus necessitates the use of specific 'Gamma-alumina' column, which is expensive and difficult to procure commercially as many of the column manufacturers do not supply this column.

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The proposed method avoids the use of a high pH mobile phase and specific 'Gamma-alumina' column and introduces a simple, accurate, precise, time saving and validated analytical method for determination of azithromycin in pharmaceutical dosage forms and in in-vitro dissolution studies.

2. Experimental

2.1. Instrumentation

The HPLC system used was a Waters chromatographic system (Waters, Milford, MA, USA) equipped with two 515 pumps, 717 autosampler and 464 pulsed electrochemical detector. Millennium[32] software (version 3.05.01) was used for data acquisition and processing. In addition, Mettler toledo AG 245 electronic balance, Remi cyclomixer and Nichipet Nichiryo (10–100 μl and 100–1000 μl) autopipettes were used in the study.

2.2. Chemicals and reagents

Azithromycin was supplied by Sarabhai Chemical Works, Vadodara (India) as gift sample. All solvents used were of HPLC grade (J.T. Baker, USA and E. Merck, India). Ammonium acetate and sodium phosphate dibasic were of analytical grade (E. Merck India Ltd. and Loba Chemie, India). Water used in the analysis was prepared by reverse osmosis (Elgastat, Elga Ltd., England) and passed through a 0.45 μm millipore filter (Millipore Company, USA). Three azithromycin products namely Azee capsules (Protec Ltd., India), Vicon tablets (Pfizer Ltd., India) and Azithral tablets (Alembic Chemical Works. Co. Ltd., India) were procured from the local market. Each product is labeled to contain 250 mg of azithromycin.

2.3. Chromatographic conditions

Azithromycin was analyzed by reversed-phase HPLC analysis using C_{18} Nova-Pak (150 mm × 3.9 mm ID and 4 μm particle size) column. The optimized mobile phase composition was ammonium acetate (0.05 M), acetonitrile, methanol and tetrahydrofuran in the proportion of 60:27:25:2.5 and always freshly prepared, filtered (Millipore filtration assembly) and sonicated (Branson 3210 sonicator, USA) before use. The flow rate of mobile phase was 1 ml/min. The column effluent was monitored using amperometric electrochemical detector with dual glassy carbon electrodes operated in the oxidative screen mode with electrode 1 set at + 0.7 V and electrode 2 set at + 0.8 V.

2.4. Preparation of calibration curve

Primary stock solutions of drug in concentration 400 μg/ml were prepared in sodium phosphate buffer (pH 6.0) and acetonitrile separately. Appropriate volumes of the above solutions were diluted to obtain secondary stock solution of 100 μg/ml. Aliquots of secondary stock solutions were taken in different volumetric flasks and diluted with mobile phase to obtain final concentration of azithromycin in the range of 1–25 μg/ml; 50 μl volume of each solution was injected for analysis. The stability of azithromycin in solution form was determined by HPLC analysis of three replicates of quality control samples (25 μg/ml), stored in a refrigerator at 4°C as well as at room temperature (25°C), every day for five consecutive days.

2.5. Validation

The method was validated by determination of the following operational characteristics: system suitability, linearity, range, lowest limit of detection, lowest limit of quantification, precision and accuracy.

The linearity of the assay was evaluated by analysis of six standard sample solutions at different concentration levels ranging from 1–25 μg/ml, each in triplicate. The correlation between peak area and concentration was established and calibration curves were obtained. The concentration of quality control samples were determined from calibration curves using model $y = mx + c$. The lowest limit of detection (LOD) is defined as lowest absolute concentration of the analyte which can be detected but not necessarily quantified under the stated experimental condi-

tion and the lowest limit of quantification (LOQ), taken as the lowest concentration of the analyte in the sample which can be determined with acceptable precision and accuracy under the stated experimental condition. In the present study, LOD and LOQ values were identified as signal to noise ratio 3:1 and 10:1, respectively.

2.6. Analysis of marketed formulations

2.6.1. Tablets or capsules assay

Five tablets were accurately weighed and crushed to a fine powder. In case of capsules, the contents of five capsules were completely removed from shells. Three accurately weighed quantities of this powder equivalent to 250 mg of azithromycin were taken in different 50 ml volumetric flasks and used for assay. About 30 ml of acetonitrile was added to it and the flask was shaken (Julabo SW21 shaker, Germany) for 30 min. The solution was then diluted to 50 ml with acetonitrile. This solution was centrifuged and 1 ml of supernatant was diluted to 50 ml with acetonitrile and mixed. Further, 2.5 ml of this solution was diluted to 10 ml with mobile phase and 50 μl of diluted sample was injected for HPLC analysis.

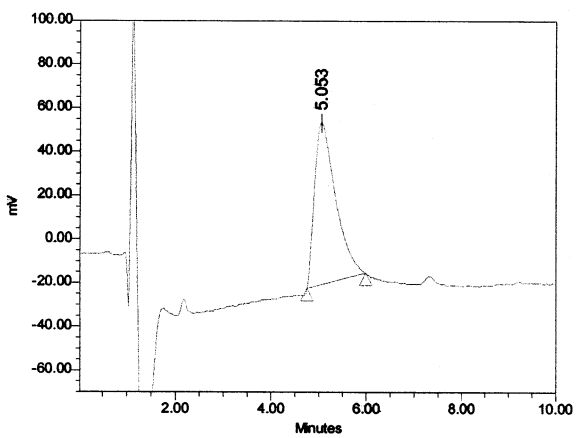

Fig. 1. Representative chromatogram of azithromycin in sodium phosphate buffer (pH 6.0).

2.6.2. In-vitro dissolution studies

Dissolution studies with three marketed products using USP23 dissolution apparatus (Electrolab, TDP-OP, India) were performed in sodium phosphate buffer (pH 6.0; 900 ml) [10] using rotating paddle method (100 rpm) at 37 ± 0.5°C temperature ($n = 3$ for each product). The samples (3.0 ml) were withdrawn at 0, 5, 10, 15, 30, 45, 60 min interval and at the same time an equivalent volume of buffer was replaced. Withdrawn samples were filtered (0.45 μm Sartorius syringe filter), suitably diluted with mobile phase and 50 μl volume of each diluted sample was injected for HPLC analysis. The cumulative % drug released in media was plotted against time, in order to determine the release profile of drug from each formulation.

3. Results and discussion

3.1. Optimization of chromatographic conditions

Initial experiments were carried out by using mobile phase comprising of ammonium acetate (0.05 M), acetonitrile and methanol in different proportions, however, satisfactory chromatographic separation could not be achieved due to poor peak shape and high retention time for azithromycin. The mobile phase compositions were modified by adding tetrahydrofuran, in order to improve peak shape and reduce retention time. Mobile phase composition of ammonium acetate (0.05 M)–acetonitrile–methanol–tetrahydrofuran (60:27:25:2.5, v/v/v/v) was finally optimized to give retention times of 5.1 and 4.6 min for azithromycin in sodium phosphate buffer (pH 6.0) and acetonitrile, respectively. The ionic strength of the mobile phase (ammonium acetate, 0.05 M) was found to be optimal for good peak shape as well as to achieve minimal background current. The pH of the mobile phase was between 7.2 and 7.4. Representative chromatogram of azithromycin in sodium phosphate buffer (pH 6.0) is shown in Fig. 1. Sodium phosphate buffer (pH 6.0) was used as dissolution medium for in-vitro dissolution studies whereas acetonitrile was used for extraction of drug from formulations [10]. System suitability was checked by evaluating dif-

Table 1
System suitability parameters

Concentration (μg/ml)	Retention time (min)	Tailing factor	Capacity factor (*k*')
1	5.20	1.508	4.212
5	5.18	1.584	4.198
10	5.15	1.522	4.159
15	5.11	1.644	4.110
20	5.08	1.651	4.087
25	5.06	1.654	4.052

ferent parameters (retention time, tailing factor and capacity factor) at different concentrations by using Millennium[32] system suitability software. These results are tabulated in Table 1. As it is clear from Table 1, all the values for different system suitability parameters checked were found to be within specified limits [11].

Azithromycin detection was accomplished with dual glassy carbon electrode in oxidative screen mode and the optimized potentials of 1 and 2 electrodes were +0.7 V and +0.8 V, respectively. Little oxidation of azithromycin occurred at the first electrode set at screen potentials of +0.7 V. Although greater response could be obtained with higher potentials than +0.8 V at second electrode, the background noise also increased. Therefore, the potential of +0.8 V was selected to provide adequate sensitivity for azithromycin determination.

3.2. Calibration range, linearity, limit of detection and limit of quantification

Linearity of response was studied by running the standard curve of azithromycin. The plot of peak area versus concentration of azithromycin in sodium phosphate buffer (pH 6.0) and acetonitrile were found to be linear in the concentration range 1–25 μg/ml. The correlation coefficient (r) was found to be always greater than 0.999 ($n = 3$). The r value was found to be real and significant at 1% level (t test with $N = 2$, i.e. 4 degrees of freedom). Table 2 enlists the calibration curve parameters for the pure drug in both solutions. The lowest limit of detection and the lowest limit of quantification were 500 ng/ml and 750 ng/ml in sodium phosphate buffer (pH 6.0), respectively.

3.3. Precision and accuracy

Precision and accuracy were assessed by performing replicate analysis of quality control samples. Four different concentration solutions within calibration range were prepared in sodium phosphate buffer (pH 6.0) and analyzed with calibration curve to determine intra-day (six replicates per concentration) and inter-day (six replicates per concentration over 1–3 days) variability. The intra-day and inter-day precision were determined as the relative standard deviation (% RSD) and accuracy as percentage relative error (% RE). Precision and accuracy results shown in Table 3 demonstrate good precision and accuracy over the concentration ranges selected.

3.4. Stability of drug in the solutions

The stability studies of azithromycin in sodium phosphate buffer (pH 6.0) and acetonitrile indicated no significant changes (all within ±5%) in drug concentrations upon storage of samples for five days at 4°C in refrigerator and at room temperature (25°C).

Table 2
Standard curve of Azithromycin

Media	Correlation coefficient	Equation of line
Sodium phosphate buffer (pH 6.0)	0.9994	$Y = 98152.36X - 53211.9$
Acetonitrile	0.9992	$Y = 99199.59X - 39082.9$

Table 3
Precision and accuracy of the HPLC analysis of Azithromycin ($n = 6$)

Theoretical concentration (μg/ml)	Concentration found (μg/ml)			% Recovery	Relative percentage error (%RE)
	Mean	SD	%RSD		
Intra-day					
08	07.724	0.16	2.1	96.56	3.4
12	12.124	0.21	1.7	101.04	1.0
18	17.501	0.20	1.1	97.23	2.7
22	21.858	0.45	2.0	99.35	0.6
Inter-day					
08	07.675	0.40	5.3	95.94	4.0
12	11.783	0.52	4.8	99.13	1.8
18	17.769	0.61	3.4	98.72	1.2
22	22.968	1.13	5.1	99.40	0.5

Table 4
Assay of Azithromycin in formulations

SN	Brand[a]	Label claim	Amount found[b] (mg)	% Recovery	% RSD
1	Azee, capsules	Azithromycin, 250mg/capsules	247.88 ± 6.69	99.15	2.699
2	Vicon, tablets	Azithromycin, 250mg/tablets	249.57 ± 3.65	99.83	1.820
3.	Azithral, tablets	Azithromycin, 250mg/tablets	246.87 ± 4.49	98.67	1.466

[a] Brand (1) Protec Ltd., India, Batch No. C90293, Manufactured Aug. 1999; (2) Pfizer Ltd., India, Lot No. 920-64011, Manufactured May 1999; (3) Alembic Chemical Works. Co. Ltd., India, Batch No. 90411, Manufactured Aug. 1999.
[b] Average of three experiments ± SD.

3.5. Application to analysis of pharmaceutical formulations

The method was applied for the determination of azithromycin content in marketed products by assaying Vicon, Azithral tablets and Azee capsules. The assay showed the drug content of these products to be within pharmacopoeial limits of label claim. The results of drug assay in three products are shown in Table 4.

Further, the developed method was used for the quantitative determination of azithromycin in in-vitro dissolution test samples obtained during the drug release studies of marketed products. The cumulative % drug released in media versus time profile for different products is shown in Fig. 2. As can be seen from Fig. 2, more than 90% drug dissolved in media from all three formulations within specified USP limit of 45 min for azithromycin capsules [10].

4. Conclusion

The proposed method obviates the need of specific 'Gamma-alumina' column and introduces the use of conventional C_{18} column using neutral pH mobile phase. Moreover, the method is sim-

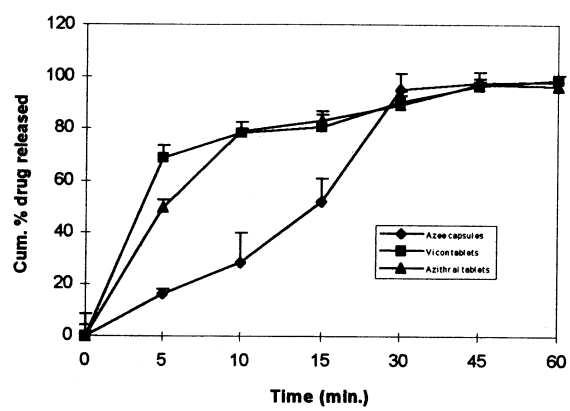

Fig. 2. Drug release profile of marketed products ($n = 3$).

ple, precise, accurate and rapid for the determination of azithromycin in pharmaceutical dosage forms and for monitoring its concentration in in-vitro dissolution studies. Hence, it can be easily adopted for the routine quality control analysis of azithromycin in pharmaceutical dosage forms.

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