J Fluoresc (2013) 23:31–39
DOI 10.1007/s10895-012-1111-8

ORIGINAL PAPER

Fluorescence Determination of Azithromycin in Pharmaceutical Formulations by Using the Synchronous Scanning Approach After its Acid Derivatization

Vanessa G. K. Almeida · Victor S. M. Braga · Wagner F. Pacheco · Ricardo J. Cassella

Received: 30 April 2012 / Accepted: 30 July 2012 / Published online: 29 August 2012

Abstract In this present work, a fluorescence method for azithromycin (9-deoxo-9a-aza-9a-methyl-9a-homoerythromycin) determination in pharmaceutical formulations is proposed. The method is based on the synchronous fluorescence ($\Delta\lambda$=30 nm, 482 nm) produced when azithromycin is derivatized in strong acidic medium (9.0 mol L^{-1} HCl). The influence of the derivatization conditions (acid concentration, reaction time and temperature) was studied. Also, the possible reaction mechanism was discussed. In the optimized conditions, the method presented a limit of detection of 0.23 mg L^{-1} and a limit of quantification of 0.76 mg L^{-1}. The developed procedure was successfully applied in the determination of azithromycin in pharmaceutical formulations.

Keywords Azithromycin · Synchronous fluorescence · Pharmaceutical formulation · Antibiotic

Introduction

Azithromycin (AZTR, Fig. 1) or 9-deoxo-9a-aza-9a-methyl-9a-homoerythromycin is an antibiotic discovered by a Croatian group of researches, initially named XZ-450. It was developed by PLIVA, in the USA, and had its approval for clinical use in 1991. Nowadays, it is available for the treatment of several adult and pediatric infections [1], including those of upper and lower respiration tract [2], skin and soft tissue, toxoplasmosis [3], as well as sexually transmitted diseases [4].

From the chemical point of view, the AZTR is classified as an azalide [5], a subclass of the macrolides. It is a semi-synthetic compound, derived structurally from Erythromycin A, in which the 9th carbonyl present in the lactone ring is replaced by a methyl-substituted nitrogen, expanding the ring to 15 members [6]. The insertion of the nitrogen distinguishes, chemically, the azithromycin from the other macrolides, which have rings with only carbon and oxygen, and increases its basicity. This structural modification blocks the internal reaction to form the hemiketal, which makes azithromycin more stable than erythromycin in the acidic conditions of the stomach, and also increases the potency, the elimination time and the tissue concentration [7].

Several techniques have been employed for the azithromycin determination in different kinds of samples, including microbiological [8–10], UV–vis spectrophotometry [11–13] and liquid chromatography coupled to different detectors such as fluorescence [14–16], electrochemical [17, 18] and mass spectrometry [19–23]. Although spectrofluorimetry was already employed for AZTR determination [24], it use is not common because of the low capacity of AZTR to absorb electromagnetic radiation in the UV-visible range of the spectrum. This method, proposed by Khashaba et al. [24], explored the oxidation of the AZTR by Ce (VI) in sulfuric acid medium. The derivative specie was excited at 255 nm and its fluorescence was observed at 348 nm. The method was applied in the analysis of pharmaceutical formulations.

Fluorescence is one of the most important types of photoluminescence in analytical chemistry. While the excitation-emission spectrofluorimetry presents high sensitivity and moderate selectivity, the synchronized scanning fluorimetry can be used in the cases where improved selectivity is required. The capacity of detection of fluorescence techniques

V. G. K. Almeida · V. S. M. Braga · W. F. Pacheco · R. J. Cassella (✉)
Departamento de Química Analítica,
Universidade Federal Fluminense,
Outeiro de São João Batista s/n, Centro,
Niterói, RJ 24020-141, Brazil
e-mail: cassella@vm.uff.br

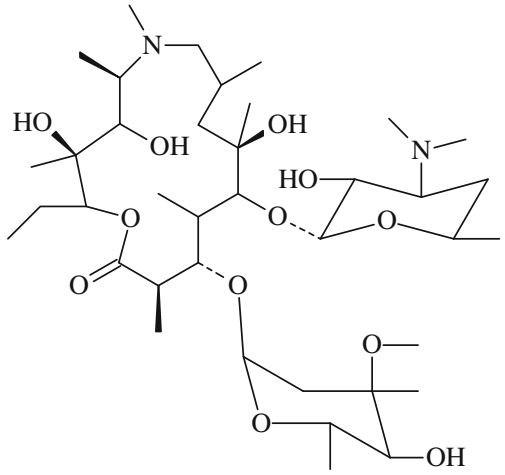

Fig. 1 Structure of the azithromycin

is, in general, greater than that observed in conventional methods based on the molecular absorption spectroscopy [25].

Some absorption-based methods for AZTR determination can be found in the current literature. In all cases the analyte was derivatized to form a substance that absorbs radiation. Paula et al. [11] developed a method for AZTR determination in pharmaceutical formulations exploring the charge transfer reaction of AZTR with quinalizarin, which formed a colored product with high absorptivity at 564 nm. Suhagia et al. [12] proposed a spectrophotometric method based on the oxidation of AZTR by potassium permanganate, producing formaldehyde, which was determined using acetyl acetone in the presence of ammonium acetate. In turn, Rachidi et al. [13] developed a new spectrophotometric method based on the measurement of the complex formed from the association between AZTR and Mo(IV)-thiocyanate complex. The colored substance was extracted with dichlorethane and measured by spectrophotometry at 469 nm.

Some other florescence methods for AZTR determination can also be found in the current literature, always coupled to liquid chromatographic systems. Bahrami et al. [14] proposed a liquid chromatographic method with fluorimetric detection of AZTR for its determination in human serum. In order to measure the fluorescence, the analyte was derivatized with 9-fluorenylmethyl chloroformate (FMOC) before injection into the chromatographic system. The product was excited at 260 nm and its emission observed at 315 nm was used for detection. The FMOC was also employed by Wilms et al. [15] and Sastre-Toraño and Guchelaar [16] for the AZTR derivatization aiming its determination by liquid chromatography with fluorescence detection.

The goal of this work was to develop a new methodology for the AZTR determination in pharmaceutical formulations employing the synchronous fluorescence scan technique. The derivatization of AZTR in strong acid medium was necessary to convert the AZTR in a fluorescent product. The experimental conditions for the conversion were optimized and the possible reaction mechanism was studied.

Experimental

Apparatus

The fluorescence measurements were carried out with a Varian Cary Eclipse spectrofluorimeter (Mulgrave, Australia), which was operated with a 10 nm spectral bandpass and a nominal resolution of 1 nm. The spectra were always recorded with a scan speed of 600 nm min^{-1} and a 10-mm quartz cuvette was employed to accommodate samples and standards.

UV-Visible measurements were performed with a Femto 800 xi double beam spectrophotometer (São Paulo, Brazil) using a 10-mm quartz cuvette. This instrument was always operated with a nominal resolution of 1 nm.

Mass spectra were recorded with a Bruker spectrometer, model HCT Ultra High Capacity Ion Trap (Bremen, Germany), employing an electrospray ionization interface.

Reagents and Solutions

All chemical reagents were of analytical grade. Deionized water (18.2 MΩ cm), from a water ultra purifier Millipore Direct-Q 3, was used to prepare all aqueous solutions. Pharmaceutical grade azithromycin (dehydrate form, 97.30 % declared purity) was supplied by *Interativo Farmácia com Manipulação* (Goiânia, Brazil). The samples of azithromycin pharmaceutical formulations were purchased randomly on local drugstores. Hydrochloric acid used in this work was of analytical grade and supplied by Tedia (Fairfield, OH, USA). Methanol of HPLC grade was employed and also purchased from Tedia.

Azithromycin stock solution (50.0 mg L^{-1}) was prepared daily by dissolving 5.0 mg of azithromycin in exactly 100 mL of methanol.

Preparation and Measurement of the Standard Solutions

The standard solutions used in the AZTR quantification were prepared by adding to a 25 mL volumetric flask, in this order, 4 mL of water, the volume of AZTR stock solution needed to achieve the desired concentration and 19.0 mL of concentrated HCl. Then, the mixture was slowly agitated in order to homogenize the solution and the volume was made up to the mark with water. Afterwards, the solution was left on the bench for 30 min to allow the conversion of the AZTR into its derivate and then measured by synchronous scanning fluorescence in the range of 300–600 nm employing a $\Delta\lambda$

of 30 nm. The intense band observed at 482 nm was used for quantitative purposes.

Pharmaceutical Samples Preparation and Measurement

Three tablets of each sample of pharmaceutical formulation (the whole content of a blister) were weighed separately in order to obtain the average weight. Then, the tablets were powdered together and a defined mass (10 mg) of the sample was mixed with 25 mL of methanol, shaken for 5 min and filtered through a membrane filter (0.23 μm). After that, 250 μL of the obtained solution was transferred to a 25 mL volumetric flask and 4 mL of water and 19.0 mL of concentrated hydrochloric acid were added to this solution to promote the derivatization of the AZTR. The volume was then made up to the mark with water and the solution was slowly agitated, in an ice-bath, to allow its homogenization. So, the final solution was left on the bench for 30 min and taken for measurement by synchronous scanning fluorescence in the range of 400–600 nm employing a $\Delta\lambda$ of 30 nm. As for the standard solutions, the fluorescence intensity at 482 nm was employed for quantitative purposes.

Results and Discussion

As mentioned previously, the main goal of this work was to develop a method for the determination of azithromycin in pharmaceutical formulations by synchronous scanning fluorescence after its derivatization in strong acidic medium. In order to achieve this goal, the effect of several variables on the efficiency of the derivatization process was evaluated. In the first part of the work, the derivatization process was monitored by UV–vis spectrophotometry, taking into account that a product with satisfactory absorption at 482 nm was yielded. The derivatization process was also studied by mass spectrometry with electrospray ionization (low fragmentation). Once the derivatization process was known, the fluorescence determination was investigated.

Influence of the HCl Concentration on the Derivatization

One of the main difficulties in the AZTR determination in different kinds of samples is related to its low absorptivity in the UV and visible ranges of the electromagnetic spectrum. This fact limits the use of several analytical techniques based on the absorption and emission of electromagnetic radiation for the determination of AZTR. This drawback can be overcome with the derivatization of AZTR in strong acidic aqueous medium. So, the acidic derivatization of AZTR with HCl was chosen to convert it into an absorbing compound that could, subsequently, fluoresce. As mentioned before, the derivatization process was monitored by molecular absorption spectrophotometry.

In this sense, the first parameter evaluated was the effect of the HCl concentration on the derivatization of AZTR. In this study the reaction time was also considered, since the reaction could not occur immediately. The experiments were performed with a 25 mg L^{-1} AZTR solution containing 5.0, 7.0 and 9.0 mol L^{-1} of HCl. The absorbance of these solutions was measured at 482 nm in a time interval of 0–180 min, always at laboratory ambient temperature (23± 2 °C). As it can be seen in the Fig. 2, the absorbance signal increased with the increase of the HCl concentration. Also, the reaction seems not to be completed in less than 30 min, even when the highest concentration of HCl (9.0 mol L^{-1}) was employed. So, the HCl concentration of 9.0 mol L^{-1} was chosen for the method and further studies about the influence of the reaction time were performed.

Influence of the Reaction Time on AZTR Derivatization

In the previous study it was observed that a minimum reaction time of 30 min was necessary to convert the non-absorbing AZTR into its absorbing derivate. This time seems to be long when an analytical procedure is considered. Also, it was necessary to verify the stability of the formed derivate in strong acidic medium. So, in order to evaluate the actual reaction time and observe whether the compound produced is stable or not in the strong acid medium, a study was performed to evaluate the effect of the reaction time with solutions containing different concentrations of AZTR in a range of 0–120 min.

Figure 3 shows the results obtained in this study for AZTR concentrations of 5, 10, 20 and 40 mg L^{-1} in a medium containing 9.0 mol L^{-1} of HCl. The obtained

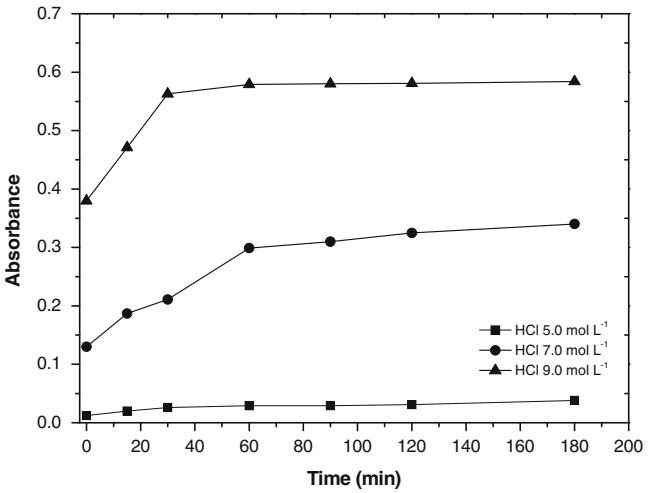

Fig. 2 Effect of the HCl concentration and the reaction time on the derivatization of AZTR. [AZTR]=25 mg L^{-1} and temperature = 23 °C

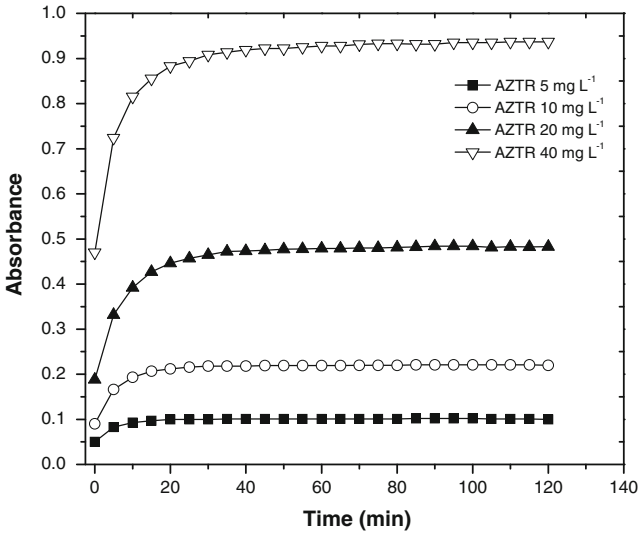

Fig. 3 Kinetic study of the reaction of azithromycin (in different concentrations) with hydrochloric acid 9.0 mol L^{-1}, at 23 °C

results showed, as expected, that the derivatization reaction depends on both time and AZTR concentration in solution. Faster derivatization was observed when the lowest concentrations of AZTR (5 or 10 mg L^{-1}) were tested. In these cases, the signal remained constant after 15 min of reaction. For the highest AZTR concentrations (20 or 40 mg L^{-1}), the time required to complete the reaction was approximately 30 min. These results indicated that a minimum reaction time of 30 min must be employed in the preparation of standard solutions and samples of AZTR before measuring by molecular absorption or fluorescence. Also, it could be verified that, after elapsed this time, the system remained unchanged for 120 min, at least, which showed that the product formed presents excellent chemical stability even in a strong acidic medium.

Influence of the Temperature on the AZTR Derivatization

As mentioned previously, a reaction time of 30 min seems to be excessively long for an analytical procedure. So, in order to get a faster AZTR derivatization, the effect of the temperature was investigated. In the experiment, solutions containing 25 mg L^{-1} of AZTR and 9.0 mol L^{-1} of HCl were put in a temperature-controlled (±1 °C) water bath, and the absorbance signals were measured at the beginning of the experiment and after 30, 60, 90, 120 and 180 min. Three temperatures were tested: 23 (laboratory ambient temperature), 40 and 70 °C. The obtained results are shown in Fig. 4. As it can be seen, the increase of the temperature did not have a positive effect on the reaction rate. The maximum signal was verified after 30 min for both 23 and 40 °C temperatures, indicating that this parameter does not have influence on the reaction time. Also, a significant decrease of the signals was always observed when the solutions were heated at 70 °C. This problem was more intense for longer reaction times, evidencing that the increase of the temperature can degrade the derivate, thus reducing the signals. So, in order to provide maximum sensitivity for the methodology under development, the reaction was always performed at laboratory ambient temperature, which varied in the range of 21–25 °C.

Investigation of the Derivatization Reaction

The derivatization reaction of AZTR in acid medium was monitored using mass spectrometry with electrospray ionization operating in the positive mode (MS-ESI(+)). The electrospray ionization was employed due to the low fragmentation imposed to the substances contained in the samples, which allowed the determination of the fragments promoted by the acid. The experiment was carried out in two steps. In the first step, an aqueous solution of AZTR (0.4 mg L^{-1}) was directly injected into the spectrometer. Secondly, the AZTR was derivatized in acid medium (40 mg L^{-1} of AZTR in 9.0 mol L^{-1} of HCl) and the products were monitored by mass spectrometry and compared with those observed without acid addition. It is important to remark that the solution yielded from the derivatization was diluted 100 times before injection into the spectrometer in order to adjust the concentration of AZTR to the same level of the non-derivatized solution (0.4 mg L^{-1}) and to avoid the injection of a solution containing very high acid concentration, which could damage the metallic parts of the instrument.

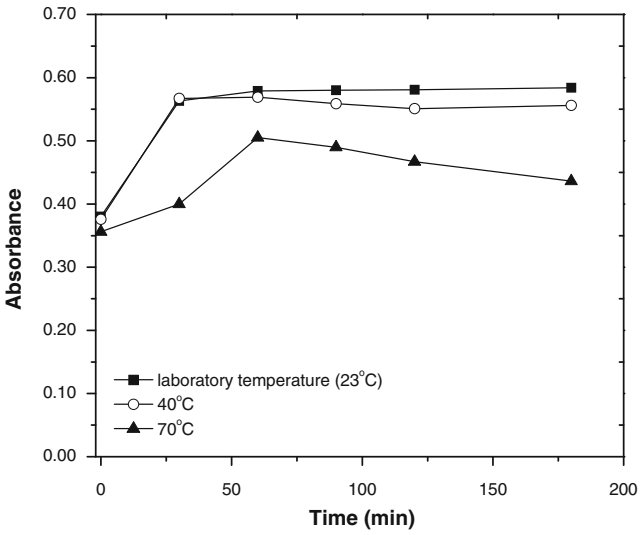

Fig. 4 Effect of the temperature on the conversion of AZTR into its absorbing form. [AZTR]=25 mg L^{-1} and HCl concentration = 9.0 mol L^{-1}

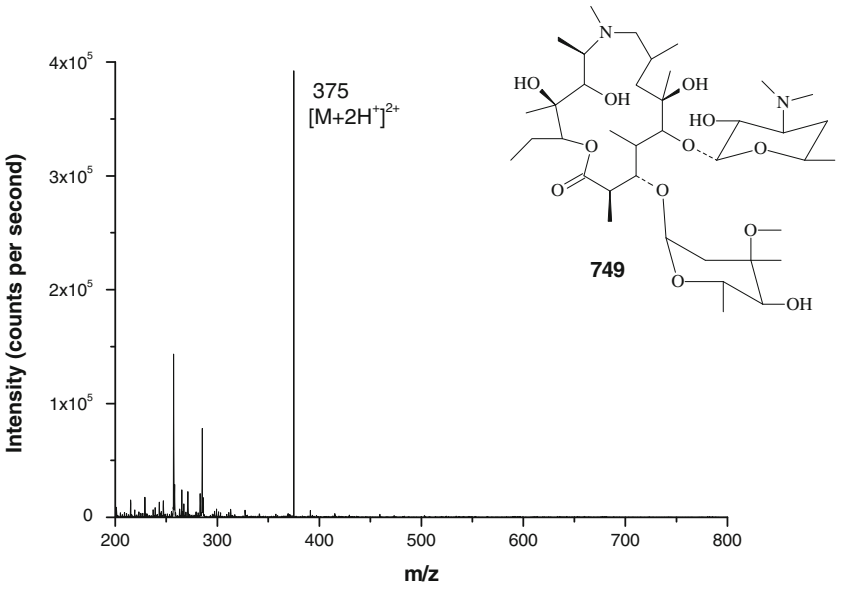

Fig. 5 MS-ESI (+) spectrum of a 0.4 mg L^{-1} aqueous solution of AZTR

Figure 5 shows the MS-ESI (+) spectrum of the aqueous solution of AZTR, in which the main peak was observed at 375 m/z. As AZTR has a molecular mass of 749 and two nitrogen atoms, one in the lactone ring and other in one of the sugar moieties, the peak at 375 m/z appears probably due to a molecular ion formed from the simultaneous protonation of the two nitrogen atoms $[M+2H]^{2+}$, as already reported by Abuin et al. [26] and Shen et al. [27]. Even though some other peaks were observed in the region of lowest m/z values in the spectrum, the predominance of the 375 m/z indicates that AZTR is practically unaltered in the aqueous solution.

In order to compare the derivatized AZTR with the non-derivatized AZTR, a MS-ESI(+) spectrum of AZTR in acid medium was obtained (Fig. 6). The difference between the spectra before and after the acid addition is clear, evidencing that the derivatization reaction has occurred. The peak at 375 m/z was not observed after acid addition, confirming that AZTR is no more present in its original form. Two other intense peaks appeared in the spectrum of the derivatized AZTR, one at 434 m/z and other at 296 m/z. The peak at 434 m/z is due to a protonated lactone fragment [26, 27] and the peak at 296 m/z can be assigned to the lactone bonded to the amino sugar (592 m/z), bi-protonated.

The mass spectra showed that the AZTR in its original form does not exist in the strong acid medium employed for derivatization. Besides, the obtained data indicated that the main reactions that occur in the system are related to the

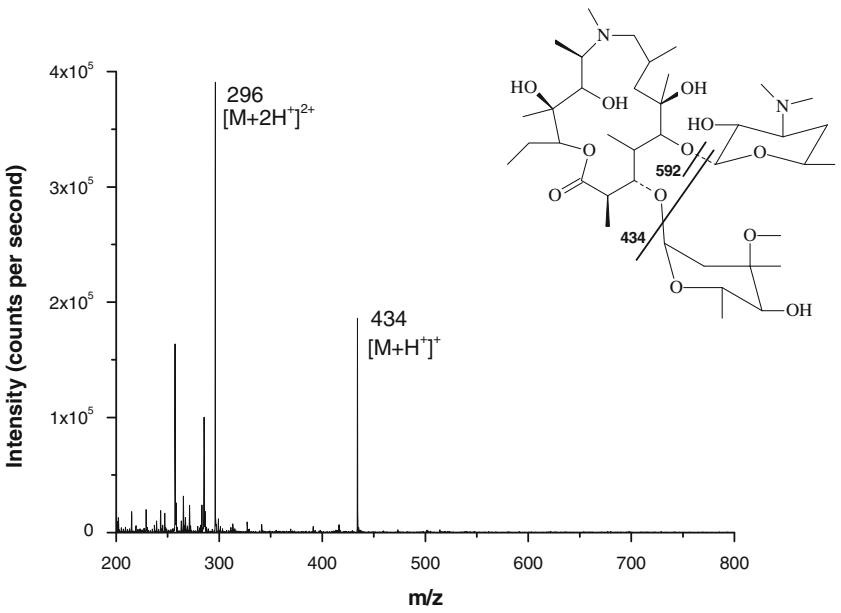

Fig. 6 Mass spectrum of derivatized AZTR in acidic solution (0.4 mg L^{-1} of Azithromycin in 9.0×10^{-3} mol L^{-1} HCl). It is important to remark that the AZTR derivatization (40 mg L^{-1}) was performed with 9.0 mol L^{-1} HCl and the solution was diluted 100 times before injection into the mass spectrometer

breaking of the bonds responsible for the attachment of the two sugar moieties to the lactone ring.

According to these observations, it is clear that the formation of the colored product is related to the detachment of the sugar moieties and to the dehydration of the OH groups present in the lactone ring, induced by the strong acid medium. The former phenomenon could lead to the appearance of conjugated double bonds in the lactone, which would explain the strong absorbance of the derivate in the visible region of the spectrum (482 nm).

Fluorescence Spectra of Derivatized Azithromycin

The conventional luminescence spectrum of a molecule consists of two spectra: (i) one related to the excitation process and (ii) other related to the emission of the absorbed radiation. The excitation spectrum can be obtained by varying the wavelength of the excitation monochromator (λ_{em}) in a given range and keeping the excitation monochromator at a fixed wavelength (λ_{ex}). The emission spectrum can be obtained using the same strategy, but scanning the emission monochromator and fixing the excitation monochromator at a particular wavelength. For a fluorescent molecule, a pair of wavelengths is observed where maximum intensity appears in the respective bands of emission and excitation spectra (λ_{em} and λ_{ex}).

The first requirement to a compound generates a luminescence spectrum is to absorb electromagnetic radiation. As AZTR does not absorb radiation, it does not produce a luminescence spectrum when in the original form. However, after acid derivatization, AZTR is converted into a substance that absorbs radiation, being also able to give a fluorescent spectrum. Taking into account that the derivatized AZTR strongly absorbs at 482 nm, its luminescence spectrum was recorded (using a 25 mg L^{-1} AZTR solution in 9.0 mol L^{-1} HCl) fixing the excitation monochromator at 482 nm and scanning the emission monochromator in the range of 485–780 nm. An intense emission band with maximum at 515 nm appeared in the spectrum, evidencing the fluorescence of the AZTR derivate. Afterwards, the excitation spectrum was registered, but now keeping the emission monochromator at 515 nm and scanning the excitation monochromator in the range of 270–510 nm. A strong light scattering (Fig. 7a) was observed when the excitation spectrum was recorded. This fact made impossible to obtain the conventional luminescence spectrum of the derivatized AZTR.

A good alternative employed to deal with luminescence when spectral interferences are present is the use of synchronous scanning fluorescence [28]. In this technique, the excitation and emission monochromator are scanned simultaneously and the emission intensity is recorded as a function of the excitation wavelength. The $\Delta\lambda$ (difference between λ_{em} and λ_{ex}) plays an important role and is responsible for the intensity and the position of the bands in the

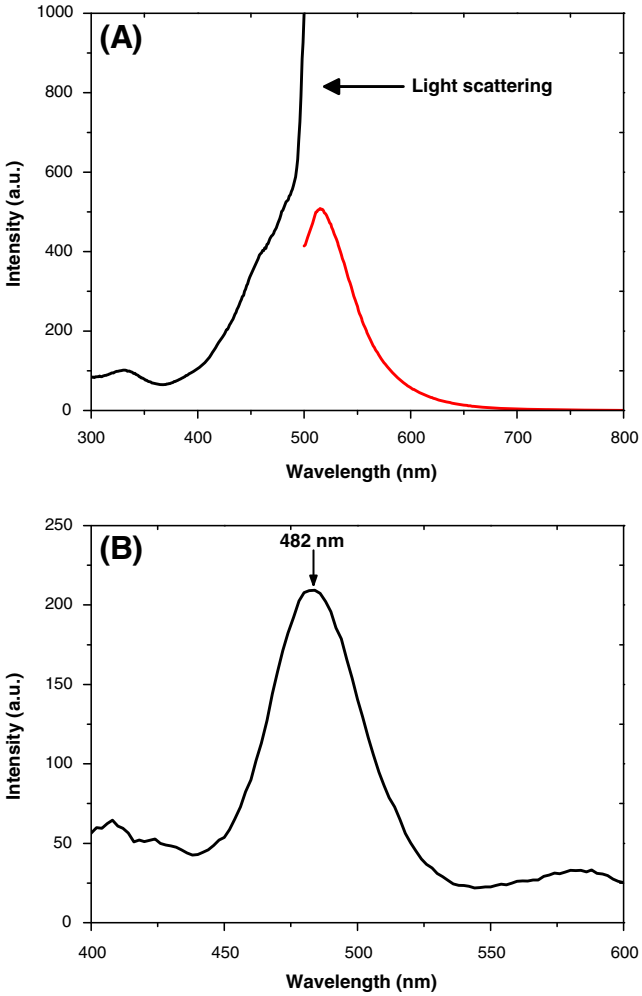

Fig. 7 Excitation and emission fluorescence spectra (**a**) and synchronous scanning spectrum ($\Delta\lambda$=30 nm) (**b**) of derivatized AZTR

synchronized spectrum. In general, sharper bands are obtained in the synchronous fluorescence spectrum in comparison with the conventional luminescence spectrum, what increases the spectral resolution [29].

Some researches [30] support that the ideal $\Delta\lambda$ should be obtained from a three-dimensional spectrum, where the three axes are the excitation wavelength, the emission wavelength and the fluorescence intensity. This graph gives a complete description of the fluorescence behavior of a compound. However, this procedure is not necessary in all cases. In the present work, an initial idea of the optimum $\Delta\lambda$ was already given by the interpretation of the luminescence spectrum. The difference between maximum excitation and emission, even in the presence of the light scattering, was 33 nm. So, in order to evaluate the influence of this parameter on the measurement of the derivatized AZTR by synchronous scanning fluorescence, the emission intensity was tested for three different $\Delta\lambda$ (30, 55 and 80 nm) at three different AZTR concentrations (2, 5 and 10 mg L^{-1}). The obtained results are shown in Fig. 8. As it can be seen, the fluorescence intensity increased with the

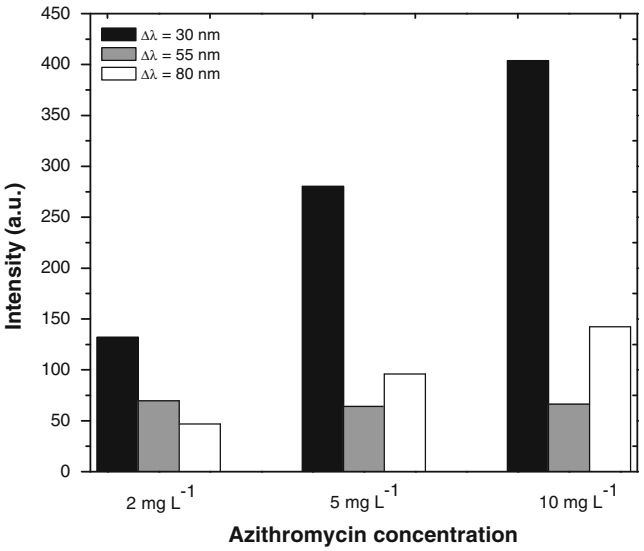

Fig. 8 Effect of the Δλ on the signal of AZTR obtained by synchronous scanning fluorescence after derivatization with 9.0 mol L^{-1} HCl

increase of the AZTR concentration only when a Δλ of 30 or 80 nm was employed. A linear relationship was observed for the two cases, evidencing that the fluorescence of the derivatized AZTR is measured in each condition. Highest sensitivity was obtained when a Δλ of 30 nm was employed, confirming the hypothesis previously raised that a good pair of excitation and emission wavelengths is represented by 482 and 515 nm, respectively. For the Δλ of 55 nm, there was no significant difference among the signals observed when using synchronous scanning fluorescence.

The results obtained in this experiment showed that the AZTR could be measured (after derivatization with 9.0 mol L^{-1} HCl) by synchronized scanning fluorescence, at 482 nm, employing a Δλ of 30 nm (Fig. 7b). These conditions were then established for the developed method, which was characterized and applied in the determination of AZTR in pharmaceutical formulations. It is important to remark that a linear relationship between fluorescence intensity and AZTR concentration was observed in the optimized conditions.

Analytical Figures of Merit of the Developed Methodology

Analytical curves for AZTR were constructed employing the optimized methodology. Good linearity was obtained in the range of 1–8 mg L^{-1} of AZTR with a typical equation of I_F=37.3 [AZTR] + 22.3 (r^2=0.998), where I_F is the fluorescence intensity, in arbitrary units, and [AZTR] is the azithromycin concentration in mg L^{-1}. It is important to notice that three standard solutions were prepared for each point of the analytical curve and the average value of I_F was used to derive the equation.

The limit of detection of the method, calculated from three times the standard deviation of ten measurements of the blank, was 0.23 mg L^{-1}, while the limit of quantification, calculated from ten times the standard deviation, was 0.76 mg L^{-1}. The precision of the method was assessed by the fluorescence measurement (synchronized scanning mode) of ten standard solutions of the same concentration (1.0 mg L^{-1}). The relative standard deviation of these measurements was 4.75 %.

Application of the Proposed Methodology in the Determination of Azithromycin in Pharmaceutical Formulations

In order to evaluate the applicability of the developed method, five pharmaceutical formulations were analyzed in relation to the AZTR content. Each tablet of the samples contained, according to the manufacturer, 500 mg of azithromycin, which corresponds to 524 mg of dihydrated azithromycin. Also some concomitants were present such as calcium phosphate, starch, sodium lauryl sulfate, magnesium stearate, polyvinyl alcohol, titanium dioxide and talc. Most of these compounds present low solubility in methanol, and for this reason, could be separated from AZTR in the extraction step (as described in the Experimental section).

Firstly, a test was performed in order to investigate if the sample matrix could cause significant interference on the AZTR determination. For this purpose, the analytical curve was compared to the standard addition curve, prepared with the extract obtained in the extraction step. As both curves presented the same slope, it was considered that matrix interferences were not present and all determinations could be carried out by employing the external calibration approach.

The analytical procedure is described, in detail, in section 2.3.2. Five samples containing AZTR were analyzed by the proposed methodology and the obtained results were compared with those indicated by the manufacturers. Such results are shown in the Table 1. It can be seen that a

Table 1 Results obtained in the determination of AZTR in pharmaceutical formulations. Values expressed as mean ± standard deviation (n=3)

Sample	Mass of di-hydrated AZTR found in each tablet (mg)	Difference[a] (%)
S_1	475±4	9.4
S_2	505±10	3.6
S_3	490±6	6.5
S_4	549±19	4.8
S_5	515±14	1.7

[a] In relation to the value declared by the manufacturer, which was 524 mg

Table 2 Results obtained in the application of the recovery test. Values expressed as mean ± standard deviation (n=3)

Sample	AZTR added (mg)	AZTR recovered (mg)	Recovery (%)
S_1	5.0	4.6±0.1	92 %
	10.0	10.4±0.2	104 %
S_2	5.0	5.0±0.5	100 %
	10.0	10.1±0.6	101 %

difference lower than 10 % was always observed when the labeled and obtained values were compared.

In order to attest the accuracy of the developed procedure, a recovery test was performed with the samples S_1 and S_2. For this purpose, the solid standard of AZTR was added to the samples (100 mg), before the extraction, in two different levels (5 and 10 mg). Afterwards, the total AZTR (sample + added) was extracted and analyzed by synchronized scanning fluorescence, employing the conditions established in the development of the method. The results are shown in the Table 2.

Conclusions

This work proposes a new method for the spectrofluorimetric determination of azithromycin in pharmaceutical formulations. The fluorescence of azithromycin could be observed after its derivatization in strong acid medium containing 9.0 mol L^{-1} of HCl. The acid concentration and the reaction time had significant influence on the conversion of AZTR into its derivate, while the increase of the temperature did not accelerate the process and caused the degradation of the fluorescent product.

The derivatization reaction was investigated by mass spectrometry and the results obtained indicated that the glycoside bonds are broken in the strong acid medium, forming a product that is able to absorb visible light. It is possible that the molecule is dehydrated in the acid medium (through OH groups attached to the lactone ring), producing a substance with a number of conjugated double bonds, which could explain the absorption of electromagnetic radiation in the visible region of the spectrum (at 482 nm).

The fluorescence of the product was evaluated and the use of the synchronized scanning mode was essential to solve the scattering of the light. Best conditions for azithromycin measurement were observed with a $\Delta\lambda$ of 30 nm. Under optimized conditions, the limit of detection of the method was 0.23 mg L^{-1} and the limit of quantification was 0.76 mg L^{-1}. The developed methodology was successfully applied in the determination of azithromycin in five pharmaceutical formulations.

Acknowledgments The authors are grateful to CNPq, CAPES and FAPERJ for the grants, fellowships and scholarships and for the financial support.

References

1. Pacifico L, Chiesa C (2002) Azithromycin in children: a critical review of the evidence. Curr Ther Res 63:54–76
2. Hoepelman IM, Mollers MJ, van Schie MH, Greefhorst APM, Schlösser NJJ, Damsté EJS, van de Moosdijk CN, Dalinghaus WH, Eland ME, Mol SJ, Rozenberg-Arska M (1998) A short (3-day) course of azithromycin tablets versus a 10-day course of amoxicillin–clavulanic acid (co-amoxiclav) in the treatment of adults with lower respiratory tract infections and effects on long-term outcome. Int J Antimicrob Agents 9:141–146
3. Degerli K, Kilimcioğlu AA, Kurt O, Tamay AT, Ozbilgin A (2003) Efficacy of azithromycin in a murine toxoplasmosis model, employing a Toxoplasma gondii strain from Turkey. Acta Trop 88:45–53
4. Passos MRL, Bravo RS, Barreto NA, Varella RQ, Santos CCC, Nascimento AVS, Morelli RH (2001) Azithromycin in the treatment of the early syphilis during pregnancy. DST-J Bras Doenças Sex Transm 13(3):33–38
5. Hoepelman IM, Schneider MME (1995) Azithromycin: the first of the tissue-selective azalides. Int J Antimicrob Agents 5:145–167
6. Lode H, Borner K, Koeppe P, Schaberg T (1996) Azithromycin—review of key chemical, pharmacokinetic and microbiological features. J Antimicrob Chemother 37:1–8
7. Treadway G (2001) Azithromycin: a new 15-membered macrolide. Jpn J Antibiot 54(Suppl A):70–76
8. Runes HR, Roncari AFF (2005) Microbiological assay for the determination of azithromycin in ophthalmic solutions. Acta Pharmacol Sin 40:544–549
9. Brazilian Pharmacopea, 4th ed., Editora Atheneu, São Paulo, 1998, p 218
10. Breier AR, Garcia CV, Oppe TP, Steppe M, Schapoval EES (2002) Microbiological assay for azithromycin in pharmaceutical formulations. J Pharm Biomed Anal 29:957–961
11. de Paula CER, Almeida VGK, Cassella RJ (2010) Novel spectrophotometric method for the determination of azithromycin in pharmaceutical formulations based on its charge transfer reaction with quinalizarin. J Braz Chem Soc 21:1664–1671
12. Suhagia B, Shah A, Rathod I, Patel H, Doshi K (2006) Determination of azithromycin in pharmaceutical dosage forms by spectrophotometric method. Indian J Pharm Sci 68:242–244
13. Rachidi M, Elharti J, Digua K, Cherrah Y, Bouklouze A (2006) New spectrophotometric method for azithromycin determination. Anal Lett 39:1917–1926
14. Bahrami G, Mirzaeei S, Kiani A (2005) High performance liquid chromatographic determination of azithromycin in serum using fluorescence detection and its application in human pharmacokinetic studies. J Chromatogr B 820:277–281
15. Wilms E, Trumpie H, Veenendaal W, Touw D (2005) Quantitative determination of azithromycin in plasma, blood and isolated neutrophils by liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonylchloride and fluorescence detection. J Chromatogr B 814:37–42
16. Sastre-Toraño J, Guchelaar H-J (1998) Quantitative determination of the macrolide antibiotics erythromycin, roxithromycin, azithromycin and clarithromycin in human serum by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride and fluorescence detection. J Chromatogr B 720:89–97

17. The United States Pharmacopeia—The National Formulary—USP 27; NF-22, Rockville, 2004, p. 198
18. Leal C, Codony R, Compañó R, Granados M, Dolors Prat M (2004) Determination of macrolide antibiotics by liquid chromatography. J Chromatogr A 910:285–290
19. Nirogi RVS, Kandikere VN, Shukla M, Mudigonda K, Maurya S, Boosi R, Yerramilli A (2005) Sensitive and selective liquid chromatography–tandem mass spectrometry method for the quantification of azithromycin in human plasma. Anal Chim Acta 553:1–8
20. Koch DE, Bhandari A, Close L, Hunter RP (2005) Azithromycin extraction from municipal wastewater and quantitation using liquid chromatography/mass spectrometry. J Chromatogr A 1074:17–22
21. Chen L, Qin F, Ma Y, Li F (2007) Quantitative determination of azithromycin in human plasma by ultra performance liquid chromatography–electrospray ionization mass spectrometry and its application in a pharmacokinetic study. J Chromatogr B 855:255–261
22. Debremaeker D, Visky D, Chepkwony HK, Van Schepdael A, Roets E, Hoogmartens J (2003) Analysis of unknown compounds in azithromycin bulk samples with liquid chromatography coupled to ion trap mass spectrometry. Rapid Commun Mass Spectrom 17:342–350
23. Barrett B, Bořek-Dohalský V, Fejt P, Vaingátová S, Huclová J, Němec B, Jelínek I (2005) Validated HPLC–MS–MS method for determination of azithromycin in human plasma. Anal Bioanal Chem 383:210–217
24. Khashaba P (2002) Spectrofluorimetric analysis of certain macrolide antibiotics in bulk and pharmaceutical formulations. J Pharm Biomed Anal 27:923–932
25. Lakowicz JR (2006) Principles of fluorescence spectroscopy, 3rd edn. Springer, NY
26. Abuin S, Codony R, Compañó R, Granados M, Dolors Prat M (2006) Analysis of macrolide antibiotics in river water by solid-phase extraction and liquid chromatography–mass spectrometry. J Chromatogr A 1114:73–81
27. Shen Y, Yin C, Su M, Tu J (2010) Rapid, sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the quantification of topically applied azithromycin in rabbit conjunctiva tissues. J Pharm Biomed Anal 52:99–104
28. Andrade-Eiroa A, de-Armas G, Estela JM, Cerda V (2010) Critical approach to synchronous spectrofluorimetry. I. Trends Anal Chem 29:885–901
29. Patra D, Mishra AK (2002) Recent developments in multicomponent synchronous fluorescence scan analysis. Trends Anal Chem 21:787–798
30. Rubio S, Gomes-Hens A, Valcarcel M (1986) Analytical applications of synchronous fluorescence spectroscopy. Talanta 33:633–640