Flow-Injection Spectrophotometric Determination of Azithromycin in Pharmaceutical Formulations Using *p*-Chloranil in the Presence of Hydrogen Peroxide

José Luiz RUFINO, Helena Redigolo PEZZA, and Leonardo PEZZA[†]

São Paulo State University—UNESP, Institute of Chemistry of Araraquara,
P. O. Box 355, 14801-970, Araraquara, SP, Brazil

A flow-injection (FI) spectrophotometric procedure exploiting merging zones is proposed for the determination of azithromycin in pharmaceutical formulations. The method is based on the reaction of azithromycin with tetrachloro-*p*-benzoquinone (*p*-chloranil) accelerated by hydrogen peroxide and conducted in a methanol medium, producing a purple-red color compound ($\lambda_{\text{max}} = 540 \text{ nm}$). The FI system and the experimental conditions were optimized using a multivariate method. Beer's law is obeyed in a concentration range of 50 – 1600 $\mu\text{g mL}^{-1}$ with an excellent correlation coefficient ($r = 0.9998$). The detection limit and the quantification limit were 6.6 and 22.1 $\mu\text{g mL}^{-1}$, respectively. No interference was observed from the common excipients, and the recoveries were within 98.6 to 100.4%. The procedure was applied to the determination of azithromycin in pharmaceuticals with a high sampling rate (65 samples h^{-1}). The results obtained by the proposed method were in good agreement with those obtained by the comparative method at 95% confidence level.

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Introduction

Azithromycin (AZ), (*N*-methyl-9a-aza-9-deoxo-9-dihydro-9a-homoerythromycin A) is a novel macrolide antibiotic and a semisynthetic acid-stable erythromycin derivative that acts on Gram positive bacteria and Gram negative bacteria (Fig. 1).^{1,2} It is used in respiratory tract infections, like pharyngitis, pneumonia, chronic bronchitis, bronchopneumonia, skin and soft tissue infections and some sexually transmitted diseases.^{2–5}

Several methods have been described in the literature for the determination of AZ in pharmaceutical formulations and biological fluids, including spectrophotometry,^{2,6–10} spectrofluorometry,¹¹ voltammetry,^{4,5,12,13} high-performance liquid chromatography using electrochemical,^{14,15} amperometric,¹⁶ fluorescence,¹⁷ UV,^{18,19} and electrospray ionization mass spectrometry,²⁰ detection and bioassay.^{21,22} Some of these methods display good detection limits and accuracy.^{5,11–18,20,22} However, most of them are time-consuming^{2,4–9,11–13,15,17,21,22} and in some cases require expensive equipment.^{11,14–20}

The United States Pharmacopeia²³ proposes a chromatographic method with amperometric detection that needs the use of a specific “Gamma-alumina” column, which is expensive and difficult to obtain commercially.

In recent years, more strict regulation related to the quality control of pharmaceuticals has led to increasing demands on automation of the analytical assays carried out in appropriate control laboratories. The flow-injection analysis (FIA) procedure became a versatile instrumental tool that has contributed substantially to the development of automation in pharmaceutical analysis due to its simplicity, low cost and

relatively short analysis time.^{24,25}

To the best of our knowledge, there are only two reported FIA methods for the determination of azithromycin in pharmaceutical formulations.^{26,27} Song *et al.*²⁶ have reported a flow-injection chemiluminescence method for the determination of AZ in pharmaceutical preparations, human urine and serum. However, despite its high sampling rate, excellent analytical sensitivity and relative low cost, this method presents drawbacks, such as the complexity of the chemiluminescence mechanism of the system utilized, the critical detection of the chemiluminescence signal within a short and strictly defined period of time and the proposed reaction is only efficient at pH 12.4. A FIA-amperometric method for the determination of AZ in pharmaceutical formulations has also been proposed.²⁷ Despite

Fig. 1 Chemical structure of azithromycin.

[†] To whom correspondence should be addressed.
E-mail: pezza@iq.unesp.br

its good analytical sensitivity, this method presents the disadvantages of relative high cost and a very low sampling rate.

Visible absorption spectrophotometry was applied in pioneering works on flow-injection analysis.^{28,29} Through all the thirty-three years of development of FIA, UV-vis spectrophotometry has been and currently is the most common detection used in FIA.³⁰ UV-vis spectrophotometry is the technique of choice for FIA pharmaceutical applications, because it offers the advantages of simple and low-cost instruments that are available at all laboratories.^{30,31}

The main purpose of this work was to develop a simple, fast and low-cost merging zones flow-injection procedure for the spectrophotometric determination of AZ in pharmaceutical formulations. This method is based on the reaction of AZ with tetrachloro-*p*-benzoquinone (*p*-chloranil) accelerated by hydrogen peroxide and conducted in a methanol medium, producing a purple-red compound ($\lambda_{\text{max}} = 540 \text{ nm}$). The main advantages of the proposed method arose from the exploitation of the intrinsic characteristics of the merging zones FIA system: the utilization of simple and inexpensive instrumentation, ease of operation and versatility, high sampling frequency and decrease of sample and reagent consumption.

Experimental

Apparatus

Flow-through measurements were carried out using an ASIA system from Ismatec (Zürich, Switzerland) equipped with a four-channel pump (IS 7610) with variable speed (1 – 50 rpm) for propelling fluids. An HP 8453A (Hewlett Packard, USA) diode array UV/Vis spectrophotometer equipped with a flow-cell of 10 mm path length and 80 μL inner volume was used for continuously monitoring the absorbance at 540 nm. The FIA peak is registered as the time-dependence of the absorbance at this wavelength.

The samples and the reagent solutions were pumped through Tygon® (2765-175) pumping tubes (i.d. 1.52 mm) and injected using a manual injector-commutator. The manual injector-commutator and the confluence in acrylic were adapted for use with organic solvents by polytetrafluoroethylene tubes (i.d. 0.8 mm) inserted in their openings. The standard connectors, loops and reaction coils were made of polytetrafluoroethylene tubes (PTFE) of 0.8 mm i.d. End-fittings and connectors (Omnifit, NY) were used.

Reagents and analytical solutions

For preparing solutions and samples, methanol-grade HPLC (Mallinckrodt, Xalostoc, Mexico) and grade-A glassware were used throughout. Analytical-reagent or pharmaceutical grade chemicals were used. The excipients or used in the interference study were of pharmaceutical grade.

p-Chloranil (Sigma, St. Louis, MO) $6.4 \times 10^{-3} \text{ mol L}^{-1}$ was freshly prepared in methanol with $4.2 \times 10^{-2} \text{ mol L}^{-1}$ of hydrogen peroxide (Merck, Germany); the solution was stable for 1 week when stored in darkness at 4°C.

Pharmaceutical-grade azithromycin dihydrate was purchased from Purifarma (Brazil, purity 99.6%). A stock standard solution of AZ in methanol was prepared at 2.0 mg mL^{-1} ; the solution was stable for a period of 4 weeks when refrigerated (4°C). Working standard solutions were prepared daily by appropriate dilution of the stock solution with methanol in order to construct an analytical curve from 50 to $1600 \mu\text{g mL}^{-1}$.

Fig. 2 Flow diagram of the FIA system used. IC, injector-commutator; L1, sampling loop (485 μL); A, sample or analytical solutions; L2, reagent loop (485 μL); R, reagent solution; C, methanol (flow 2.0 mL min^{-1}); W, wastes; RC, coiled reactor ($140 \text{ cm} \times 0.8 \text{ mm}$ i.d.); x, confluence point; PP, peristaltic pump; D, detector (540 nm). Black area, next position of the injector-commutator. Downward-pointing black arrow indicates the movement of the central part of the IC.

Optimization of variables

The optimization of all variables was performed by using a multivariate methodology. The variables were studied first using the fractional factorial design, and subsequently the two most significant variables were studied using the response surface methodology.³² All statistical calculations were performed using Statistic 6.0 software.

General procedure

The FIA system for AZ determination is depicted in Fig. 2. At the position specified in the figure, a sample volume is selected by a loop (L1), and the reagent volume is selected by a second loop (L2). After loop-based injection, AZ and *p*-chloranil solutions are loaded by a porter (methanol) merged at the confluent point x, and react inside the reactor coil (RC) forming a purple-color product. Passage of the colored compound through the flow-injection spectrophotometric cell results in a transient signal that is recorded as absorbance (540 nm), which is proportional to the AZ content in the sample. After an absorbance maximum measurement, the injector-commutator is switched back to the initial position starting another cycle.

Samples preparation

Five commercial samples of pharmaceutical formulations (tablets) containing 500 or 525 mg of AZ from different batches and different brands were purchased in local drugstores in Araraquara city (Brazil) and analyzed by the proposed method.

Ten tablets of each commercial brand pharmaceutical to be studied were weighed exactly and ground to a fine powder. A portion of this powder, equivalent to approximately 50 mg of AZ, was accurately weighed. The sample was dissolved with 10 mL of methanol and diluted with methanol in a 25-mL volumetric flask. In the sequence, this solution was filtered through Whatman 42 filter paper. The first few milliliters of the filtrate were discarded, and an aliquot of 5.0 mL of the filtrate was transferred to a 10-mL volumetric flask and the volume was completed with methanol. All samples were analyzed according to the recommended procedure.

Scheme 1

Table 1 Variables with those levels studied and the optimum values

Variable	Low level	High level	Optimum value
[<i>p</i> -Chloranil] × 10 ⁻³ mol L ⁻¹	4.17	8.34	6.4
[H ₂ O ₂] × 10 ⁻² mol L ⁻¹	4.23	6.34	4.23
Reactor/cm	100	140	140
Loops/cm	80	100	96.5
Flow/mL min ⁻¹	1.3	2.0	2.0

Results and Discussion

The molecular interactions between the electron donors and the acceptors are generally associated with the formation of intensely colored charge-transfer complexes and radical ions, which absorb radiation in the visible region of the spectrum.^{33,34} Spectrophotometric methods based on these interactions are usually simple and convenient because of the rapid formation of complexes.³⁴⁻³⁷ Azithromycin (electron donor) reacts instantaneously with *p*-chloranil (π-acceptor) in the presence of hydrogen peroxide, resulting in the formation of an intense purple-red product at room temperature, which exhibits an absorption maximum at 540 nm. This reaction is markedly accelerated by hydrogen peroxide; however, without hydrogen peroxide, the reaction is very slow at room temperature.

According to literature concerning the different functional groups present in AZ, the tertiary amino group on the desosamine sugar residue is the most easily oxidizable.^{4,12} The oxidation of tertiary amines with hydrogen peroxide in aqueous or organic media generates tertiary amine N-oxides.^{38,39} The oxygen atom of tertiary amine N-oxides (N-oxides) has been shown to work as a very effective electron donor.^{40,41} The formation of charge-transfer complexes of N-oxides with iodine⁴⁰ and boron trifluoride⁴² has been described in the literature.

These previous considerations suggest that, probably, AZ is oxidized by hydrogen peroxide to its respective N-oxide, which donates an electron to *p*-chloranil (CL) to yield radical ions *via* the dissociation of a charge-transfer complex (Scheme 1). In agreement with previously proposed mechanisms for similar reactions,^{43,44} the predominant chromogen with CL was the purple-red radical anion CL^{•-}, which was probably formed by the dissociation of an original donor-acceptor (DA) complex with the N-oxide (Scheme 1). The dissociation of DA complex is promoted by the high ionizing power of the solvent methanol.

Optimization of variables

Due to the fact that more than one variable is potentially important, and that it would be difficult to optimize the conditions through a uni-variant optimization procedure, the experimental conditions were obtained using a chemiometric experimental design. Factorial designs are widely used in experiments involving several factors where it is necessary to investigate the effects of the factors on a response variable.

The fractional factorial designs are among widely used types

Fig. 3 Pareto diagram for visualizing the effects of the FI and chemical variables on the absorbance measurements ($\lambda = 540$ nm) using a 2⁽⁵⁻¹⁾ factorial design.

of designs in screening experiments. These are experiments in which many factors are considered with the purpose of identifying these factors that have large effects. The screening experiments are usually performed early in a response surface study when it is likely that many of the factors initially considered have little, or no, effect on the response.³² Moreover, by using the experimental design it is possible to evaluate the interaction of factors, which have a significant effect on the absorbance using a small number of experiments.

Initially, a 2⁽⁵⁻¹⁾ fractional factorial design was carried out, which allowed simultaneously studying five factors that could have an important effect on the absorbance signal. The factors of interest were the flow rate, the loop of the sample and the reagent, the size of the reactor coil, CL and the hydrogen peroxide concentration. In this design, the variables were studied at two levels: low (-1) and high (+1). For this design, 16 experiments were necessary, which were realized in triplicate and randomized to eliminate any environmental variation. The variables and their levels are summarized in Table 1. The highest and lowest values of each variable were defined based on preliminary experiments. The flow rate of the carrier stream, C, was studied by varying the rotation speed of the peristaltic pump.

As a result of the fractional factorial design, a Pareto chart was drawn (Fig. 3) in order to visualize the estimated effect of the main variables. The Pareto chart gives a graphical presentation for these effects, and it allows looking at both the magnitude and the importance of an effect. The importance of each variable depends on their signs and values. Positive signs indicate that the absorbance signal is increased with an increase of the value of the respective variable within the range studied, while negative signs indicate that the absorbance signal is favored with a decrease of the variable. If the effect is smaller than 5% (*i.e.* the resulting graph does not go over the vertical line), the variation of the response caused by changing the variable is smaller than the experimental error. Therefore, the variable is considered not to be significant.^{45,46}

The flow rate was considered not to be significant. To obtain a reasonable analytical frequency, the flow rate of the carrier selected for subsequent experiments was (15 rpm) 2.0 mL min⁻¹.

The variables that have significant effects in the represented Pareto chart of the Fig. 3 (loops-size, size of the reactor coil, hydrogen peroxide and CL concentrations) were optimized

Fig. 4 Pareto diagram for visualizing the effects of the FI and chemical variables on the absorbance measurements ($\lambda = 540$ nm) using a $2^{(4-1)}$ factorial design.

Fig. 5 Three-dimensional plot of the optimized response surface, showing the absorbance as a function of the *p*-chloranil concentration and the size of loops.

using the present experimental design, $2^{(4-1)}$ fractional factorial. It can be observed in Fig. 4 that two variables (hydrogen peroxide concentration and reactor coil) were considered not to be significant, but two other variables (loops size and *p*-chloranil concentration) showed positive significant effects. The responses of the models, the R^2 values, were greater than 98%, implying that the models were well-fitted by the data at the 95% confidence level for the response of the absorbance.

Afterwards, the above-mentioned variables (loops size and *p*-chloranil concentration) were optimized by the response surface methodology. These variables were studied at five levels, including four central points for statistical validity within the range of -1.41 to $+1.41$, which corresponds to loops with a size range of 80–100 cm and *p*-chloranil with a concentration range of 4.0×10^{-3} – 7.0×10^{-3} mol L⁻¹.

Figure 5 depicts the response surface with contours of constant Z plotted against the CL concentration and the loops. It clearly shows that the system produces a stationary point that has the maximum estimated response. By analyzing the fitted surface, it is possible to identify that the points referring to the best conditions for the maximum response were loops-size of 96.5

Fig. 6 Transient signals related to the AZ determination. From the left (A), the recorded peaks refer to seven analytical solutions (50, 100, 200, 400, 600, 1000 and 1600 $\mu\text{g mL}^{-1}$) plus five samples (A, B, C, D and E) processed three times.

cm and a CL concentration equal to 6.4×10^{-3} mol L⁻¹. The quadratic regression model is give by:

$$Z = -0.61223 + 0.08974x - 0.00574x^2 + 0.02139y - 0.00010y^2 - 0.00017xy,$$

where Z represents the response factor corresponding to the absorbance value. The factors x and y are the *p*-chloranil concentration and the loops size, respectively.

The responses of the model, the R^2 values, were greater than 95%, implying that the model was well-fitted by the data at the 95% confidence level for the response of the absorbance. Table 1 gives the optimum values of the variables studied.

Analytical data

The developed analytical method was validated by evaluating the linear dynamic range, precision, accurate, ruggedness, limit of detection (LOD) and limit of quantification (LOQ) as well as by applying the standard addition technique.^{47,48}

Under the optimized experimental conditions, a linear calibration curve was constructed from 6.50×10^{-5} to 2.06×10^{-3} mol L⁻¹ (50 to 1600 $\mu\text{g mL}^{-1}$). A least-squares treatment of the calibration data ($n = 7$) yielded the following regression equation: $A = 0.0216(\pm 0.0075) + 436.34(\pm 0.70)C$, where A is the absorbance of the peak at 540 nm and the C azithromycin concentration in mol L⁻¹. The correlation coefficient was 0.9998, indicating excellent linearity of the calibration curve. The typical transient signals, in triplicate, corresponding to a linear calibration curve for AZ and injections, in triplicate, of five samples of tablets are shown in Fig. 6.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to Skoog *et al.*,⁴⁸ using the following expressions: $\text{LOD} = 3\sigma/b$ and $\text{LOQ} = 10\sigma/b$, where σ is the standard deviation of 20 measurements of the blank, and b is the slope of the analytical curves. The LOD and the LOQ were 8.46×10^{-6} mol L⁻¹ and 2.82×10^{-5} mol L⁻¹ (6.6 and 22.1 $\mu\text{g mL}^{-1}$), respectively. The flow systems with chemiluminescence²⁶ and amperometric²⁷ detection present very low LODs (0.04 $\mu\text{g mL}^{-1}$ and 0.76 $\mu\text{g mL}^{-1}$, respectively). Nevertheless, as is evident from the above-mentioned analytical data, the proposed FI spectrophotometric procedure is sufficiently sensitive (LOD = 8.46×10^{-6} mol L⁻¹) to permit the determination of AZ in

Table 2 Determination of azithromycin in pharmaceutical formulations

Sample	Label value ^a	Proposed method	Comparative method ¹⁹	<i>t</i> value (2.78) ^c	<i>F</i> value (19.00) ^c
		Found ^b	Found ^b		
A	525	527.3 ± 1.8	529.4 ± 0.9	1.78	4.00
B	524	522.7 ± 1.3	520.9 ± 0.7	2.00	3.10
C	500	508.5 ± 2.2	511.9 ± 0.7	2.61	9.17
D	500	502.9 ± 2.5	505.5 ± 1.2	1.66	4.42
E	500	505.1 ± 0.5	506.4 ± 1.1	1.90	4.84

a. Content for tablet: mg tablet⁻¹.

b. Average ± standard deviation (SD) of three independent analysis.

c. Theoretical values of *t* and *F* at 95% confidence level.

pharmaceuticals.

The precision and accuracy were evaluated by comparing the results obtained from the analysis of pharmaceutical formulations by the proposed method with those obtained using a HPLC method, described in the literature.¹⁹ The susceptibility of the developed analytical method to changes was tested to evaluate the ruggedness of the method. For this purpose the experimental designs were employed where the variables were tested.

The matrix interference can introduce systematic errors in analytical determinations. In order to investigate the presence of matrix effects in the proposed method, a recovery study was carried out. In this study, 100, 200, 300 and 400 µg mL⁻¹ of AZ reference solutions were added to three selected pre-analyzed pharmaceuticals (samples A, C, E). The recoveries obtained by the standard-addition method ranged from 98.6 to 100.4%, indicating the absence of any significant matrix effects on the proposed FI procedure.

The effects of the common excipients present in commercial pharmaceutical formulations, such as microcrystalline cellulose, hydroxypropylmethylcellulose, lactose, titanium dioxide, dibasic calcium phosphate, sodium lauryl-sulfate, sodic croscarmellose and magnesium stearate were carefully evaluated. The effects were considered to be interference when the signal showed an error of more than 3% in determination of the drug. No interference was observed in the presence of these substances.

Analytical applications

The applicability of the proposed method for the determination of AZ in pharmaceutical preparations was examined by analyzing marketed products. The results were statistically⁴⁹ compared with those obtained by the comparative HPLC method,¹⁹ and are summarized in Table 2. In all cases, the calculated *t* and *F* values are less than the theoretical ones at 95% confidence level, indicating that there is no significant difference between either method regarding the precision and the accuracy in the determination of AZ in pharmaceutical formulations. The proposed flow system provides a sampling frequency of 65 samples h⁻¹.

Conclusion

The proposed flow-injection merging zones procedure is a valuable strategy for the determination of azithromycin in pharmaceutical formulations, and could be an advantageous alternative to other available methods, because it is inexpensive, simple, precise and accurate; allows rapid determination at low operating costs; shows adequate selectivity, and a low limit of

detection; and requires minimum amounts of sample and reagent.

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