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Short communication

Microbiological assay for azithromycin in pharmaceutical formulations

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Abstract

The validation of a microbiological assay, applying the cylinder–plate method, for the determination of the antibiotic azithromycin is described. Using a strain of *Micrococcus luteus* ATCC 9341 as the test organism, azithromycin at concentrations ranging from 0.1 to 0.4 μg ml^{-1} could be measured in capsules and suspensions. A prospective validation of the method showed that it was linear ($r = 0.998$), precise (RSD = 1.40—capsules; RSD = 1.19—powder for suspension and RSD = 1.73—oral suspension) and accurate (it measured the added quantities). We conclude that the microbiological assay is satisfactory for quantitation of in vitro antibacterial activity of azithromycin.

Keywords: Azithromycin; Microbiological assay; Cylinder–plate method; Micrococcus luteus

1. Introduction

Azithromycin [9-Deoxo-9a-aza-9a-methyl-9a-homoerythromycin A dihydrate] (Fig. 1) [9] is an active 15-membered ring azalide antibacterial agent. Structurally, azithromycin is related to the macrolide erythromicyn, but its properties are different from those of the prototype macrolide. Azithromycin has a broad spectrum of activity against common gram-negative pathogens and great pharmacokinetics characteristics that appear ideal for the treatment of respiratory tract infections, skin infections and sexually transmitted diseases [1–3]. Assays reported in the literature for the determination of azithromycin in biological fluids include HPLC using atmospheric pressure chemical ionization [4], coulometric and amperometric detection [5], and fluorescence detection [6], and microbiological diffusion method on an 8 × 8 Latin Square to find out which of the test microorganisms used show the highest sensitivity [7].

For the measurements in pharmaceutical dosage forms, the only method reported in literature is HPLC, using eletrochemical detection [8,9]. The USP [9] method use a pH 11.0 mobile phase, what makes necessary to use an specific Gamma-alumina column, which is very expensive and not common. Besides, the eletrochemical detection ad-

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vocated by both methods is not the most usual in the HPLC equipments. The microbiological assay can reveal subtle changes not demonstrable by chemical methods [9]. Moreover, the microbiological assay makes possible the evaluation of the potency, witch is very important to antibiotics analysis. There are no official microbiological cylinder–plate assays described neither in the official codes nor in the literature to determine azithromycin in pharmaceutical formulations. The activity of antimicrobial agents may be demonstrated under suitable conditions by their inhibitory effect on microrganisms [9]. The activity of azithromycin in vitro has been tested against various microrganisms. These studies have shown that azithromycin has potent in vitro activity against *Micrococcus luteus* as well against other bacterias, like *Escherichia coli* and *Bacillus pumilus* [7].

The aim of this study was to validate an agar diffusion method through the parameters linearity, precision and accuracy, to quantify azithromycin in capsules and suspensions.

2. Materials and methods

Azithromycin substance reference (95.66%) was obtained from Teuto (São Paulo, Brazil) while pharmaceuticals containing azithromycin were obtained commercially. Azithromycin capsules were claimed to contain 250 mg (as anhydrous base) of the drug and the following excpients: corn starch, lactose, sodium lauryl sulfate and magnesium stearate. The suspension were claimed to contain 40 mg ml^{-1} (as anhydrous base) of the drug and the following excpients: sugar, xanthan gum, sodium phosphate tribasic and flavouring. Analytical reagents grade chemicals were used.

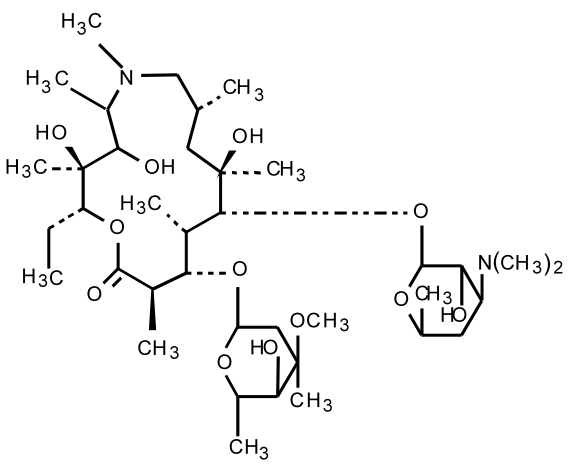

Fig. 1. Structure of azithromycin.

2.1. Preparation of azithromycin reference substance

The standard solution in methanol (1 mg ml^{-1}) was diluted in potassium phosphate buffer, pH 8.0 and assayed at concentrations of 0.1, 0.2 and 0.4 μg ml^{-1}.

2.2. Preparation of the samples

2.2.1. Capsules

Ten capsules were opened and its content was weighed. An amount of powder equivalent to 25 mg of azithromycin was transferred to 25 ml volumetric flask with 15 ml methanol and shaken for 20 min, followed by marking up to volume with methanol. After filtration, the dilutions were made with potassium phosphate buffer, pH 8.0, to give a final concentrations of 0.1, 0.2 and 0.4 μg ml^{-1}. This sample was evaluated in triplicate. This procedure was performed two times.

2.2.2. Powder of suspension

The samples were prepared by the same method used for capsules.

2.2.3. Reconstituted suspension

The samples were prepared using 5 ml of the suspension that was transferred to 20 ml volumetric flask with 10 ml methanol and shaken for 20 min, followed by marking up to volume with methanol. After filtration, the dilutions were made with potassium phosphate buffer, pH 8.0, to give the same final concentrations used above.

This sample was evaluated in triplicate. This procedure was performed two times.

2.3. Organism and inoculum

The cultures of *M. luteus* ATCC 9341 were cultivated on Grove Randall number 1 agar (Merck) at freezer and pealed to another Grove Randall number 1 agar (24 h before the assay) that was kept in stove at 37 °C. The bacteria was suspended in Grove Randall number 3 broth (Merck) using a glass homogenizer. A diluted cultures suspensions of $25 \pm 2\%$ turbidity were obtained at 580 nm, using a suitable spectrophotometer (Analyser-Model 800, São Paulo, Brazil) and a 10 mm diameter test tube as an absorption cells against Grove Randall number 3 broth a blank. Portions of 1 ml of the inoculated Grove Randall number 3 broth were added to 100 ml of Grove Randall number 11 agar (Merck) at 47 °C ± 2 °C and used as inoculated layer.

2.4. Cylinder–plate assay

The agar was composed of two separate layers. The Grove Randall number 11 agar (20 ml) was poured into 100×20 mm^2 petri dish for the base layer. After solidification this layer portions of 5 ml of inoculated Grove Randall number 11 agar was poured to the base layer. Six stainless steel cylinders of uniform size ($8 \times 6 \times 10$ mm^3) were placed on the surface of inoculated medium. Three alternated cylinders were filled with 200 μl of reference concentrations solutions and the other three with the concentrations samples solutions. After incubation (37 °C for 24 h) the zone diameters (in mm) of the growth inhibition were measured using a caliper (Mitutoyo). Eight plates were used in each assay.

2.5. Calculation

To calculate the activity of aziyhromicyn in capsules and suspensions, the Hewitt [10] equation was used. The assay were statistically calculated by the linear parallel model and by means of regression analysis and verified using analysis of variance [9,10].

2.6. Method validation

The method was validated by determination of linearity, precision and accuracy [9,11].

2.6.1. Linearity

In order to assess the validity of the assay three doses of the reference substance were used. The calculation of regression line by the method of least squares was employed.

2.6.2. Precision

Repeatability (intra-assay) was determined by assaying samples of capsules and suspensions, at same concentration, during the same day and under same experimental conditions.

2.6.3. Accuracy

This was determined by adding known amounts of azithromycin reference substance to the samples at the beginning of the process. Amounts of 2, 2 and 4 mg of azithromycin were placed in 200, 100, and 100 ml volumetric flask, respectively, where 1.0, 1.0 and 2.0 ml of azithromycin reference solution (200 μg ml^{-1}) were added (in this order). Dilutions were made in potassium phosphate buffer, pH 8.0, to give a final concentrations of 0.11, 0.22 and 0.44 μg ml^{-1}. The solutions were submitted the cylinder–plate assay described above. The percentage recovery of azithromycin reference added was calculated using the equation proposed by AOAC [12].

3. Results and discussion

In this work experimental 3×3 design using three dose levels for each standard and sample were used following the procedure described in Brazilian and European Pharmacopoeias [13,14]. The calculation procedure normally assume a direct relationship between the observed zone diameter and logarithm of applied dose. The corresponding mean zone diameters for reference solutions were: 16.46 mm (RSD = 0.19) for low dose (0.1 μg ml^{-1}), 18.59 mm (RSD = 0.48) for medium dose (0.2 μg ml^{-1}) and 20.75 mm (RSD = 0.81) for high dose (0.4 μg ml^{-1}). The

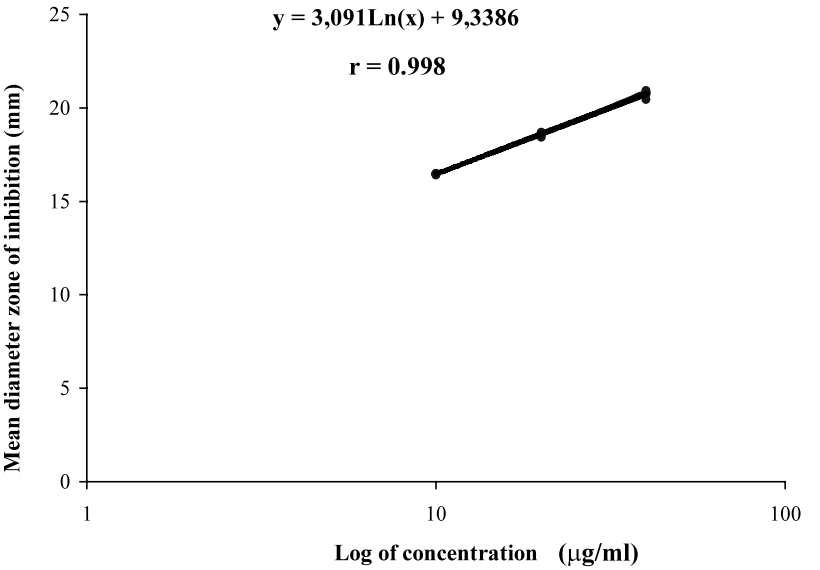

Fig. 2. Calibration curve for azithromycin, obtained by the microbiological cylinder–plate assay.

calibration curve for azithromycin was constructed by plotting log of concentrations ($\mu g\ ml^{-1}$) versus zone diameter (mm) and showed good linearity on the 0.1–0.4 $\mu g\ ml^{-1}$ range (Fig. 2). The representative linear equation for azithromycin was $y = 3.091\ \text{Ln}\ x + 9.3386$, where x is log dose and y is zone diameter. The coefficient of regression was $r = 0.998$.

The experimental values obtained for the determination of azithromycin in samples are present in Table 1. According Brazilian, European, and British Pharmacopoeias [13–15], if a parallel-line model is chosen, the two log dose-response lines of the preparation to be examined and the reference preparation must be parallel and they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. The assays were validated by means of the analysis of variance, as described in these official codes. There are no deviation from parallelism and linearity with results obtained here ($P < 0.05$).

The precision and accuracy of the assay were demonstrated. The precision is usually expressed as the RSD of a series of measurements [11]. The repeatability shows mean RSD of 1.40 for capsules, 1.19 for powder for suspensions and 1.73 for reconstituted suspensions (Table 1).

The accuracy express the agreement between the accepted value and the value found [11]. The

Table 1
Experimental values obtained for the determination of azithromycin in samples by the microbiological cylinder–plate assay

Sample[a]	Experimental amount* (mg)	% Level	RSD
Capsules	243.83	97.53	1.40
	237.63	95.05	
	242.50	97.00	
	235.13	94.05	
	237.15	94.86	
	240.00	96.00	
Powder for suspension	196.80	98.40	1.19
	198.64	99.32	
	201.88	100.94	
	201.32	100.66	
Reconstituted suspensions	193.30	96.65	1.73
	197.26	98.63	
	190.90	95.45	
	188.88	94.44	
	190.00	95.00	

[a] Theorical amount: 250 mg per capsule and 200 mg per 5 ml of suspension.

* Each value is the mean of eight analysis.

Table 2
Experimental values obtained in the recovery test for azithromycin in samples, by the microbiological cylinder–plate assay

Sample	Amount of reference (mg)		% Recovery[a]
	Added	Recovered	
Capsules	0.01	0.0101	101.21
	0.02	0.0204	102.08
	0.04	0.0413	103.22
Powder for suspension	0.01	0.0096	95.54
	0.02	0.0191	95.25
	0.04	0.0400	100.1
Reconstituted suspension	0.01	0.0096	95.57
	0.02	0.0196	97.83
	0.04	0.0390	97.53

[a] Each value is the mean of 10 analysis.

mean recovery were found to be 102.17% for capsules, 96.96% for powder for suspensions and 96.98% for reconstituted suspensions (Table 2).

Although the biological assays have a high variability, the analysis of the obtained results could demonstrate that the proposed method is very useful to the determination of this drug in pharmaceutical dosage forms.

4. Conclusion

The results indicated that the microbiological cylinder–plate assay hold linearity, precision and accuracy at concentration ranging from 0.1 to 0.4 $\mu g\,ml^{-1}$ being an acceptable alternative method for the routine quality control of azithromycin in the formulations studied.

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