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Research Article

Quantification of three Macrolide Antibiotics in Pharmaceutical lots by HPLC: Development, Validation and Application to a Simultaneous Separation

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ABSTRACT

A new validated high performance liquid chromatographic (HPLC) method with rapid analysis time and high efficiency, for the analysis of erythromycin, azithromycin and spiramycin, under isocratic conditions with ODB RP_{18} as a stationary phase is described. Using an eluent composed of acetonitrile –2-methyl-2-propanol –hydrogenphosphate buffer, pH 6.5, with 1.5% triethylamine (33:7: up to 100, v/v/v), delivered at a flow-rate of 1.0 mL min^{-1}. Ultra Violet (UV) detection is performed at 210 nm. The selectivity is satisfactory enough and no problematic interfering peaks are observed. The procedure is quantitatively characterized and repeatability, linearity, detection and quantification limits are very satisfactory. The method is applied successfully for the assay of the studied drugs in pharmaceutical dosage forms as tablets and powder for oral suspension. Recovery experiments revealed recovery of 97.13–100.28%.

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INTRODUCTION

Macrolides are an important class of antimicrobial compounds. Of the macrolide antibiotics currently in clinical use, erythromycin, azithromycin and spiramycin are the most widely prescribed, against infectious diseases caused by a number of different microorganisms (Elks and Ganellin, 1991).

Erythromycin, azithromycin and spiramycin are complex carbohydrates. The common selection of these substances which have molecular mass's in the range of 689 to 842, is macrocyclic lactone ring containing 14, 15 or 16 atoms with neutral and/or amino sugars linked via glycosidic bonds (Omura, 1984). The chemical structures of erythromycin (ERY), azithromycin (AZI) and spiramycin (SPI) are depicted in Figure 1.

Analysis of macrolide antibiotics has been performed by paper chromatography, thin–layer chromatography as well as gas and liquid chromatography (LC) among these methods, LC is the most popular technique used for the analysis of macrolides (Lahane et al., 2014; Kanfer et al., 1998).

Several HPLC methods for the individual analysis of macrolides in a variety of matrices including raw material, pharmaceutical dosage forms, biological fluids and various tissues have been reported (Kanfer et al., 1998; Nasr and Tscchappler, 1996;

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Kauss et al., 2012; Cao et al., 2013), merely for erythromycin and occasionally for the other macrolides. Unfortunately, most official methods for the analysis of macrolides are mainly antimicrobial assays (Mahmoudi et al., 2015; British Pharmacopoeia, 1998; Official Methods for Residual Substances in Livestock Products, 1994), which they suffer from many disadvantages as the long incubation periods and the lack of sensitivity. Other reported HPLC methods, including electrochemical detection (Duthu, 1984), fluorescence detection by pre-column derivatization (Bahrami et al., 2005) and liquid chromatography–mass spectrometry or LC–MS/MS (Gonzalez de la Huebra et al., 2004; Shen et al., 2010) have been used for the individual quantitative determination of ERY, AZI and SPI. These methods were time-consuming, tedious, and dedicated to sophisticated and expensive analytical instruments. Many LC methods have been applied to multiresidue analysis of macrolides in biological samples (Gonzalez de la Huebra et al., 2007; Dubois et al., 2001), and no reversed–phase LC methods have been reported for the simultaneous determination of the three macrolides in routine pharmaceutical dosage forms.

In this manuscript, a novel validated, rapid, precise and robust HPLC method by using response peak-height methodology and ODB stationary phase was described for the analysis and quantitative determination of erythromycin, azithromycin and spiramycin in raw material and pharmaceutical dosage forms. In addition, simultaneous separation was also applied to support the economical suitability of this method.

2. MATERIALS AND METHODS

2.1. Apparatus

LC experiments were performed using a LC–10ATVP pump (Shimadzu, Japan) equipped with an injector model Rheodyne (C.C, U.S.A) and a 20µL loop, a SPD –10AVP UV–vis detector (Shimadzu, Japan) set at 210 nm. The column was immersed in a water–bath, heated by a memert D–91126 thermostat (FRG, Germany). Separation was achieved with ODB RP_{18} column, 250 mm × 4.6 mm, packed with silica uptisphere 5 µm (Interchim, France).

[A]

[B]

[C]

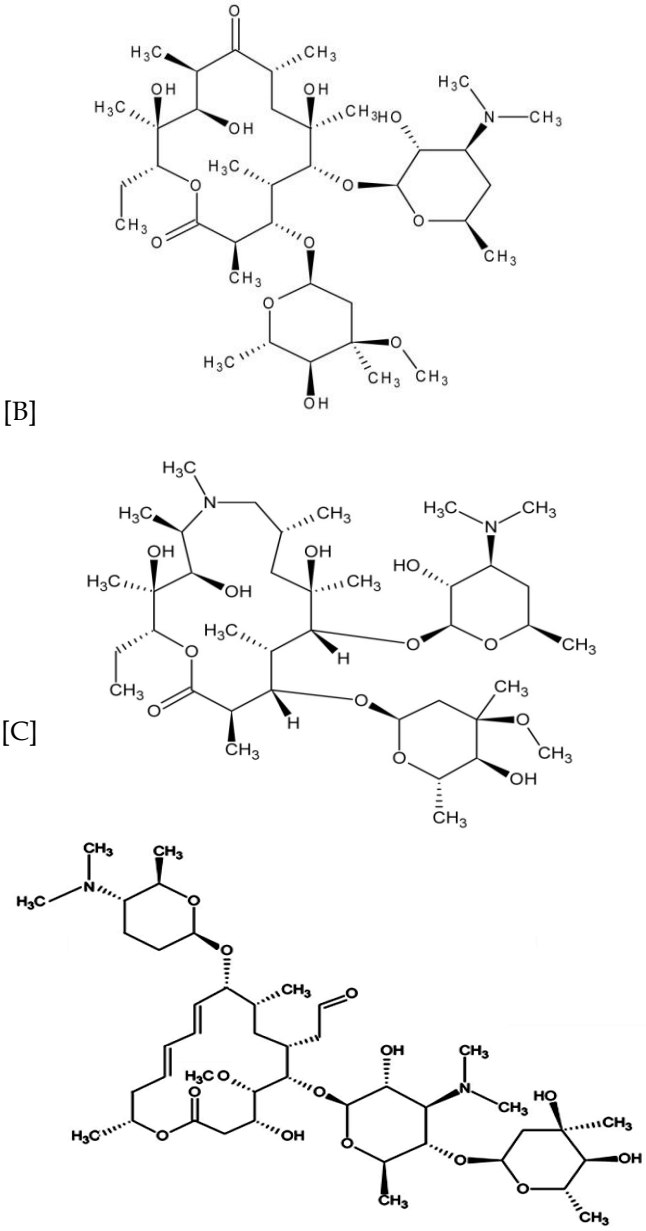

Fig.1. *Chemical structures of [A] erythromycin, [B] azithromycin and [C] spiramycin.*

2.2. Materials and reagents

Pharmacopeial standards of erythromycin and spiramycin were purchased from Sigma (Steiheim, Germany), and working standard of azithromycin was kindly provided from SAIDAL–GROUPE (Saidal, Algeria), while pharmaceuticals containing these macrolide antibiotics were obtained commercially. Erybesan® film-coated tablets (Sandoz, Algeria): labelled to contain erythromycin stearate as 500 mg/tablet; Azimycine® powder for oral suspension (Saidal, Algeria): labelled to contain azithromycin as 200 mg/5mL; Rovamycin® tablets

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(Saidal, Algeria): labelled to contain spiramycin as 1.5 MIU/tablet (equivalent of 1.0 IU is 0.3µg).

Dipotassium hydrogenphosphate, 2-methyl-2-propanol, acetonitrile (ACN), triethylamine and absolute ethanol were of analytical–reagent grade from SIGMA–ALDRICH (Steiheim, Germany). Methanol was of chemical grade from the same source. The following mobile phase was finally used: acetonitrile–2-methyl-2-propanol–0.025M potassium phosphate buffer (pH 6.5) (33: 7: up to 100, v/v/v), the flow–rate was 1.0 mL min^{-1}. The phosphate buffer solution was filtered through a Millipore GS 0.22 µm filter (Milford, MA, USA). Deionized and double-distilled water was used throughout all experiments.

2.3. Preparation of Standard Solutions

Stock standard solutions of ERY and AZI were prepared by dissolving 400 mg of each compound in 100 mL of 0.025 M dipotassium hydrogen phosphate (pH 6.5)–acetonitrile (7:3, v/v), while a weighed quantity of 100 mg of SPI was dissolved in 100 mL of water–acetonitrile (7:3, v/v). The standard solutions were kept at 4°C in amber glass vessels. From each standard solution a series of dilution was prepared quantitatively in appropriate solvent to obtain standard solutions having concentration ranges of linearity. The solutions were prepared freshly every day and used as working standards.

2.4. Sample Preparation

2.4.1. Erybesan® Tablets

The sample preparation was done as follows based on a previous paper (Khashaba, 2002). Ten tablets were weighed and finely powdered. A weighed portion equivalent to the weight of one tablet was transferred to a 100 mL volumetric flask, sonicated for 5 min with about 10 mL phosphate buffer(pH 6.5)–acetonitrile (7:3, v/v) then the solution was diluted to volume with the same solvent. The mixture was mixed well, allowed any insoluble matter to settle then filtered through a Millipore GS 0.22 µm filter. A measured volume of the filtrate was diluted quantitatively with the same solvent to yield a sample solution having a working concentration assumed to be 4.0 mg mL^{-1}. This sample was evaluated in triplicate. This procedure was performed two times.

2.4.2. Azimycine® Powder for Oral Suspension

An amount of powder equivalent to 100 mg of azithromycin was transferred to 25 mL volumetric flask with 15 mL absolute ethanol and shaken for 20 min, followed by marking up to volume with the same solvent. The procedure was then completed as in Section 2.4.1 to yield a sample solution having a concentration assumed to be 4.0 mg mL^{-1} of azithromycin. This sample was evaluated in triplicate. This procedure was performed two times.

2.4.1. Rovamycin® Tablets

An amount equivalent to 450 mg of spiramycin was transferred to 200 mL volumetric flask with 50 mL water–acetonitrile (7:3, v/v) and shaken for 20 min, followed by marking up to volume with the same solvent. After filtration, from this solution a series of dilution was prepared quantitatively in double distilled water to give a final concentration of 1.0 mg mL^{-1} of SPI. This sample was evaluated in triplicate. This procedure was performed two times.

2.5. Method Validation

The method was validated by determination of linearity, sensitivity, precision, accuracy and specificity (US Pharmacopoeia, 2000; Validation on analytical procedures, 1996). Each day, a quantity of about 25 mg of macrolide was weighed and dissolved in appropriate solvent. Independent dilutions were then undertaken to cover our analytical dynamic range.

2.5.1. Linearity

In order to assess the validity of the assay seven doses of the reference substance were used. Standards at concentration ranges of: 0.002–4.8, 0.004–4.8 and 0.0003–1.2 mg mL^{-1} for erythromycin, azithromycin and spiramycin, respectively, were prepared from their stock standard solutions. A 20 µL volume of these solutions was injected into the column. The calculation of regression line by the method of least squares was employed.

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2.5.2. Precision and Accuracy

Precision and accuracy were assessed by performing replicate analysis of quality control samples. Three different concentration solutions within calibration range (80, 100 and 120%) were prepared and analyzed with calibration curve to determine intra-day (5 replicates per concentration) and inter-day (5 replicates per concentration over 1–3 days) variability.

2.5.3. Interference Studies and Stability

The previous tests proposed with the samples in comparison to the standard should detect excipients and/or probable impurities and in this case the contents would propositional to the peak-height. The Student's *t*-test was performed to compare the macrolide standard and sample values.

Different aliquots of standard preparation and solutions were treated with 1.0 M hydrochloride, 1.0 M sodium hydroxide, hydrogen peroxide (3.0%) and exposed to natural light and UV lamp during 24 h, neutralized and suspended in mobile phase were then analyzed by the procedure described above in order to study method selectivity.

Drug stability of macrolides were evaluated at low and high concentrations of standards after short and long times of storage at room temperature for 4 and 8 h, at 4°C for one, two and three weeks. Stability studies were estimated by comparing the detector response of tested solutions (after storage) with that of freshly prepared ones (measured at zero time).

In the case of the simultaneous study sample, several volumes of ERY, AZI and SPI working samples were added to 10 mL flask. After slightly mixing, the obtained homogenized mixture was filtered. Then, a 20 µL of the filtrate solution was injected into the HPLC system.

3. RESULTS AND DISCUSSION

3.1. Method Development

Macrolides are basic compounds having one or several amino sugars in their molecule and strongly affected by residual silanols groups remaining in the column packing material. So, the choice of adequate conditions for a HPLC separation is governed by the ionizable group, i.e. amino sugar, of the three macrolides (Edder et al., 2002). Previous results, obtained for troleandomycin and erythromycin, indicated that a neutral mobile phase pH favoured the separation of macrolides on X Terra RP_{18} (Chepkwony et al., 2001a, 2001b, 2001c). Therefore, mobile phases containing different amounts of acetonitrile, as organic modifier (x%, v/v), 0.2 M phosphate buffer pH 7.0 (5%, v/v) and water (95–x %, v/v) were used as starting conditions for method development. In order to improve the separation and peak symmetry, the chromatographic variable acetonitrile (%), buffer pH, buffer concentration and temperature were investigated.

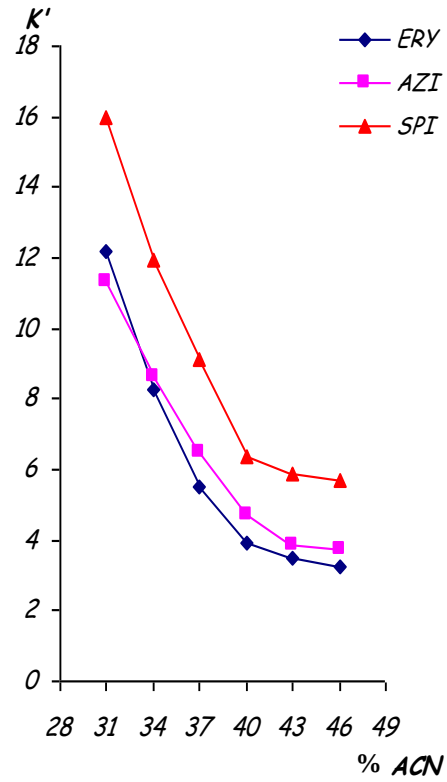

Fig.2. *Influence of the concentration of acetonitrile (ACN) in the mobile phase on the capacity factors (K') of ERY, AZI and SPI. Mobile phase : acetonitrile- 0.01 M potassium phosphate buffer (pH 7) (x: up to 100, v/v); stationary phase : ODB RP_{18} 5µm (250×4.6 mm I.D.) ; flow-rate : 1.0 ml min^{-1} ; temperature : ambient ; detection : UV at 215 nm .*

Several organic modifiers were investigated. The influence of the concentration of acetonitrile (CH_3CN) in the mobile phase on the selectivity is shown in Figure 2. The capacity factors decreased rapidly with increasing amounts of acetonitrile. The separation of ERY–AZI remained satisfactory over the range 31% to 46%. The separation SPI–ERY was not achieved, and the use of another organic modifier

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such as 2-methyl-2-propanol, tetrahy-drofuran or methanol was necessary. Addition of some 2-methyl-2-propanol as a second organic modifier reduced the analysis time but did not influence the selectivity, so the amount of 2-methyl-2-propanol could be decreased, which improved the separation SPI–ERY. Amounts of 33% v/v of acetonitrile, and 7 % v/v of 2-methyl-2-propanol were chosen to be as optimal in terms of selectivity and analysis time.

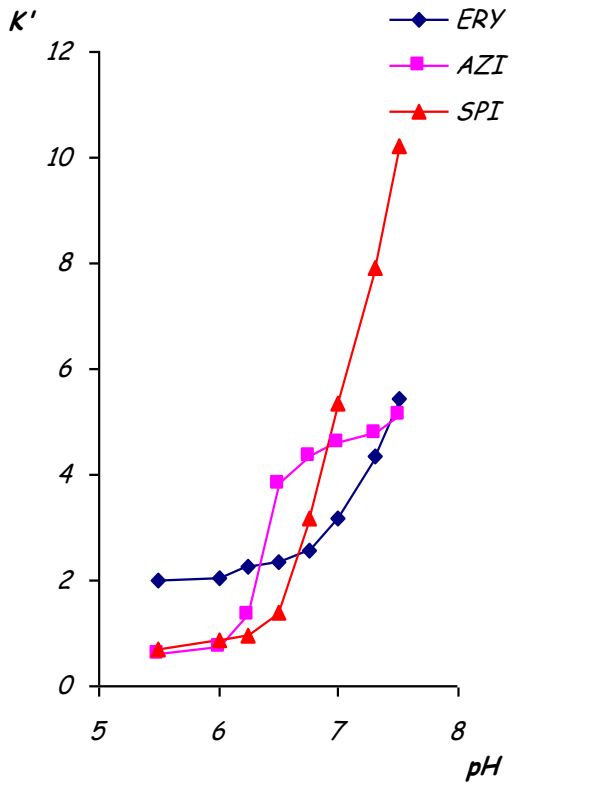

Fig.3. *Influence of mobile phase pH on the capacity factors (K') of ERY, AZI and SPI. Mobile phase: CH_3CN - 2-methyl-2-propanol- 0.01 M potassium phosphate buffer (pH x) (33:7: up to 100, v/v/v). See fig.2 for experimental conditions.*

Mobile phase pH is an important parameter that can be manipulated to optimise selectivity in HPLC as it enhances mobility differences between the solutes and the stationary phase (KLalloo et al., 1997). The influence of the pH on the selectivity was investigated between 5.5 to 7.5 (Figure 3). Below pH 6.5 the capacity factors decreased rapidly with loss of selectivity. At pH 7.0 the separation ERY–AZI was good, but SPI was no longer separated from ERY and the peak corresponding to ERY was slightly distorted, because of co-elution with SPI. At pH above 7.0 the separation was poorer, and column efficiencies decreased by 40% relative to values below pH 7.0. The results indicated that in the pH range 6.0 to 7.0, small pH changes did not significantly affect the separation and therefore pH 6.5 was chosen for further work. The effect of the concentration of potassium phosphate buffer (pH 6.5) was negligible in range 0.01–0.04 M. An increasing in the concentration of potassium phosphate buffer decreases the capacity factors. A possible explanation is that, due to the formation of ion pair with macrolide. 0.025 M buffer concentration was therefore chosen as the best compromise because it afforded optimal separation for all the components with better peak symmetry.

It is known that temperature is a useful parameter that sometimes provides profound changes in selectivity. Because column temperature has proved to be useful variable in reversed–phase LC separations (Dolan, 2000). Therefore, the influence of the column temperature was examined over the range: 25–50 °C. It was observed that an increase in temperature led to an increase in retention times. This chromatographic behaviour has previously been observed with macrolides (Dolan, 2000; Cachet et al., 1987). It is probable that the hydrophobicity of these antibiotics increases with temperature, because of poorer solvation, and this leads to stronger interaction with the apolar stationary phase. This phenomenon correlates well with the lower solubility of these substances in aqueous solutions at higher temperatures (Chepkwony et al., 2001d).

At 25°C elution was rapid with complete loss of selectivity. Increasing the temperature improved the selectivity but also led to longer retention times. Cachet et al. also previously reported this observation (Cachet et al., 1987). A temperature of 30°C was chosen as optimum for the separation. This temperature is very important for baseline–resolution, better peak symmetry and shorter retention times. Triethylamine was used as a shielding agent, which usually improves peak shape in compounds with amino groups.

The macrolides were dissolved in appropriate solvent and their UV spectra were measured. The UV absorption maxima were in the vicinity of 215 nm for ERY, 210 nm for AZI and 230 nm for SPI, and 210 nm was chosen for the simultaneous detection. The final composition of the mobile phase chosen is acetonitrile– 2-methyl-2-propanol– 0.025 M

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potassium phosphate buffer (pH 6.5), with 1.5% triethylamine (33: 7: up to 100, v/v/v).

3.2. Assay Validation

3.2.1. Linear Working Range

The linearity of method was determined by injection of various standard concentrations. Linear plots with good correlation coefficients (*r*) (more than 0.999) were obtained in the concentration ranges of 0.002–4.8, 0.004–4.8 and 0.0003–1.2 mg mL^{-1} for the ERY, AZT and SPI, respectively. Typically, the regression equation for the calibration curve was found to be y = 28551.54 x + 899.31 (*r*=0.9998) for erythromycin, y = 44279.89 x + 20.87 (*r*=0.9995) for azithromycin and y = 503657.84 x + 5557.96 (*r*=0.9998) for spiramycin, where x are peak–height values and y the concentration values in mg mL^{-1}. The *r* values were found to be acceptable and significant with confidence intervals at *P*=0.05.

3.2.2. Limit of Detection and Limit of Quantification

Limit of detection (LOD) and limit of quantitation (LOQ) levels are only valuable in analytical methods for purity, and they give no important information in assay determinations. Nevertheless, to present the sensitivity of the developed method, the minimum level at which the investigated compound can be reliably detected and quantified was determined experimentally. By steadily diluting the samples, for an injection of 20 µL, the LOQ with a signal-to-noise ratio of 10 was: 0.07% for ERY; 0.05% for SPI and 0.09% for AZI. The LOD with a signal-to-noise ratio of 3 was: 0.02% for ERY and SPI and 0.03% for AZI. The chromatogram peaks resembling these concentrations were all clearly distinguishable from noise peaks. These results indicate the acceptable sensitivity of the proposed method comparing to some reported HPLC methods (Chepkwony et al., 2000, 2001d; Yang et al., 2009).

3.2.3. Method Precision

The precision is evaluated in terms of repeatability (Intra-day precision) and intermediate precision (Inter-day precision) expressed by relative standard deviations (%RSD). The repeatability RSD values of ERY (4.0 mg mL^{-1}), AZI (4.0 mg mL^{-1}) and SPI (1.0 mg mL^{-1}) were 0.75%, 0.97% and 0.79%, respectively (Table 1). As shown in Table 2, the inter-day precisions were less than 1.11%, 1.29% and 1.24% for ERY, AZT and SPI, respectively. This level of precision is suitable for the routine quality control analysis of pharmaceutical dosage forms.

3.2.4. Accuracy and Recovery

For pharmaceutical studies, the most widely used approach to test accuracy is recovery study. It was tested in the same linearity assay. Tables 1 and 2 summarize the recoveries of drugs. Results obtained indicate good recoveries (mean recoveries greater than 90%) and confirm the accuracy of the proposed method (%RSD less than 5%).

3.2.5. Robustness and Ruggedness

It was examined by evaluating the influence of change in mobile phase pH, flow rate, percentage of acetonitrile and column oven temperature on the method suitability and sensitivity. None of these variables significantly affected the relative detection intensity (Table 3). This provides an indication of the reliability of the proposed method during normal usage and so it could be considered robust (US Pharmacopoeia, 2000). The ruggedness study was done by applying the proposed method to the assay of studied macrolides using the same operational conditions but using different lab and different elapsed time. Results obtained due to lab-to-lab and day-to-day variations were found reproducible as % RSD did not exceed 4%.

3.2.6. Specificity and Interference Studies

The specificity of the method was performed by comparison of the chromatograms of drug standard and sample solution. The chromatographic parameters show a good correlation between the results of the two determinations (i.e. ERY, y = 0.988 x + 0.062; n = 12; *r* = 0.97). This interference study indicated no interfering peak around the studied retention times; also baseline showed no significant noise. Furthermore, the Student's *t*-values of ERY, AZI and SPI calculated for assay of the commercial lots and the recovery study, 1.34, 1.78 and 1.14, respectively, are below tabulated values (t_{n-1}, α /2 from tables= 2.571, for five freedom degrees). These results showed that the proposed method can be considered specific.

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Table 1. *Within–day precision and accuracy of the HPLC analysis of ERY, AZI and SPI*

Drug	Theoretical Concentration (mg mL^{-1})	Experimental Concentration[a] (x 10^{-1} mg mL^{-1})	Precision (RSD)[b] (%)	Accuracy[c] (%)
ERY	4.80	4.7030 ± 0.43	0.91	97.98
	4.00	3.9948 ± 0.30	0.75	99.87
	3.20	3.2038 ± 0.27	0.84	100.12
AZI	4.80	4.7237 ± 0.36	0.77	98.41
	4.00	3.9628 ± 0.38	0.97	99.07
	3.20	3.2090 ± 0.37	1.14	100.28
SPI	1.20	1.1879 ± 0.12	1.01	98.99
	1.00	1.0004 ± 0.08	0.79	100.04
	0.80	0.7905 ± 0.07	0.89	98.81

[a] Mean ± SD (n=5)
[b] Relative standard deviation (RSD) =SD/mean×100
[c] Accuracy=experimental/theoretical×100

Table 2. *Between–day precision and accuracy of the HPLC analysis of ERY, AZI and SPI*

Drug	Theoretical Concentration (mg mL^{-1})	Experimental Concentration[a] (x 10^{-1} mg mL^{-1})	Precision (RSD)[b] (%)	Accuracy[c] (%)
ERY	4.80	4.6622 ± 0.51	1.10	97.13
	4.00	3.9592 ± 0.37	0.93	98.98
	3.20	3.1747 ± 0.33	1.04	99.21
AZI	4.80	4.6915 ± 0.60	1.28	97.74
	4.00	3.9380 ± 0.35	0.89	98.45
	3.20	3.1782 ± 0.37	1.17	99.32
SPI	1.20	1.1732 ± 0.19	1.01	97.77
	1.00	0.9915 ± 0.10	0.99	99.15
	0.80	0.7835 ± 0.09	1.23	97.94

[a] Mean ± SD (n=5)
[b] Relative standard deviation (RSD) =SD/mean×100
[c] Accuracy=experimental/theoretical×100.

Representative chromatograms of erythromycin and their degradation products (represented as an example of the studied macrolide antibiotics) are shown in Figure 4, which shows a good resolution with no interference peaks from endogenous components at the retention time of ERY, indicating the selectivity of measurement. Similar significant degradation was obtained for AZI and SPI samples, and all degradation products were separated from their macrolide peak. Further, to confirm the specificity of this analytical method, the homogeneity and peak purity of each macrolide peak in the samples were estimated based on a photodiode-array detector (DAD) scan from 200 to 400 nm. The obtained peak purity factors were comparable with the purity threshold calculated by DAD software automatically (i.e. ERY, peak purity factor was 994.134 and purity threshold calculated by DAD software was 996.781).

3.2.7. Stability of the assay preparation

The stability study of ERY, AZI and SPI showed that, there are no significant difference in drug concentrations upon storage of samples, in refrigerator (4 °C) for 21 days, and at room temperature (25°C) for 8 h, with a %RSD remained to be less than 5% (< 2.5%; n = 5).

Over 200 tested solutions were analyzed by this method without any significant loss of resolution. No change in the column efficiency and back pressure

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Table 3. *Results of robustness study*

Factor	Level	Mean % assay (n = 3)			% R.S.D. of results		
		ERY	AZI	SPI	ERY	AZI	SPI
pH of mobile	6.2	99.1	99.4	100.2	0.77	0.98	0.85
phase	6.4	100.5	99.3	99.8	1.04	1.12	0.98
Flow rate	0.9	99.7	98.7	100.7	0.63	1.00	0.79
(mL min^{-1})	1.1	100.3	98.9	99.5	0.91	1.03	1.02
Column oven	25	99.2	99.6	100.2	0.83	0.99	0.89
temperature (◦C)	35	100.1	98.8	97.0	1.09	1.01	1.18
% of	31	99.3	98.7	98.3	0.79	1.11	0.78
acetonitrile	35	100.2	101.0	99.5	0.95	1.09	1.10

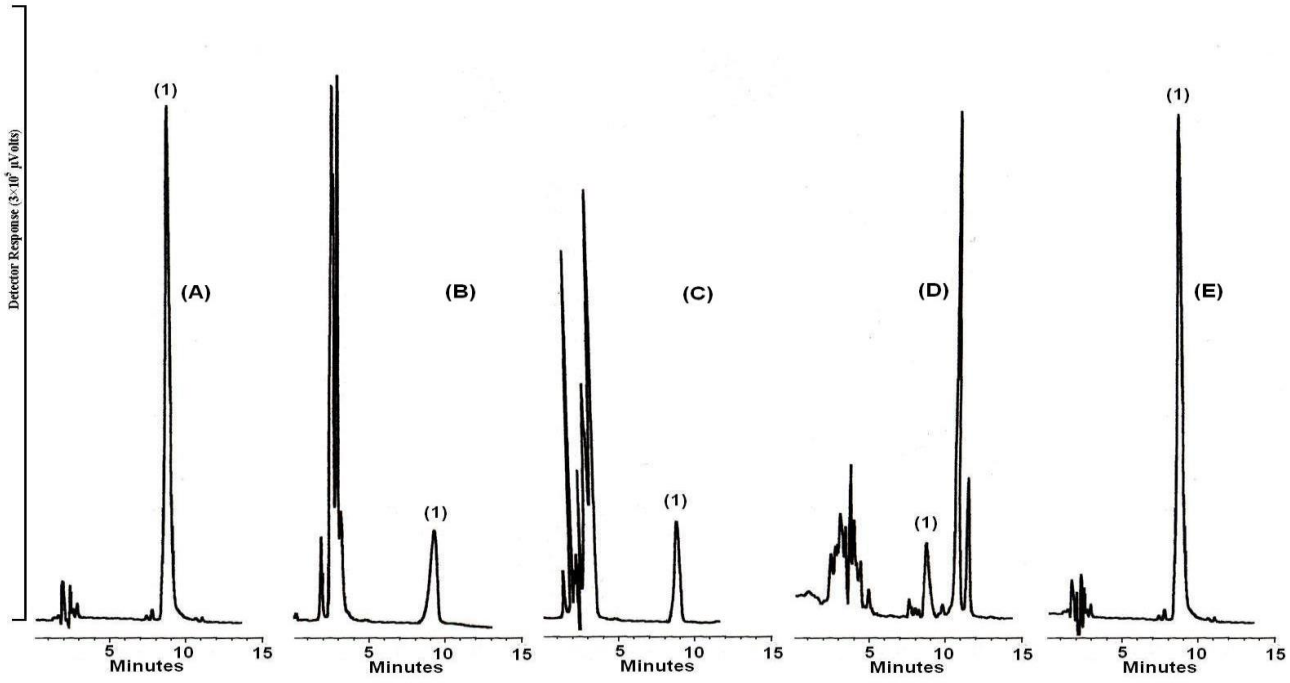

Fig.4. *Representative chromatograms corresponding to erythromycin (1), as an example of the studied macrolide antibiotics, tested as standard (A) and as raw material treated with acid (B), alkali (C), 3.0%-hydrogen peroxide (D) and, exposed to natural light and UV lamp (E). Mobile phase: CH_3CN- 2-methyl-2-propanol - 0.025 M potassium phosphate buffer (pH 6.5) (33: 7: up to 100, v/v/v); stationary phase: ODB RP_{18} 5µm (250×4.6 mm I.D.); flow-rate: 1.0 mL min^{-1}; temperature: 30°C; detection: UV at 210nm.*

was also observed over the entire study time, thus proving its suitability.

3.3. Application to analysis of certain commercial lots

The suggested HPLC method was applied for the quantitative determination of ERY, AZI and SPI content in marketed products by assaying Erybesan® tablets, Azimycine® powder for oral suspension and Rovamycin® tablets. The assay showed the drug content of these products to be within pharmacopoeial limits of label claim. This indicates an excellent concordance between experimental and nominal values. The results of drug assay in products are shown in Table 4. Using this method, a good recovery (98.42–99.89%) was obtained without any interference from coexisting substances.

In industry, quantification of ERY, AZI and SPI by HPLC method is precise but rather expensive and inconvenient. For example, HPLC apparatus is quite expensive and a great deal of organic solvents will also be used during analysis, and the analysis time is also quite long (about 10 min to 1 h per sample), a lot of money and time would be cost. On the other hand, a large number of reports in the literature (Lahane et

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al., 2014; Kanfer et al., 1998) have been published for the simultaneous analysis of antibiotics in several tissues and biological fluids (liver, meat, plasma…etc.), by which samples may be screened for residual amounts of substances caused by incorrect use of these drugs in veterinary practices. However, there are few reports described in the literature for the simultaneous determination of macrolides in dosage forms by HPLC and, to our knowledge, none of them includes erythromycin, azithromycin and spiramycin. Up to now, no HPLC methods have been reported for quantification of these macrolides in pharmaceutical dosage forms of the tested local markets. Consequently, the developed method was applied for the simultaneous separation of these analytes. Figure 5 shows a typical chromatogram of a mixed solution of the studied macrolides in their commercial available dosage forms. As can be seen from this figure, the proposed method has the advantages of being virtually simple, rapid and reliable for quantification practices.

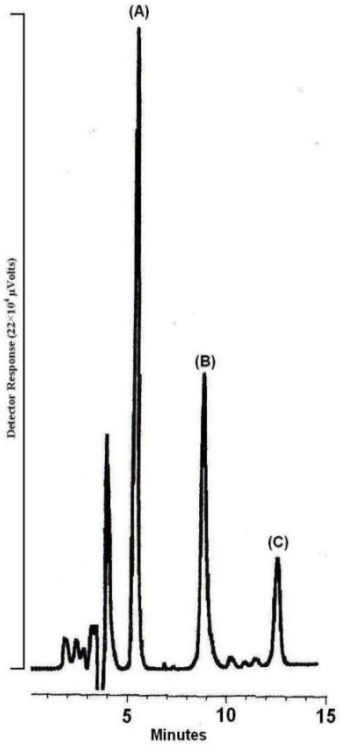

Fig.5. *Chromatogram corresponding to simultaneous analysis of SPI (A), ERY (B) and AZI (C). See fig.4 for experimental conditions.*

Table 4. *Assay of Erythromycin, Azithromycin and Spiramycin in their pharmaceutical formulations*

Description[a]	Label claim	Proposed method		Reported method[e]
		Recovery[b] (%)	RSD (%)	Recovery (%)
1. Erythromcin		99.08	0.78	99.21
a. Pure ERY				
t-value		1.89		
F-value		2.71		
b. Erybesan®, tablets	500mg/tablets	98.42	2.42	98.51
t-value[d]		1.52		1.75
F-value[d]		1.28		
2. Azithromycin		98.94	0.89	99.01
a. Pure AZT				
t-value		1.59		
F-value		2.17		
b. Azimycine® powder for oral suspension	200mg/5mL	99.89	2.19	99.89
t-value[d]		1.98		1.81
F-value[d]		1.17		
3. Spiramycin		100.48	0.81	99.97
a. Pure SPI				
t-value		1.77		
F-value		1.31		
b. Rovamycin®, tablets	450mg/tablets[c]	98.91	1.98	99.88
t-value[d]		1.42		1.65
F-value[d]		1.21		

[a] (1) Sandoz, Algiers, Algeria, Lot No.25, Manufactured Jun. 2012; (2) Saidal, Medea, Algeria, Lot No.136, Manufactured Jan. 2011; (3) Aventis Pharma Saidal, Algiers, Algeria, Lot No. RM001J, Manufactured Aug. 2009.
[b] Average of five experiments.
[c] Label claim is 1.5 MIU/tablet (equivalent of 1.5MIU is 450 mg)
[d] Theoretical value at 95% confidence limit and n=5 for *F* is 6.26 and *t* is 2.776.
[e] Ref. (Chepkwony et al., 2001d) for Spiramycin and Ref. (British Pharmacopoeia, 2007) for both Erythromycin and Azithromycin.

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3.4. Comparison of the proposed method with the current assays

The performance of the proposed method was judged by comparing with other reported methods (British Pharmacopoeia, 2007; Chepkwony et al., 2001d). Then, pharmaceutical formulations of the studied drugs were analyzed by the proposed method and the current assays, and results indicate the high accuracy and precision, as can be seen from Table 4. Mean values were obtained with a Student's *t*- and *F*-tests at 95% confidence limits for five degrees of freedom (Miller and Miller, 1993). The results obtained were compared statistically by the student's *t*-test (for accuracy) and the variance ratio *F*-test (for precision) with those obtained by the reference methods on samples of the same batch (Table 4). The values of *t*- and *F*-tests obtained did not exceed the theoretical tabulated value indicating no significant difference between the methods compared.

CONCLUSIONS

The ODB RP_{18} stationary phase (silice uptisphere, 5 µm) shows good selectivity toward erythromycin, azithromycin and spiramycin. The isocratic method presented here is suitable for the quantitative determination and to separate ERY, AZI and SPI from each other. This new method is simple, linear, precise, accurate and rapid. Hence, it can be easily adopted for the routine quality control analysis of these antibiotics in their pharmaceutical dosage forms.

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