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LC method for telithromycin in tablets: A stability-indicating assay

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ABSTRACT

A liquid chromatographic (LC) method for the quantitative determination of telithromycin, the first member of the ketolides, which is a new class of macrolides, was developed. Analytical parameters were studied according to International Conference on Harmonization (ICH) guidelines. An Ace RP-18 octadecyl silane column (250 mm × 4.6 mm, 5 μm) maintained at 50 °C was used as the stationary phase, and methanol and 0.067 M potassium monobasic phosphate buffer pH 4.0 (55:45, v/v) were used as the mobile phase with UV detection at 265 nm. In forced degradation studies, the effects of acid, base, oxidation, UV light and temperature were investigated showing no interference in the drug peak.

The method was linear ($r = 0.9999$) at concentration ranging from 10.0 to 40.0 μg/mL, precise (intra-day relative standard deviation [RSD] and inter-day RSD values < 2.0%), accurate (mean recovery = 100.76%), specific and robust. Detection and quantitation limits were 0.0027 and 0.0082 μg/mL, respectively. The results showed the proposed method is suitable for its intended use.

The validated method may be used to quantify telithromycin tablets and to determine the stability of the drug. The method is able to separate telithromycin from its degradation products and tablet excipients for its sensitivity and reproducibility.

These results are in accordance with a previous microbiological assay study, which used the same tested conditions showing that the methods can be interchangeable.

1. Introduction

The emergence of antibiotic-resistant bacterial strains is driving the search for new antimicrobial agents and will hopefully lead to the widespread application of new antibiotics in the future. A number of new agents have reached the market in recent years. They show significantly improved activity against bacteria that have acquired resistance or showed limited susceptibility to older agents (Ackermann and Rodloff, 2003).

Telithromycin (Fig. 1) 3-De[(2,6-dideoxy-3-*C*-methyl-αLribohexoppyranosyl)oxy]-11,12-dideoxy-6-*O*-methyl-3-oxo-12,11-[oxycarbonyl[4-[4-(3-pyridinil)-1*H*-imidazol-1-1-yl]butyl]imino] erythromycin; [191114-48-4]; $C_{43}H_{65}N_5O_{10}$; mol. wt. 812.00 is the first antibiotic belonging to a new class of 14-membered ring macrolides, named ketolides, to achieve clinical use. This new addition to the macrolide-lincosamide-streptogramin$_B$ (MLS$_B$) group was developed specifically for the treatment of community-acquired respiratory tract infections (The Merck Index, 2001; Ackermann and Rodloff, 2003).

The discovery of ketolides, derived from erythromycin incorporating a C-3 ketone modification, revealed a class of compounds with excellent activity against some macrolide-resistant bacteria, especially clinically important respiratory tract pathogens, such as *Streptococcus pneumoniae.* The positive results showed by the use of ketolides could be responsible for the increase of macrolide antibiotics research in the pharmaceutical industry (Heninger et al., 2004).

All macrolide structures and their ketolide-derivatives are based on a macrolactone ring, being the most therapeutically relevant macrolides comprising a 14-, 15- or 16-membered ring. Erythromycin A, a natural antibiotic isolated from *Streptomyces erythreus* consists of a 14-membered lactone ring with two attached sugar groups: L-cladinose at C3 and desosamine at C5.

Telithromycin is structurally differentiated from the macrolides in three ways, each of which is associated with specific improvement in antimicrobial properties: L-cladinose at the position C3 of the macrolactone ring has been replaced by a keto function. This modification enables telithromycin to bind to its

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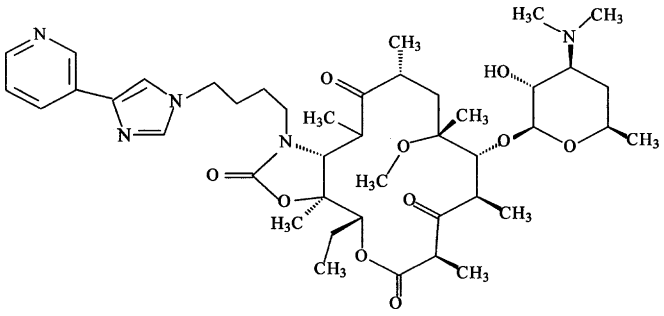

Fig. 1. Chemical structure of telithromycin.

target without tripping the inducible resistance to macrolide-lincosamide-streptogramin$_B$ (MLS$_B$) drugs that are exhibited by many pathogens groups; C6 position was modified by the addition of a methoxy group avoiding the hemiketalization of the C6 position with the 3- and 9-keto groups, thereby conferring excellent acid stability, particularly on gastric pH values; and the addition of a large aromatic N-substituted carbamate extension at C11/C12, which is responsible for the improved ribosome-binding affinity of telithromycin contributing to the improved interaction of telithromycin with MLS$_B$–resistant ribosomes, compared with erythromycin A and newer macrolides (Graul and Castañer, 1998; Zhanel et al., 2002).

Assays reported in the literature for the determination of the telithromycin in biological fluids include high-performance liquid chromatography-mass spectrometry (LC-MS) (Perret et al., 2002), LC with fluorescence detection (Traunmuller et al., 2005) and microbiological assays (Lingerfelt and Champney, 1999). With the results, the authors have made a critical analysis of *in vitro* and *in vivo* activities, pharmacokinetic and pharmacodynamic considerations concerning the use of this new agent for the treatment of respiratory infections. Other researchers have described a sensitive method for the examination of 20 macrolides and ketolides in bulk, using an LC method with a volatile mobile phase, allowing the recovery and subsequent analysis of these antibiotics (Lingerfelt and Champney, 1999). A microbiological assay in pharmaceutical dosage form applying cylinder-plate method was performed and described in a previous study (Vaucher et al., 2006). The microbiological assay can reveal subtle changes not demonstrable by chemical methods. This assay makes it possible to evaluate the potency, which is very important in the analysis of antibiotics (USP, 2006).

However, there are no studies describing quantification methods of this drug in tablets. Due to this, a method was validated by linearity, detection limit (LOD), quantitation limit (LOQ), accuracy, repeatability, intermediate precision, specificity and robustness. The system suitability was carried out to verify if the resolution and reproducibility of the chromatographic system were adequate for the analysis to be done (British Pharmacopoeia, 2005; USP, 2006). The ICH emphasizes that the test of the drugs, which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy, must be done by validated stability-indicating testing methods (ICH, 1995). Then, the aim of the present work was to develop and validate a rapid, inexpensive and selective liquid chromatography method for routine quality control analysis of telithromycin in tablets. Stress testing of the drug was also conducted, as required by the International Conference on Harmonization (ICH, 2003) to support the suitability of the method. The method was compared with a previous microbiological assay study that used the same tested conditions.

2. Materials and methods

2.1. Materials

LC grade methanol, sodium hydroxide, hydrochloric acid, hydrogen peroxide, potassium dihydrogen phosphate and orthophosphoric acid (reagent grade) were purchased from Merck (Darmstadt, Germany). Distilled water purified by a Millipore Milli-Q® UF-Plus apparatus (Millipore) was used to prepare the mobile phase. Telithromycin film-coated tablets were claimed to contain 400 mg (as the anhydrous base) of the drug and the following inactive ingredients: corn starch, croscarmellose sodium, hypromellose, lactose monohydrate, magnesium stearate, microcrystalline cellulose, polyethylene glycol, povidone, red ferric oxide, talc, titanium dioxide and yellow ferric oxide.

The telithromycin reference standard (99.3%) and the pharmaceutical dosage form were kindly supplied by Aventis Pharma (São Paulo, Brazil).

2.2. Chromatographic system

The assay was developed and validated using a Shimadzu liquid chromatograph (Kyoto, Japan) equipped with a model LC-10ADvp binary pump, SIL-10ADvp auto sampler, CTO-10ACvp column oven, SPD-M10Avp photodiode-array detector, SCL-10Avp system controller and CLASS-VP 6.12 SP2 manager system software which was used to control the equipment and to calculate data and responses from the LC system. Chromatographic analyses were performed in an Ace RP-18 octadecyl silane column (250 mm × 4.6 mm i.d., particle size 5 μm) maintained at 50 C. Telithromycin was determined by UV detection at 265 nm using photodiode array detector (PDA detector). Peak identity was confirmed by retention time comparison and comparison of the spectra obtained from the PDA detector.

The mobile phase was composed of methanol and M 0.067 potassium monobasic phosphate buffer adjusted to pH 4.0 with orthophosphoric acid (55:45, v/v). It was prepared daily, filtered through a 0.45 μm membrane filter (Millipore) and degassed using the degasser of the chromatographic system prior to use. The flow rate of the mobile phase was 1 mL/min and the injection volume was 20 μl.

2.3. Preparation of sample for LC analysis

The stock solution of telithromycin reference standard (500 μg/mL) was prepared in methanol since the drug is freely soluble in this solvent. The working standard solution (25 μg/mL) was obtained by dilution of the stock solution in the mobile phase.

Twenty tablets were weighed and the average weight was calculated. Tablets were crushed to a fine powder. A quantity of the powdered tablets, equivalent to 25 mg of telithromycin, was transferred to 50 mL volumetric flasks. Then, 25 mL of methanol was added, shaken for 10 min by a mechanical shaker, and more methanol was added until the solution reached 500 μg/mL. The solution was filtered through a 0.45 μm membrane filter (Millipore) and an aliquot of the filtrate was diluted with mobile phase to 25 μg/mL, as the working sample solution. Samples prepared to identical conditions, but protected from light, were used as a control.

2.4. Validation method

2.4.1. Linearity, limits of detection (LOD) and quantitation (LOQ)

Appropriate amounts of telithromycin stock solution (500 μg/mL) were diluted with mobile phase to give concentrations of 10.0,15.0, 20.0, 25.0, 30.0, 35.0 and 40.0 μg/mL.

On three different days each concentration was injected in triplicate and calibration plots were prepared. Linearity was evaluated by linear least-squares regression analysis.

LOD and LOQ were calculated directly from the calibration plot. Limits of detection (LOD) and quantification (LOQ) were calculated as $3.3\rho/S$ and $10\rho/S$, respectively, where ρ is the standard deviation of intercept and S is the slope of the calibration plot (ICH, 2003).

Precision: The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by assaying samples, at the same concentration and during the same day. The intermediate precision was studied by comparing the assays on different days (3 days). Six sample solutions (25 μg/mL) were prepared and assayed daily.

2.4.2. Accuracy

Accuracy was determined by the recovery of known amounts of telithromycin reference standard added to the samples in the beginning of the process. An accurately weighed amount of powdered tablets, equivalent to 25 mg of telithromycin was transferred to 50 mL volumetric flask and dissolved in methanol (final concentration of 500 μg/mL). Aliquots of 2.5 mL of this solution were transferred into 50 mL volumetric flasks containing 0.5, 1.0 and 1.5 mL of telithromycin standard solution (500 μg/mL) and then mobile phase was added to make up the volume giving final concentrations of 30, 35 and 40 μg/mL respectively, corresponding to 120, 140 and 160% of the nominal analytical concentration (25 μg/mL).

2.4.3. Specificity

Chromatographic runs of a placebo solution and forced degradation studies were performed in order to provide an indication of stability. The stress conditions employed were: heat, light, acid, base, and oxidant media. Samples were analyzed against a freshly prepared control sample (with no degradation treatment) and under light protection. All solutions were injected in triplicate. The peak purity was determined using the tools of the software Class VP by checking the similarity between all spectra of the telithromycin chromatographic peak with the apex of the same peak.

The excipient solution was submitted to the same degradation conditions in order to demonstrate no interference. Specific conditions are described below.

2.4.3.1. Effect of heat. An amount of powder equivalent to 20 mg telithromycin was transferred to a 20 mL volumetric amber flask with methanol (1 mg/mL). 2 mL of this solution was placed in a stopped 1 cm quartz cell, and wrapped in aluminum foil in order to protect from light. The sample was kept in an oven at 80 °C for 24 h to study the effect of heat. After the specified time, this solution was diluted to 50 μg/mL with mobile phase.

2.4.3.2. Effect of UV Light. An amount of powder equivalent to 20 mg telithromycin was transferred to a 20 mL volumetric flask with methanol (1 mg/mL). 2 mL of this solution was placed in a stopped 1 cm quartz cell. It was exposed to UV chamber (100, 18, and 17 cm) with mirrors and a UV fluorescent lamp, model 30 W Ecollume ZW emitting radiation at 254 nm for 4 and 24 h. Protected samples, wrapped in aluminum foil in order to protect from light were submitted to identical conditions and used as control. After the degradation treatment, the samples were diluted to 50 μg/mL with mobile phase.

2.4.3.3. Effect of oxidation. To promote oxidation, an amount of powder equivalent to 10 mg of telithromycin was transferred to 10 mL volumetric amber flasks, in order to protect from light, and then 3 mL of methanol was added in order to dilute the sample. The volume was completed with a 3% hydrogen peroxide solution, and left to react for 1, 2, 3, 5 and 8 h. After that, the solutions were filtered and diluted with mobile phase. A control solution, arranged in volumetric amber flasks was prepared under the same procedure except for the addition of telithromycin.

2.4.3.4. Effect of acid and alkaline hydrolysis. An amount of powder equivalent to 10 mg of telithromycin was transferred to 10 mL volumetric amber flasks in order to protect from light. 3 mL of methanol was added until the volume was completed with 0.1 M HCl, 0.1 M NaOH, respectively. Then, the solutions were refluxed for 1 h. After that, samples were left to equilibrate at room temperature, the pH was adjusted, and then diluted with mobile phase to 50 μg/mL.

2.4.4. Robustness

Robustness was evaluated by small variations in the pH values of the aqueous phase of the mobile phase, as well as by column temperatures. The stability of analytical solutions and reference standard stock solutions was also determined. The stability of the solutions was assessed by monitoring the peak area.

Standard working solutions were analyzed immediately after their preparation as well as 6, 12 and 24 h later. The solutions were kept at 25 °C, either exposed to day light or in the dark. The method robustness was assessed by deliberately modifying the operating conditions of the former and measuring chromatographic parameters such as retention time, tailing factor, number of plates, capacity factors. In addition, samples were analysed at each modified condition to assess any impact on assay results.

2.4.5. System suitability

System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such (USP, 2006). To verify the system suitability of the analytical method during the validation, the solution exposed to direct UV radiation (254 nm) during 4 h was used. This solution was chosen because it formed a photo degradation product with retention time next to telithromycin. The parameters available were theoretical plates, retention factor, asymmetry, resolution between the telithromycin and photo degradation peaks and, system repeatability (Shabir, 2003; USP, 2006).

3. Results

3.1. Selection of chromatographic conditions

Acceptable separations, with a retention time of 6.3 min for telithromycin, were obtained using an Advanced Chromatography Technologies Ace RP-18® octadecyl silane column (250 mm × 4.6 mm, particle size 5 μm), a mobile phase composed by methanol, and 0.067 M potassium monobasic phosphate buffer adjusted to pH 4.0 with orthophosphoric acid (55:45, v/v) was adopted. The temperature employed was 50 °C. Samples protected from light were used as a control and showed the same results.

3.2. Linearity, limits of detection (LOD) and quantitation (LOQ)

Good linearity was observed over the concentration range evaluated (10.0–40.0 μg/mL). The slope and intercept of the calibration plot (±standard deviation, $n = 3$) were 15225.66 ± 0.59 and 5587.83 ± 12.53, respectively, and the correlation coefficient was 0.9999. The validity of the assay was verified by analysis of variance. This revealed that the regression equation was linear ($F_{calculated} = 7147.26 > F_{critical} = 4.96$; $P = 0.05$) with no deviation from

Table 1
Experimental values of the telithromycin obtained for a commercially available sample by using the LC method.

	Intra-day precision ($n = 6$)	
	Label claim(%)[a]	RSD (%)
Day1	101.9	1.0
Day2	100.4	0.2
Day3	100.9	0.4
Inter-day precision ($n = 18$)		
Label claim(%)		RSD (%)
101.0		0.8

[a] Each values the mean of 3 analyses.

linearity ($F_{calculated} = 0.180 < F_{critical} = 3.71$; $P = 0.05$). LOD and LOQ were 0.0027 and 0.0082 μg/mL, respectively.

3.3. Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as relative standard deviations (RSD) of a set of results. Results presented in Table 1 indicate good repeatability and low inter-day variability (RSD maximum 1.0%).

3.4. Accuracy

The accuracy of the method was evaluated and the mean recovery obtained for each level. At each level of telithromycin concentration three determinations were performed. The mean levels (100.76%) showed that the method is accurate (Table 2).

3.5. Specificity

Telithromycin solutions were submitted to forced degradation studies to induce degradation. When submitted to 24 h at 80 °C, the telithromycin content exhibited a slight decrease, and a single degradation peak was detected in the initial chromatograph run (Fig. 2B). Under 4 h of UV radiation (254 nm), the telithromycin content exhibited some decrease and additional peaks were detected quicker than in the other conditions (Fig. 2C).

Total degradation was observed when exposed for 24 h. The results obtained in this preliminary stability study indicate that telithromycin is very susceptible to photo degradation. The extent of degradation was higher and more peaks arose with the increase in exposure time.

The same was observed when the oxidation was promoted. When the increase of the exposition time occurred, the extent of degradation was higher and additional peaks emerged. In the first hour, in 9.7 min a degradation peak was already observed, as the telithromycin content decreased (Fig. 3A). An increase in this peak along the hours was also observed. After 5 h, other peaks started to appear. Peaks in the range from 1.0 to 2.0 min were attributed to hydrogen peroxide and detected in the blank solution.

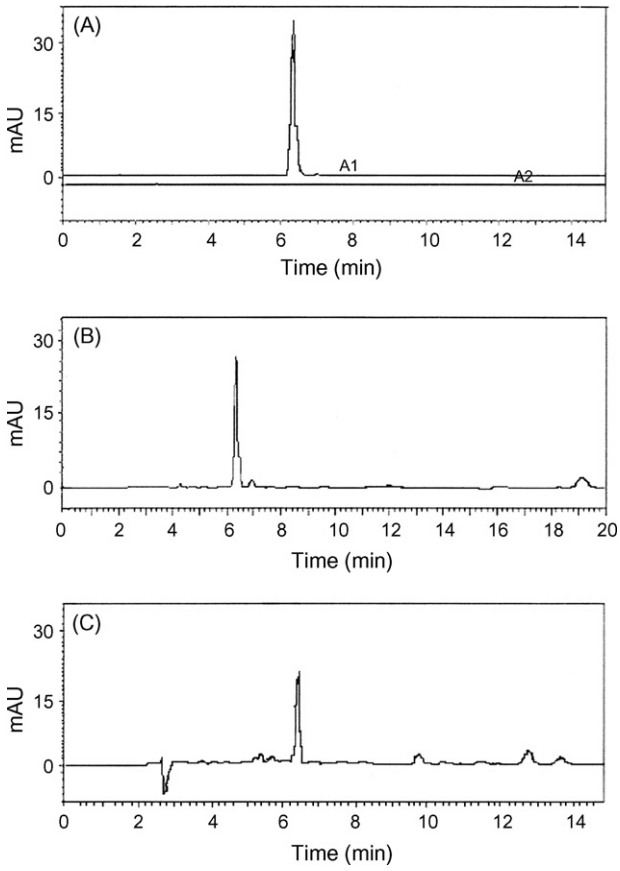

Fig. 2. Chromatograms obtained from telithromycin: a reference substance (25 μg/mL) (A1), and excipients solution (A2); after heat degradation(80 °C/24 h) (B); after UV light degradation(254 nm/24 h) (C).

When acid degradation was promoted, no degradation peaks were detected and the telithromycin content remained constant. When submitted to a basic condition with 0.1 M sodium hydroxide, the degradation was more intense and more peaks were detected. Using a different wavelength, it is possible to observe additional peaks however the peaks are not visible when telithromycin chromatographic run wavelength was used. Chromatographic runs after acid and alkaline stress are presented in Fig. 3B and C, respectively.

The LC method did not suffer interference by the formulation excipients, since no other peaks occurred in the same telithromycin retention time (Fig. 2A). The same was observed after the excipient solutions have been submitted to degradation conditions. No peak was observed in the chromatograms.

3.6. Robustness

In order to study the robustness of the proposed method, deliberate modifications were made in pH values of the aqueous phase to mobile phase and column temperature, as shown in Table 3.

Table 2
Experimental values obtained in the recovery test for telithromycin from tablets solutions spiked with standard solution.

Sample concentration (μg/mL)	Concentration of added standard (μg/mL)	% Recovery[a]	RSD (%)	Mean % Recovery
25.0	5.0	101.0	1.2	
25.0	10.0	101.4	0.8	100.76
25.0	15.0	99.9	0.7	

[a] Mean of 3 determinations.

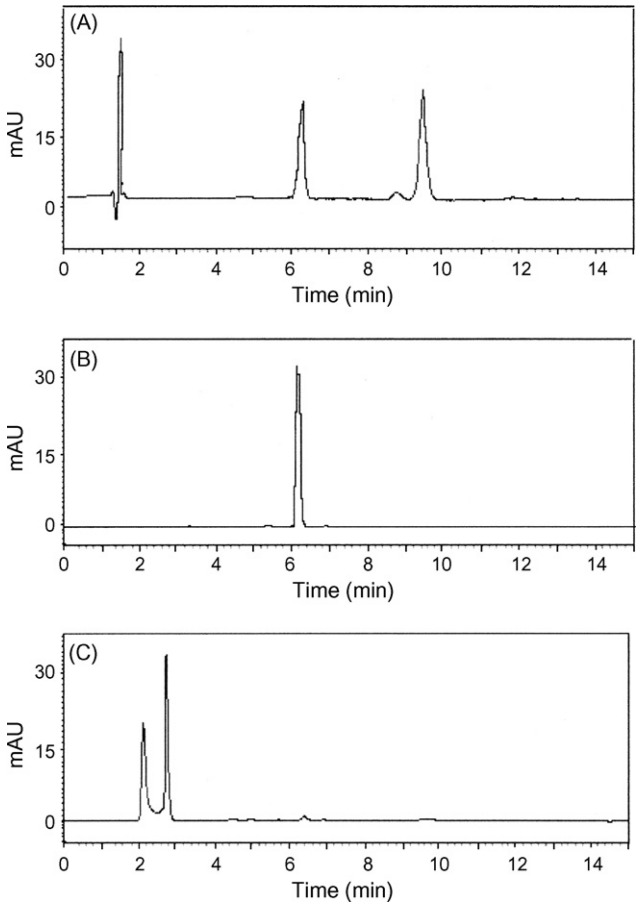

Fig. 3. Chromatograms obtained from telithromycin: after attempted oxidative degradation (3% H_2O_2/8 h) (A); after acid degradation (B); after alkaline degradation (C).

3.7. System suitability

The system suitability was verified through common parameters to telithromycin peak (R_t = 6.35) and its photo-degradation product peak (R_t = 5.35), respectively (Fig. 2C). The approximate results were: theoretical plates (N = 8686.57 and 8675.77), retention factor (k = 3.1 and 2.4), peak asymmetry or tailing factor (T = 1.25 and 1.35) and resolution between telithromycin and its photo degradation product (R = 2.3). The area peak RSD of five injections of telithromycin peak and photo degradation product peak were 0.26 and 0.23%, respectively, demonstrating the injection repeatability. The values for these parameters were satisfactory in accordance with the literature (FDA, 1994; Shabir, 2003).

4. Discussion

The LC procedure was optimized to develop a stability indicating method to resolve the degradation products from the drug. Chromatographic conditions were chosen after the test of different mobile phases with different proportions of organic solvent. The reversed phase chromatography was performed with various mixtures such as methanol–water, acetonitrile–water and phosphate buffers. The pH value of the aqueous phase was checked over a range (pH 3.5–4.5) before mixing with acetonitrile or methanol. The retention time observed (Rt = 6.3 min) allowed a rapid determination of the drug, which is important for routine analysis. Methanol was used to dissolve the samples because it showed the best values of theoretical plates and asymmetry, ease of preparation and cost.

Different brands of chromatographic columns were tested to optimize the separation, and the effect of mobile phase composition was examined.

Preliminary trials were done using different column temperatures, and acceptable separations along with a retention time of 6.3 min were observed (Fig. 2-A1). The importance of column temperature control is well known for its influence on resolution, selectivity, system pressure, and column stability. In order to demonstrate the influence of the temperature in the resolution (R), the temperature was gradually changed, (ranging from 25 to 50 °C, with a 5 °C increment), and as the temperatures increased, greater resolutions values were observed.

The best resolution values were obtained when the temperature was 50 °C (R = 2.11) being that, the minimum acceptable resolution value is R = 1.5 (Snyder et al., 1997; Dolan, 2002). Such observations indicated the use of this temperature condition for the present assay. Additionally, the assay of clarithromycin, another macrolide, is performed using this temperature (USP, 2006).

Low LOD and LOQ values are indicative of the high sensitivity of the method.

The mean (%) among the samples determined by the LC method (101.0%) was compared statistically with the mean (%) determined by the microbiological assay (99.8%) using the Student's t-test,

Table 3
Results of the robustness of the LC method.

Variations		Retention time	Tailing factor	Theoretical plates	Capacity factor	Assay of label claim (%)	RSD (%)
Mobile phase proposed[a,b] (55:45, v/v) Aqueous phase	**50 °C** **pH 4.0**	6.55	1.16	10323	1.59	100.4	0.8
Mobile phase proposed[a,b] (55:45, v/v) Aqueous phase	**50 °C** **pH 3.8**	6.52	1.25	10326	1.55	101.1	0.9
Mobile phase proposed[a,b] (55:45, v/v) Aqueous phase	**50 °C** **pH 4.2**	6.60	1.28	10401	1.56	100.4	0,8
Mobile phase proposed[a,b] (55:45, v/v) Aqueous phase	**48 °C** **pH 4.0**	6.64	1.16	9898	1.67	100.1	0.9
Mobile phase proposed[a,b] (55:45, v/v) Aqueous phase	**52 °C** **pH 4.0**	6.55	1.17	10410	1.60	101.14	0.78

[a] Metanol: 0.067 M potassium monobasic phosphate buffer; pH adjusted with orthophosphoric acid.
[b] Oven temperature.

which indicated there is no significant difference between the two methods at 0.05 significant level. Thus, the methods can be considered equivalent. The microbiological assay makes the evaluation of the potency possible. Besides, it requires no specialized equipment as well as no toxic solvents.

The chromatographic peak purity tool, applied to the telithromycin peak, demonstrated that it was pure in all cases, confirming the absence of other substances coeluting in the same retention time. Since the main telithromycin peak could not be attributed to any other peak, the method is confirmed as highly suitable for stability studies.

To be considered specific, an assay method should demonstrate that it can separate and quantify the drug from a physical mixture of the drug, degradation products and excipients.

The results obtained in the acid degradation are in accordance with the current literature about the structural modifications of the C6 position in the lactone ring. This modification proves the excellent acid stability of telithromycin (Graul and Castañer, 1998; Zhanel et al., 2002).

The specificity of the proposed LC method was also studied by analysis of degraded samples, using the microbiological assay as a comparison method. The results obtained by microbiological assay demonstrate that a decrease in the potency of telithromycin in the conditions used occurs. Telithromycin was less active at 80 °C with a mean potency of 76.76% after 24 h, whereas a mean potency of 6.42% was observed when the drug solution was kept at 3% H_2O_2 for 12 h. The drug solution exposed to ultraviolet light (254 nm) for 24 h showed total loss of the activity (Vaucher et al., 2006). In the LC method, total degradation was observed when the telithromycin solution was kept at 3% H_2O_2 for 12 h and after the UV light exposition for 24 h. It was impossible to detect the peaks with the retention time (Rt = 6.3) in both degradation conditions but more peaks appeared in the chromatographic run. The telithromycin solution submitted to 24 h at 80 °C showed 10% of loss in its concentration of the remaining drug.

The results by LC method and microbiological assay are in accordance when the same degraded conditions are used.

The robustness study has been proved that in every employed condition, the chromatographic parameters agreed with established values and the assay data remained acceptable (ICH, 2003).

The change of ±1 unit of pH value around 4.0 (pH of mobile phase) had no impact on chromatographic performance and did not generate any extra peaks. This indicates that, in the range of pH 3.5–4.5, results remain within acceptable stability.

5. Conclusions

The LC method developed is very simple and results confirm suitable accuracy, specificity and precision. Therefore, the method could be useful for both routine analytical and quality control assay of telithromycin in tablets and it could be a very powerful tool to investigate chemical stability of telithromycin. LC methods have been widely employed in pharmaceutical analysis and they have gained popularity in stability studies due to their high-resolution capacity, sensitivity and specificity. The results verified by the LC method and microbiological assay were similar. Hence, they can be used alternatively in routine quality control of telithromycin in the formulation studied.

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