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Short communication

Azithromycin potency determination: Optimal conditions for microbiological diffusion method assay

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1. Introduction

The use of erythromycin includes a whole range of problems: the increase in the resistance of Gram-positive strains, slow bactericidal action, associated gastrointestinal disturbance, allergic reactions and hepatotoxic effects [1,2]. Therefore, the number of novel 14-, 15- and 16-membered macrolides has been increasing over the past few years [3]. The result of the extensive research carried out in the laboratories of Pliva is a new 15-membered macrolide termed 'azithromycin'. Azithromycin is a representative of a new macrolide group, named azalides [4–6]. Its lacton ring has been extended by the introduction of nitrogen, which resulted in significant changes of its pharmacokinetic properties and spectrum of action [7,8].

Administered orally, azithromycin shows pharmacokinetic properties superior to those of erythromycin A [9]. Azithromycin is readily absorbed, reaches higher serum levels, rapidly achieves high concentrations in human tissues, can be administered over a significantly shorter period and has a longer half-life [10–12]. Azithromycin does not cause gastrointestinal disturbances as often as 14-membered macrolides [13]. The azalides are extremely active against Gram-negative organisms and exert better bactericidal effect [14].

This paper represents part of the research on azithromycin carried out to determine its potency by the microbiological diffusion method assay on an 8 × 8 Latin Square with two standard and two sample concentrations. The object is to standardize the method, and find out which of the test microorganisms used show the highest sensitivity and under which conditions. As this is a new antibiotic for which no relevant data regarding the conditions of microbiological assay were

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available, it was necessary to test the classical method and to select the most suitable test microorganisms. The sensitivity results for 10 different test microorganisms were compared.

2. Materials and equipment

2.1. Tested azithromycin sample and Azithromycin dihydrate Pliva working standard

The standard and sample stock solution were prepared by dissolution in methanol and then further diluted with phosphate buffer solution pH 8.0.

2.2. Test microorganisms

Sarcina lutea ATCC 9341, *Bacillus pumilus* NCTC 8241, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538 P, *Staphylococcus epidermidis* ATCC 12228, *Pseudomonas aeruginosa* NCTC 10490, *Proteus morganii* NCTC 235, *Streptococcus faecalis* ATCC 8043, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 10536 [15].

2.3. Agar medium

Inoculated medium (250 ml) with drilled in wells. Content of agar medium (water to 1000 ml) and final pH value: **No.1**, Ph 6.5–6.7 (Peptone 6 g, Panc. digest of casein 4 g, Beef extract 1.5 g, Yeast extract 3 g, Glucose 1 g, Agar 15 g); **No.2**, pH 7.7–7.9 (Peptone 6 g, Panc. digest of casein 4 g, Beef extract 1.5 g, Yeast extract 3 g, Glucose 1 g, Agar 15 g); **No.3**, pH 6.4–6.6 (Peptone 6 g, Beef extract 1.5 g, Yeast extract 3 g, Glucose 1 g, Tryptone 4 g, Agar 15.0 g); **No.4**, pH 6.0–6.2 (Peptone 9.4 g, Beef extract 2.4 g, Yeast extract 4.7 g, Glucose 10 g, Sodium chloride 10 g, Agar 25 g); **No.5**, pH 7.1–7.3 (Peptone 5 g, Beef extract 1 g, Yeast extract 2 g, Sodium chloride 5 g, Agar 15 g); **No.6**, pH 6.5–6.7 (Peptone 6 g, Beef extract 1.5 g, Yeast extract 3 g, Agar 15 g); **No.7**, pH 7.4 (Peptone 5 g, Beef extract 1.5 g, Yeast extract 3 g, Glucose 1 g, Agar 15 g); **No.8**, pH 6.5–6.6 (Peptone 6 g, Beef extract 1.5 g, Yeast extract 3 g, Glucose 1 g, Agar 15 g).

2.4. Phosphate buffer solution

Dissolve 16.73 g dipotassium hydrogen phosphate and 0.52 g potassium dihydrogen phosphate in 1000 ml of distilled water; pH = 8.

2.5. Equipment

Glass plates, 310 × 310 mm in size, with aluminium frames and an aluminium lid; 64-well assay plate punch (Autodata); Optomax V system for image analysis (Analytical Measuring System).

3. Procedures

3.1. Optimal conditions of azithromycin potency determination by diffusion method assay

Eight solid media were tested under the same cultivation conditions and comparatively monitored for the bacterial growth and culture properties of 10 strains of microorganisms. The diffusion method assay was used for each strain to optimise the conditions for azithromycin potency determination, and in that respect the following was carried out.

3.1.1. Testing and selection of solid medium suitable for azithromycin potency determination

Standard solution (80 μl) in the concentrations of: 100, 80, 40, 20, 10, 5, 2.5, 1.5, 1, 0.5 μg azithromycin ml^{-1} were added to the wells (8 mm in diameter) drilled in the 4-mm thick medium inoculated with each of the studied test microorganisms. After incubation at 35–37°C/16–18 h, the following indices were evaluated:

1. Image of the zones, i.e. sharpness of the inhibition zone limits;
2. Readability on Optomax V system for image analysis;
3. Ratio of inhibition zone diameters obtained with various concentrations, since the assay sensitivity increases with the increase of the ratio of the inhibition zone diameter;
4. Minimal detectable concentrations, since the assay sensitivity increases with the decrease in the concentrations of tested solutions and standard solution.

Table 1
Optimal conditions for diffusion method assay of azithromycin potency on 8×8 Latin Square

Test microorganism	Agar medium	Volume of standardized inoculum in ml to be added to each 250-ml agar medium	8×8 Latin Square	
			High dose level (μg azithromycin ml^{-1} solution)	Low dose level
Sarcina lutea ATCC 9341	2	5 MF I	1	0.5
Bacillus pumilus NCTC 8241	2	2 MF I	5	2.5
Bacillus subtilis ATCC 6633	7	2 MF I	10	5
Staphylococcus aureus ATCC 6538 P	2	12.5 MF II	10	5
Staphylococcus epidermidis ATCC 12228	7	10 MF I	10	5
Pseudomonas aeruginosa NCTC 10490	2	12.5 MF II	100	50
Proteus morganii NCTC 235	2	12.5 MF II	100	50
Streptococcus faecalis ATCC 8043	2	10 MF I	100	50
Klebsiella pneumoniae ATCC 10031	2	20 MF II	10	5
Escherichia coli ATCC 10536	2	0.2 MF I	10	5

MF I, II, Degree of dilution of test microorganism suspension according McFarland standard.

Each of the above indices has been evaluated from 1 (maximum) to 8 (minimum), and the medium selected regarding their sum.

3.1.2. The inoculum standardization

The 20 plates were prepared. Medium (250 ml) inoculated with 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 18, 20 ml of standardized inoculum. The inocula were previously diluted so as to obtain the turbidity corresponding to that of the McFarland standards I (3×10^8 cfu ml^{-1}) and II (6×10^8 cfu ml^{-1}). Standard solution (80 μl) was added in the following concentrations: 100, 80, 40, 20, 10, 5, 2.5, 1.5, 1, 0.5 μg azithromycin ml^{-1} into the wells on each plate. The plates were then incubated at 35–37°C/16–18 h. The volume of the diluted inoculum was determined that was to be added to a specified amount of medium so that sufficiently large and sharply limited inhibition zones, readable on the Optomax V system for image analysis, could be achieved with the lowest standard concentration.

3.1.3. Determination of suitable concentrations for azithromycin potency determination

Among concentrations that produced zones readable on the Optomax V system, those which showed the highest ratio in inhibition zone diameters were selected, this being the precondition for higher sensitivity of the diffusion method.

3.2. Azithromycin potency determination on 8 × 8 Latin Square under optimal conditions

To determine the azithromycin potency, the inoculum of each test microorganism was diluted, so that the turbidity produced corresponded to that of the McFarland standard shown in Table 1.

The standard solution was prepared by weighing the azithromycin dihydrate working standard into a 100-ml volumetric flask in such an amount as to produce a standard solution content of 50000 μg azithromycin/100 ml, then dissolved and made up to the volume with methanol (standard

solution). The standard solution was further diluted with phosphate buffer solution (pH 8.0) to produce higher and lower concentrations (Table 1).

The sample solution was prepared by weighing 52.1 mg of the tested sample into a 100-ml volumetric flask, and then dissolved and made up to the volume with methanol (sample solution). The sample solution was further diluted with phosphate buffer solution (pH 8.0) to the concentrations corresponding to standard solution concentrations for each test microorganism.

Statistical analysis for the 8×8 Latin Square was carried out by a computer program for data analysis regarding the measured inhibition zone diameters, which corresponds to the requirements of BP [16].

3.3. Selection of test microorganisms producing highest precision and accuracy of determination

The most suitable microorganisms for azithromycin potency determination were selected using the results of the azithromycin potency assay ($n = 6$), and on the basis of the following indices.

3.3.1. Standard concentrations

The stock standard solution was diluted by the phosphate buffer solution (pH 8.0) to produce two standard concentration solutions in the ratio of 2:1 (higher and lower concentration). The selected azithromycin solution concentrations produced sharply limited inhibition zones. As the concentrations decrease, so the microorganisms show greater sensitivity to the tested antibiotic.

3.3.2. Error limits of the assay

The error limits of the assay represent experimental data. With microbiological determinations a range of 2 standard deviations—the reliability interval at the level of 95% probability—is considered satisfactory.

3.3.3. Potency deviations from mean value in individual determinations

The individual assay deviations from the mean value of all the assays on the 10 test microorganisms were calculated on the basis of the azithromycin potency results obtained. Where the deviation was minor the obtained potency was closer to the actual value, and the conditions in which it was determined provided greater accuracy and precision of determination.

3.3.4. Comparing the ratio between the measured inhibition zone diameters of standard solution

The greater the ratio of inhibition zone diameters between higher and lower standard concentrations, the higher the sensitivity of determination.

3.3.5. Zone image, i.e. zone limit sharpness

The test microorganisms were also scored on the basis of readability on the Optomax V system. The higher the contrast between the inhibition zones and those parts of media into which antibiotic solution had not diffused in sufficiently high concentrations to inhibit microorganism growth, the easier the readability.

3.4. Azithromycin potency determination on the selected group of test microorganisms

Measurements ($n = 30$) done on an 8×8 Latin Square using the group of optimal test microorganisms (*Bacillus pumilus* NCTC 8241, *Sarcina lutea* ATCC 9341, *Escherichia coli* ATCC 10536) confirmed the results of the experiments and their proper selection.

3.4.1. Statistical evaluation of the results obtained by diffusion method assay

The results obtained were compared by t-test and F-test [17].

3.4.2. Repeatability of sample preparation

Repeatability of sample preparation was determined on 30 samples of azithromycin from one batch on the selected group of test microorganisms. Sample solutions ($n = 30$) were prepared, determined and the azithromycin potency was calculated. Repeatability of sample preparation was evaluated by determining the relative standard deviation (RSD).

Table 2
Optimal concentration for diffusion method assay of azithromycin potency

Test microorganism	Potency of azithromycin standard solutions (μg azithromycin ml^{-1})										
	0.1	0.5	1	1.5	2.5	5	10	20	40	80	100
Sarcina lutea ATCC 9341	a	a	*	*	*	*	*	*	*	*	*
Bacillus pumilus NCTC 8241			*	*	a	a	*	*	*	*	*
Bacillus subtilis ATCC 6633				*	*	a	a	*	*	*	*
Staphylococcus aureus ATCC 6538 P					*	a	a	*	*	*	*
Staphylococcus epidermidis ATCC 12228			*	*	*	a	a	*	*	*	*
Pseudomonas aeruginosa NCTC 10490									a	*	a
Proteus morganii NCTC 235								*	a	*	a
Streptococcus faecalis ATCC 8043		*	*	*	*	*	*	*	a	*	a
Klebsiella pneumoniae ATCC 10031				*	*	a	a	*	*	*	*
Escherichia coli ATCC 10536				*	*	a	a	*	*	*	*

*, Azithromycin concentration in solutions which give sharply limited zones; a, Azithromycin concentration in solutions which give sharply limited zones and the largest ratio between diameter of inhibition zones.

4. Results

4.1. Optimisation of conditions for azithromycin potency determination by diffusion method assay

In order to select the most suitable media for each test microorganism in the azithromycin content assay by microbiological diffusion method, the solid media were scored. Solid medium **No.2** was found to be the most suitable one for the majority of test microorganisms (Table 1).

Table 1 shows adequate volumes of standardized inocula determination ($n = 10$) the comparison according to the McFarland standards.

The measurements showed that the detectable inhibition zones, with a sufficient ratio between the higher and lower concentration solution diameters (Table 2), were obtained with *Sarcina lutea* ATCC 9341 at concentrations of 1 and 0.5 μg azithromycin ml^{-1}; *Bacillus pumilus* NCTC 8241 at 5 and 2.5 μg ml^{-1}; with *Pseudomonas aeruginosa* NCTC 10490, *Proteus morganii* NCTC 235, *Streptococcus faecalis* ATCC 8043 at 100 and 50 μg ml^{-1} and with other test microorganisms at concentrations of 10 and 5 μg azithromycin ml^{-1}.

Due to the research, the optimal conditions for azithromycin potency assay were determined. Because of the great number of parameters, these are summarised in Table 1.

4.2. Azithromycin potency determination on an 8×8 Latin Square under optimal conditions

The azithromycin potency determination ($n = 6$) tested the possibility of measuring the azithromycin potency using the studied test microorganisms under conditions (Table 1) that were found to be optimal for them. The results of azithromycin activity in Table 3 confirmed that the assay conditions were properly selected. The mean value of the azithromycin potency determination with all test microorganisms was 960.4 μg azithromycin mg^{-1} within the range 951.4–967.8 μg azithromycin mg^{-1}. According to the BP and Ph. Eur. [16,18], these values were within the error limits of the tests (95.0–105.0%) with the majority of test microorganisms.

4.3. Selection of test microorganisms which produced the highest precision and accuracy of determination

The determined azithromycin levels in the solutions of higher and lower concentrations are shown in Table 1. It can be noted that the lowest azithromycin concentrations (1 and 0.5 μg ml^{-1}) produced measurable inhibition zones on an 8×8 Latin Square with *Sarcina lutea* ATCC 9341. The highest concentrations (100 and 50 μg ml^{-1}) were required for potency determination with *Pseu-*

Table 3
Some parameters for the selection of test microorganisms which produced the highest precision and accuracy of determination

Test microorganism	Parameter			
	A	B	C	D
Sarcina lutea ATCC 9341	961.8	96.5–103.7	1.38	1.127
Bacillus pumilus NCTC 8241	963.6	96.6–103.5	3.17	1.158
Bacillus subtilis ATCC 6633	957.2	95.6–104.6	−3.22	1.134
Staphylococcus aureus ATCC 6538 P	959.2	95.7–104.5	−1.23	1.137
Staphylococcus epidermidis ATCC 12228	964.0	94.9–105.4	3.64	1.136
Pseudomonas aeruginosa NCTC 10490	958.1	96.7–103.5	−2.25	1.196
Proteus morganii NCTC 235	963.3	95.3–104.9	2.87	1.197
Streptococcus faecalis ATCC 8043	957.6	94.6–105.7	−2.78	1.127
Klebsiella pneumoniae ATCC 10031	967.8	95.0–105.3	7.38	1.149
Escherichia coli ATCC 10536	951.4	96.8–103.3	−8.95	1.182

A, Azithromycin activity obtained by 8×8 Latin Square under optimal conditions (mean value of $n = 6$), μg azithromycin mg^{-1}; B, Error limits, %; C, Potency deviations from mean value in each determination, μg azithromycin mg^{-1}; D, The ratio of zone inhibition diameter between the highest and the lowest standard dose level by 8×8 Latin Square.

domonas aeruginosa NCTC 10490, *Proteus morganii* NCTC 235, *Streptococcus faecalis* ATCC 8043.

Error limits of the diffusion method assay on an 8×8 Latin Square ($n = 6$) are shown in Table 3. The narrowest error limits appeared in the azithromycin potency determination with *Escherichia coli* ATCC 10536.

Table 3 shows individual potency deviations from the mean value. The least potency deviation was obtained with *Staphylococcus aureus* ATCC 6538 P and *Sarcina lutea* ATCC 9341.

The best results obtained by measuring the inhibition zone ratios between higher and lower concentrations (Table 3) of azithromycin standard solutions were obtained with *Proteus morganii* NCTC 235, *Pseudomonas aeruginosa* NCTC 10490, *Escherichia coli* ATCC 10536 and *Bacillus pumilus* NCTC 8241.

During evaluation of the aforementioned parameters for selecting the optimal test microorganism, satisfactory results were obtained with *Pseudomonas aeruginosa* NCTC 10490. However, this strain produced an unsatisfactory sharpness of the inhibition zone limit, and hence an unsatisfactory zone image. Thus the zone image proved to be one of the key indices for the selection of a limited group of test microorganisms.

Taking into consideration the data obtained, i.e. the evaluation of indices (1 = maximum, 10 = minimum) a points system was obtained. Table 4 gives a rank order of microorganisms for the selection of a smaller group suitable for azithromycin potency determination. A group of microorganisms with the lowest score showed higher precision and accuracy in azithromycin potency determination compared to other studied bacterial strains. A narrow group, recommended for azithromycin potency determination by diffusion, comprises: *Sarcina lutea* ATCC 9341, *Bacillus pumilus* NCTC 8241 and *Escherichia coli* ATCC 10536.

4.4. Azithromycin potency determination using the selected group of test microorganisms

Fig. 1 shows the results of azithromycin potency determination by diffusion method using the most sensitive test microorganisms on an 8×8 Latin Square.

4.4.1. Statistical evaluation of the results obtained by diffusion method assay

The azithromycin potency standard deviations obtained by an 8×8 Latin Square, and compared by the *F*-test were below 1.9 for the recommended test microorganisms. The values

Table 4
Score of test microorganism sensitivity for azithromycin assay

Test microorganism	I	II	III	IV	V	Sum
1. *Bacillus pumilus* NCTC 8241	2	3	6	4	2	17
2. *Sarcina lutea* ATCC 9341	1	4	2	10	1	18
3. *Escherichia coli* ATCC 10536	7	1	10	3	4	25
4. *Pseudomonas aeruginosa* NCTC 10490	10	2	3	2	10	27
5. *Staphylococcus aureus* ATCC 6538 P	7	5	1	6	9	28
6. *Proteus morganii* NCTC 235	10	7	5	1	8	31
7. *Klebsiella pneumoniae* ATCC 10031	7	8	9	5	5	34
8. *Bacillus subtilis* ATCC 6633	7	6	7	8	7	35
9. *Staphylococcus epidermidis* ATCC 12228	7	9	8	7	6	37
10. *Streptococcus faecalis* ATCC 8043	10	10	4	10	3	37

I, Azithromycin concentration in solutions which give sharply limited zones; II, Error limits of the assay; III, Potency deviations from mean value in each single measurement; IV, Comparison of the ratio diameter of inhibition zones between high and low standard dose levels; V, Image of zones on the plates.

obtained by *t*-test, for the diffusion method on an 8×8 Latin Square were below 2.0, which confirmed that there were no significant differences between the results of azithromycin potency assay with the selected group of test microorganisms.

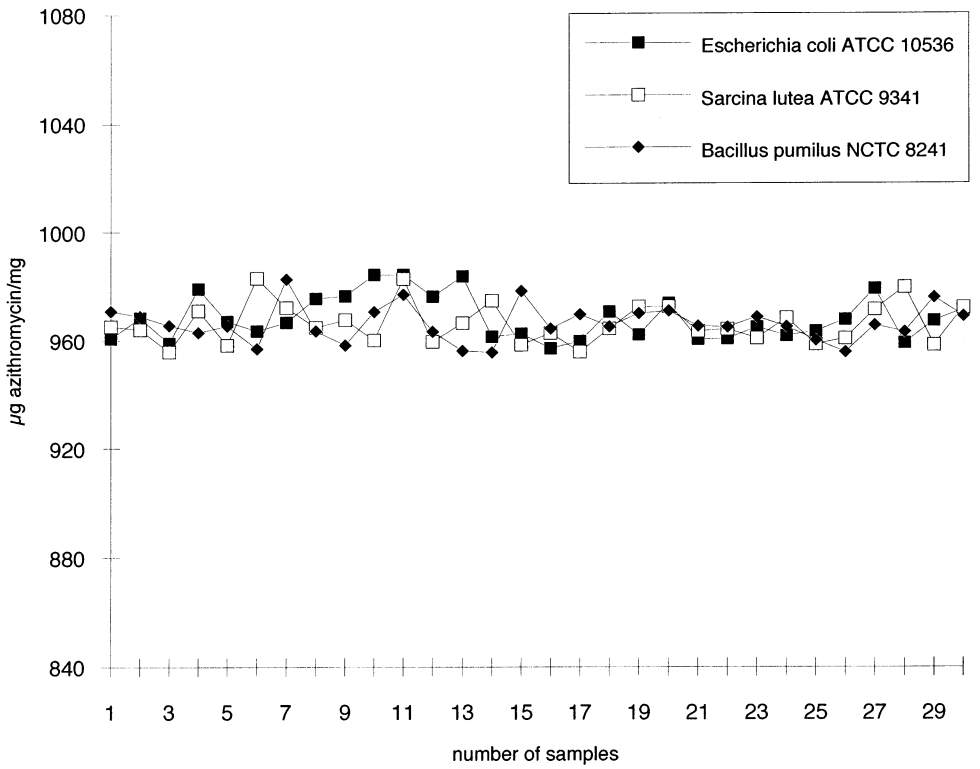

Fig. 1. Results of azithromycin activity obtained by 8×8 Latin Square.

4.4.2. Repeatability of sample preparation

Repeatability of sample preparation was satisfactory with respective RSD of 0.8% for *Sarcina lutea* ATCC 9341, RSD = 0.7% for *Bacillus pumilus* NCTC 8241 and RSD of 0.9% for *Escherichia coli* ATCC 10536.

5. Discussion

The above results showed that *Sarcina lutea* ATCC 9341, *Bacillus pumilus* NCTC 8241 and *Escherichia coli* ATCC 10536 represented the group recommendable for the analysis of azithromycin by the diffusion method assay. These test microorganisms provided satisfactory precision and accuracy of results tested in 30 successive measurements by 8 × 8 Latin Square assay (Fig. 1).

Some pharmacopoeias give official methods for determination of erythromycin potency. The USP recommendation as to the diffusion method on Petri dishes and relevant test microorganism is *Sarcina lutea* ATCC 9341 [19]. For potency determination by the diffusion method on 8 × 8 Latin Square and Petri dishes, Ph. Eur. suggest *Bacillus pumilus* (NCTC 8241, CIP 76,18) and *Sarcina lutea* ATCC 9341 [18]. In the same instances BP suggests *Bacillus pumilus* NCTC 8241 [16].

All the aforesaid recommendations are in agreement with our findings, although our research includes a completely new antibiotic. As azithromycin and erythromycin belong to the same antibiotic group, our results showed that the test microorganisms used were similarly sensitive to both of them. The recommended group of microorganisms was also supplemented by a new member *Escherichia coli* ATCC 10536.

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