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LC determination of impurities in azithromycin tablets

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Abstract

A LC method with UV detection for determining azithromycin impurities in tablets as pharmaceutical form has been developed. It is to be employed in routine and stability tests. A linear gradient elution was employed starting with 47% A and 53% B to reach 28% A and 72% B at 48 min. Mobile phase A was KH_2PO_4 10 mM (H_2O) at pH 7.00. B was a mixture methanol:acetonitrile 1:1 (v/v). UV detection was performed at 210 nm. The chromatographic column was Phenomenex Synergi® MAX-RP 4 μm 250 × 460 mm kept at 50 °C. Six impurities were separated and identified and it was possible to quantify five out of the six with reasonable accuracy and precision.

Keywords: Azithromycin; Impurities; HPLC; Pharmaceuticals

1. Introduction

Azithromycin is an azalide, a subclass of macrolide antibiotics, for oral administration. Azithromycin has the chemical name (*2R*,*3S*,*4R*,*5R*,-*8R*,*10R*,*11R*,*12S*,*13S*,*14R*)-13-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-a-L-*ribo*-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-b-D-*xylo*-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one. Azithromycin is derived from erythromycin; however, it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring, as can be seen in Fig. 1. Its molecular formula is $C_{38}H_{72}N_2O_{12}$, and its molecular weight is 749.00. Azithromycin, as the dihydrate, is a white crystalline powder with a molecular formula of $C_{38}H_{72}N_2O_{12}\cdot 2H_2O$ and a molecular weight of 785.0.

This 15-membered expanded lactone ring results in improved acid stability and oral bioavailability compared with erythromycin. It binds to the 50 S ribosomal subunits of susceptible bacteria and suppresses protein synthesis. Azithromycin appears to bind to the same receptor as erythromycin. These drugs may be bactericidal or bacteriostatic. It is also used to treat bacterial upper and lower respiratory tract infections, skin and skin structure infections, and sexually transmitted diseases. Azithromycin represents a significant improvement in the treatment of selected community-acquired infections [1].

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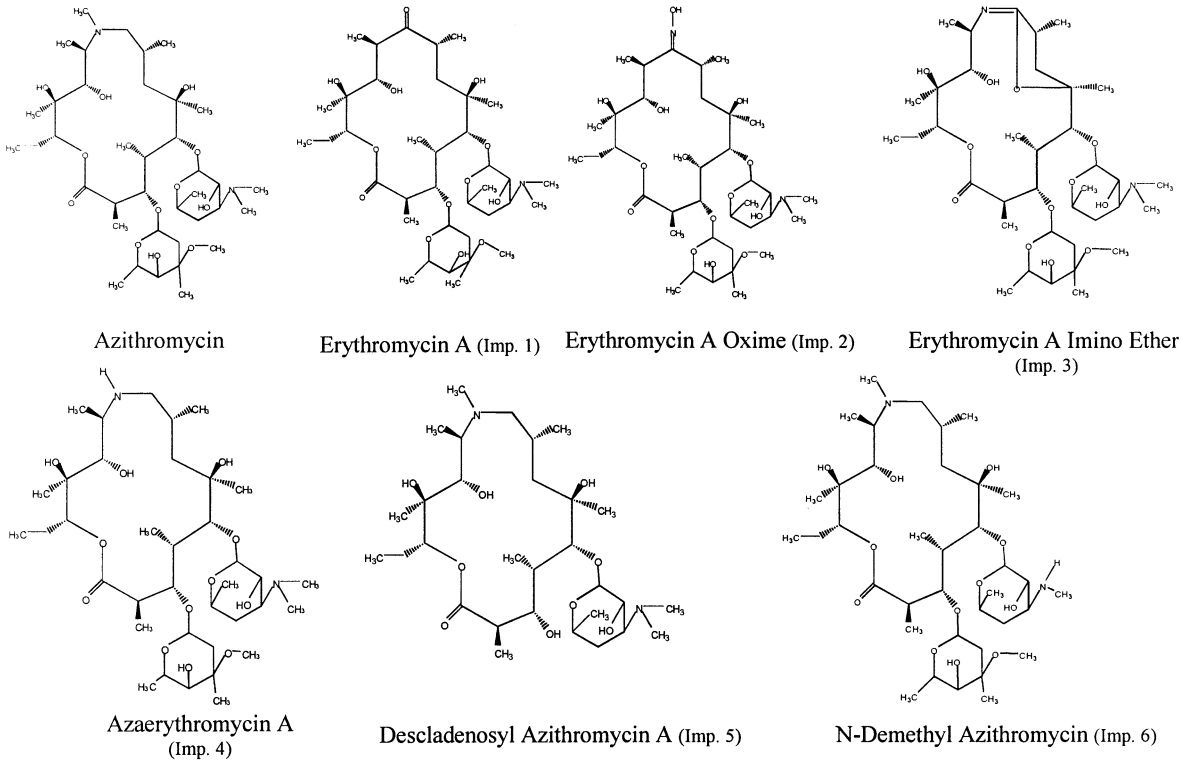

Fig. 1. Structures of azithromycin and related impurities.

Fig. 1 shows the structures of the azithromycin and the related impurities that were examined. As can be seen, azithromycin and the related impurities resemble each other. Their origin and instability leads to a situation in which small amounts of structurally related compounds and byproducts may be present together. It is very difficult to determine small amount of degradation products in a vast excess of parent drug, and even more so when the compounds do not present a chromophore as this makes their detection more difficult. These problems hinder the analysis of these antibiotics.

Microbiological methods can assay the parent drug together with its active metabolite(s), but they do not detect the presence of inactive related impurities. Some review articles published recently have covered literature on the analysis of macrolide antibiotics [2–4]. Azithromycin has been measured in biological fluids by HPLC using electrochemical, [1,5–7], and fluorescence [8] for detection. However, there are few methods described in the literature for its determination in pharmaceutical dosage forms and, to our knowledge, none of them includes related substances evaluation.

The USP method [9], describes the use of a high pH mobile phase (pH 11.0) and therefore it requires the use of specific 'Gamma-alumina' column, which is expensive and difficult to obtain commercially.

A reversed-phase HPLC method has been described [10] with amperometric electrochemical detection and a neutral mobile phase. It permits the use of conventional C_{18} columns. Another HPLC method with UV detection at 215 nm has also been described [11].

A spectrofluorimetric method has been described [12] for the analysis of several macrolides including azithromycin. The method is based on the oxidation by cerium (IV) in the presence of sulphuric acid and monitoring the fluorescence of

cerium (III) formed. It permits the measurement of the actives in pure and formulated dosage forms.

Porous monodisperse poly(glycidylmethacrylate-divinylbenzene) microspheres have proved to be well suited for the separation of macrolide antibiotics by isocratic CEC with high efficiency and resolution [13], but it cannot be considered a routine technique in pharmaceutical laboratories yet.

Nevertheless, none of them allows the separation and quantitation of azithromycin impurities. On the other hand, an amperometric electrochemical detector is not available in many laboratories.

The objective of the present work was the development and validation of a HPLC method with UV detection for determining azithromycin impurities in tablets as pharmaceutical form to be employed in routine and stability tests.

2. Experimental

2.1. Apparatus

An Agilent technologies HPLC 1100 series (Las Rozas, Madrid, Spain) provided with an automatic injector, a diode-array detector and a column oven was employed. The chromatographic analysis was performed on Phenomenex Synergi® MAX-RP 4 μm column (Micron Analítica, Madrid, Spain) with 250 × 460-mm length and kept at 50 °C.

A linear gradient elution was employed starting with 47% A and 53% B to reach 28% A and 72% B at 48 min. Then, the system recovered the initial conditions in 1 min. Mobile phase A was KH_2PO_4 10 mM (H_2O) brought up pH 7.00 with KOH. B was a mixture methanol:acetonitrile 1:1 (v/v). The flow rate was 1.2 ml/min and the injection volume was 70 μl. UV detection was performed at 210 nm and peaks were identified with retention times as compared with standards and, during method development, by spiking.

2.2. Chemicals

Standards of azithromycin and impurities as well as tablets and placebo of the speciality were kindly provided by CINFA, S.A. (Pamplona, Spain). KH_2PO_4 was from Sigma (St. Louis, USA), triethylamine was from Merck (Darmstadt, Germany), and other organic solvents from Scharlau (Barcelona, Spain).

2.3. Optimization of HPLC method

Selectivity under RP-HPLC conditions can be controlled by mobile phase composition, pH, temperature and stationary phase chemistry. These parameters were varied to achieve the separation.

2.4. Standard solutions and sample preparation

Samples were dissolved in solvent A:acetonitrile 35:65 (v/v), being solvent A: phosphate buffer pH 7.0, 10 mM prepared from KH_2PO_4 and made up to the corresponding pH with KOH. For quantitation 1161.3 mg of the pulverized tablets (one tablet weight) were made up to 50 ml with the solvent previously described. After water bath sonication for around 5 min samples were filtered with 0.45-μm nylon filters prior to the injection.

A stock solution of azithromycin was prepared with 52 mg in a 5 ml volumetric flask and the same solvent. The working standard (azithromycin prepared in the same concentration range as impurities) was prepared with 40 μl of stock solution of azithromycin made up to 10 ml with the samples-solvent. The quantitation was performed with the response factors previously calculated for each impurity related to azithromycin and determined during method validation.

For the validation, stock solutions of every impurity were individually prepared with 10 mg exactly weighed and dissolved in 5 ml volumetric flasks with methanol.

2.5. Validation

Selectivity was tested by injecting the placebo of the pharmaceutical speciality and checking that

there was no interfering peak and by spiking the drug substance and the drug product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix. Moreover, identification of each impurity was confirmed with migration time as compared with those of pure standards and by spiking. The spectra do not give any information, because these compounds do not present characteristic spectra.

For impurity methods, linearity is determined by preparing standard solutions at five concentration levels over a range such as 0.05–0.25 wt.%. As impurities standards were not available with enough purity (one impurity was contaminated with others) they were divided into three groups which were studied separately. Group A: impurities 1 and 2; group B: impurities 3, 5 and 6; and group C: impurity 4. In this case standards ranged from 0.01 to 0.2 mg/ml and they were prepared in 10-ml volumetric flasks containing 104 mg of azithromycin with 52, 100, 210, 310, 420 and 1000 μl of the individual impurities solution and they were leveled off with the samples-solvent. Six points were prepared instead of five just in case there were sensitivity problems in the lower value. Samples linearity was tested in the same way but with the proportional weight of the excipients of the speciality (126.5 mg) added to each flask.

For pharmaceutical studies, the most widely used approach to test accuracy is the recovery study, which is performed by spiking analyte in blank matrices. It was tested in the same linearity assay. The percent recovery and R.S.D.s were then calculated.

Intra-assay precision data were obtained by repeatedly analysing, in one laboratory on 1 day, six aliquots of a homogeneous sample, each of which was independently prepared according to the method procedure. Data to evaluate intermediate precision were obtained by repeating the intra-assay experiment on a different day with a newly prepared mobile phase and samples.

Limits of quantification must be established with the lower concentrations in which the method can be validated with enough precision and accuracy.

Standards stability was tested by running the same sample corresponding to the medium point in the linearity assay for 0 and 1 day and with the same mobile phase. Between runs, solutions were stored at 4 °C. The initial area was considered 100% and recoveries in the following days were evaluated.

Finally, as the method was proposed not only for impurity evaluation in the initial active, but also for stability assays, a forced degradation was performed. In order to validate the stability indicating power of the analytical procedure, azithromycin standard and samples were treated with acidic, basic, oxidative media, heat and light.

3. Results and discussion

3.1. Optimization of HPLC method

HPLC method development frequently begins with a type of column described as medium in hydrophobicity and silanol activity, such as NovaPak C18, 4 μm, 3.9 × 150 mm (Waters, Madrid, Spain), but in view of the difficulties due to the aspect of the peaks and their overlapping, two other columns with different characteristics were tested: (a) Discovery RP amide C16 and (Supelco, Madrid, Spain) (b) Synergi MAX-RP C12 (Phenomenex, Madrid, Spain), both 4.6 × 250 mm. Problems when analysing basic drugs, such as azithromycin, are well known in the pharmaceutical industry, since these compounds strongly interact with polar ends of HPLC column packing materials, causing severe peak asymmetry and low separation efficiencies. High purity silica backbone and advances in bonding technology have alleviated the tailing problem of polar compounds in HPLC to a significant extent.

Discovery column minimises the interaction between polar groups and silanols because these silanols are protected with the amide group used for linking the hydrocarbonated chain, thus providing better peak symmetry. On the other hand our work group had previous experience with loratadine impurities [14] about the ability of Synergi MAX-RP C12 to retain and separate compounds with very related structures.

Synergi packing, in spite of being C12, presented higher retention times than Discovery RP amide C16. It could be explained by the pore size which is smaller in the Synergi than in the Discovery packing and that provides a higher surface area (500 m^2/g versus 200 m^2/g according to the manufacturers). It results in a phase with hydrophobic retention and methylene selectivity superior to Discovery RP amide C16 column.

Regarding the mobile phase, due to the lack of other chromophore than the ester group and, therefore, the need to work at a low wavelength (215 nm), acetonitrile was considered as organic solvent instead of methanol. On the other hand, pH was adjusted in the highest value (pH 7) permitted to avoid problems with silica dissolution but to obtain the lower ionization degree in the amino groups to increase retention. Moreover, the stability of azithromycin and related compounds is lower in acidic media. Temperature was increased to facilitate mass exchange with the corresponding decrease of peak broadening and increase in sensibility. Fifty degree celsius was a compromise, because at 70 °C the peaks were narrower, but column life was quite short. The use of organic modifiers such as triethylamine, which usually improves peak shape in compounds with amino groups, gave no result with azithromycin impurities and increased the baseline noise, therefore it was not employed. Finally a linear gradient was needed to separate more polar compounds while eluting azithromycin in an adequite time.

After the optimization, the chromatogram showed the profile that can be seen in the Fig. 2. Impurities have been zoomed and azithromycin peak, which is a large peak at 45 min has not been included to make the figure more clear. The lower chromatogram shows the typical excipients of the pharmaceutical form, while in the upper chromatograms each impurity has been separately added to this sample. Peaks were identified with their migration time related to a pure standard of the same compound and by spiking, because UV spectra of all the analytes are very similar.

3.2. Validation

Main validation parameters of the method are summarised in Table 1. Both standards and samples showed a good linearity for the six

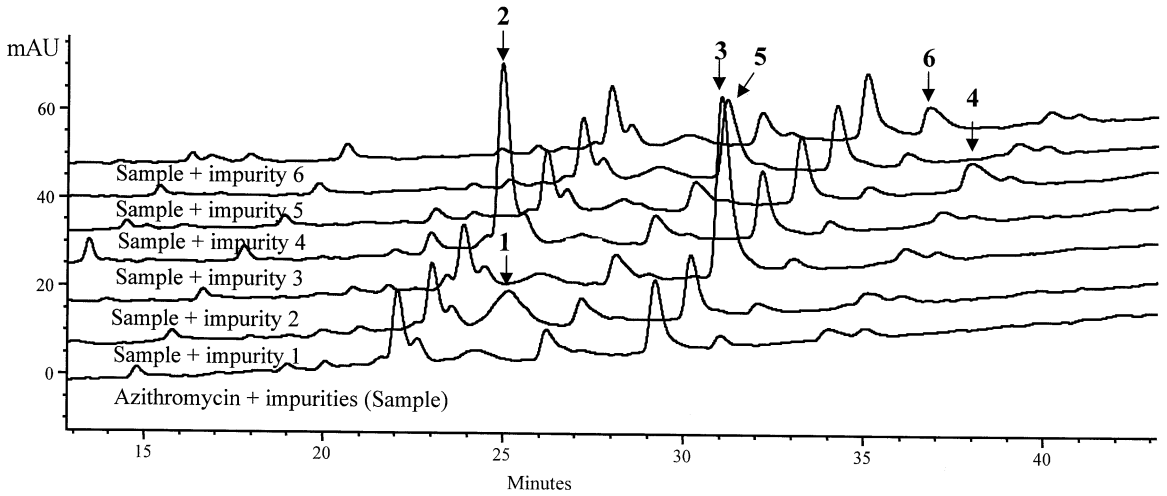

Fig. 2. Lower line: azithromycin plus impurities added at 0.1% w/w. In upper lines each impurity has been spiked to identify. A linear gradient elution was employed starting with 47% A and 53% B to reach 28% A and 72% B at 48 min. Mobile phase A was KH_2PO_4 10 mM (H_2O) at pH 7.00. B was a mixture methanol:acetonitrile 1:1 (v/v). UV detection was performed at 210 nm. The chromatographic column was Phenomenex Synergi® MAX-RP 4 μm 250 × 460 mm.

Table 1
Main validation parameters of the method

Parameter		Imp. 1	Imp. 2	Imp. 3	Imp. 4	Imp. 5	Imp. 6
Linearity							
Range (mg/ml)		0.0104–0.2080	0.0052–0.2000	0.0052–0.2000	0.0104–0.2000	0.0052–0.2000	0.0104–0.2000
Standards	Intercept ± C.I.	8 ± 15	8 ± 12	32 ± 23	10 ± 12	−7 ± 21	3 ± 4
	Slope ± C.I.	2697 ± 150	8458 ± 129	7173 ± 242	1402 ± 129	4521 ± 225	1226 ± 46
	r	0.996	0.999	0.998	0.987	0.996	0.997
Samples	Intercept ± C.I.	4 ± 23	20 ± 16	32 ± 18	4 ± 30	−0.03 ± 16	22 ± 12
	Slope ± C.I.	2878 ± 219	8206 ± 172	6644 ± 190	1392 ± 321	3942 ± 163	1104 ± 126
	r	0.992	0.999	0.999	0.917	0.997	0.978
Accuracy							
Samples	Recovery (%)	90.3	95.3	88.38	95.3	95.6	107.7
	R.S.D. (%)	25	15	15	49	13	14
Precision							
Intra-assay	*n*	6	6	6	6	6	6
	Mean (mg/ml)	0.043	0.030	0.021	0.157	0.024	0.048
	R.S.D. (%)	9.4	4.1	12.5	13.5	4.1	16.8
Intermediate	*n*	12	12	12	12	12	–
	Mean (mg/ml)	0.031	0.018	0.013	0.067	0.039	–
	R.S.D. (%)	30.2	33.3	36.6	13.3	8.6	–

C.I., confidence interval.

analytes with correlation coefficients over 0.99 except for impurities number 4 and number 6 in samples, which were only over 0.9. In both cases it was mainly due to the low signal obtained from these compounds which made integration more difficult. No appreciable bias was found.

Recoveries did not statistically differ from 100% (t-test, $P < 0.05$) in any case, although R.S.D.s for recoveries were high, ranging from 13 to 49%.

R.S.D. values ranged from 4.1 to 16.8% in the *intra*-assay method precision. In some cases they are high, but they could be considered acceptable for the level and characteristics of the analytes. Intermediate precision was not acceptable, but it was due to the instability of the compounds. As only a very small amount of most of the impurities was available, they were dissolved and the solution kept at −20 °C. Nevertheless on the second day of analysis, areas were smaller in most cases and different peaks appeared. Therefore, inter-assay precision could not really be tested properly. For quantitation through the stability study impurities will be measured with the response factors calculated on the first day related to a diluted azithromycin solution, included in Table 2.

Experimental limits of quantification were established in 0.1% (0.01 mg/ml) for impurities number 1, 4 and 6 and in 0.05% for impurities number 2, 3 and 5 (0.005 mg/ml). These limits are the lowest concentration values of the impurities measured in the validation and passing the acceptance criteria. Therefore, they are more reliable than the values obtained with mathematical approaches.

In relation to the stability test, all the impurities showed a response at least a 25% lower after 24 h than at the initial time, therefore they can not be considered stable and solutions must be freshly prepared or response factors related to a diluted

Table 2
Response factors for azithromycin impurities

Impurity	Response factor
1	1.23
2	0.39
3	0.80
4	2.18
5	0.81
6	2.05

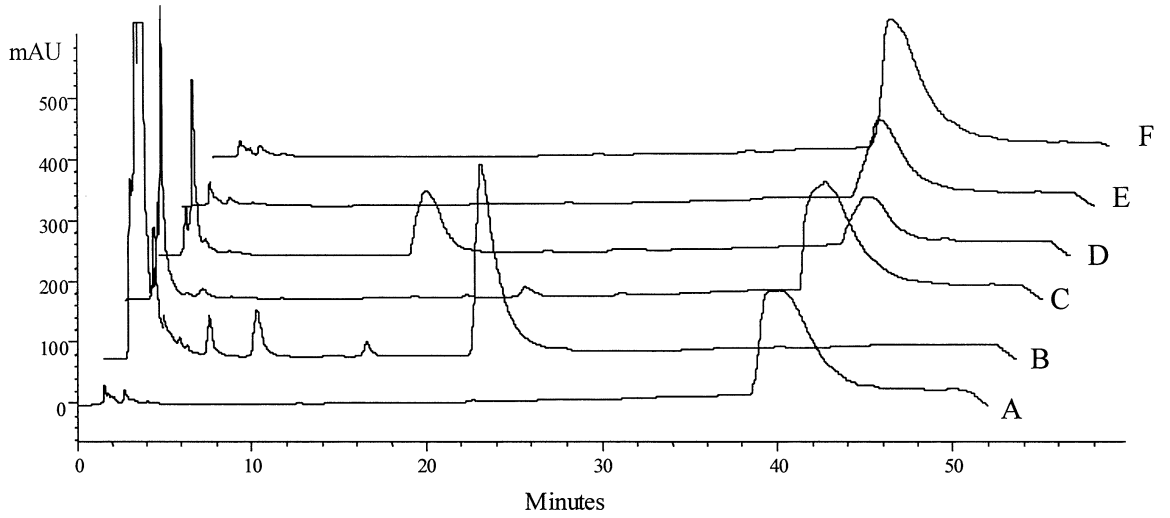

Fig. 3. Stability testing: (A) Sample under ambient conditions. (B) Acidic media. (C) Oxidant media. (E) Light. (F) Heat. For conditions see the text.

solution of azithromycin employed for quantification.

Degradation products obtained with forced degradation conditions shown in Fig. 3. Azithromycin was stable over 24 h when treated with light or heat. It was slightly degraded in basic media and it was completely degraded in acidic media. Degradation products were not identified, but the peaks at 24 and 25 min could correspond to impurities 1 and 3.

4. Conclusion

A LC method with UV detection has been developed which permits the separation and identification of six impurities of azithromycin. Five of them (impurities number 1, 2, 3, 5 and 6) could be quantified with enough reliability, while impurity number 4 has R.S.D. values for the recoveries too high to permit its reliable quantification.

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