Isocratic Liquid Chromatographic Method for the Analysis of Azithromycin and Its Structurally Related Substances in Bulk Samples

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Abstract

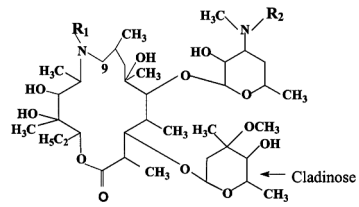
An isocratic liquid chromatographic method with UV detection at 215 nm, which is suitable for the analysis of azithromycin (AZT) in bulk samples, is described. AZT is separated from its synthesis intermediates and a degradation product as well as from six unknown impurities on an XTerra RP_{18} column at 70°C using a mobile phase consisting of acetonitrile–pH 6.5 0.2M K_2HPO_4–water (35:10:55, v/v/v) at 1.0 mL/min. The XTerra stationary phase contains methyl groups that are incorporated in the bulk structure of the material. This allows for special selectivities. Robustness is evaluated by a full factorial design experiment. The method shows good selectivity, repeatability, linearity, and sensitivity.

Introduction

Azithromycin (AZT) is a semisynthetic macrolide antibiotic with a 15-membered lactone ring derived from erythromycin A (EA). A Beckmann rearrangement of the 9-oxime derivative of EA followed by reduction and *N*-methylation results in this ring-expanded compound (1–3). Decladinosylazithromycin (dclAZT) is an acid degradation product of AZT. Figure 1 shows the structure of AZT and its related substances.

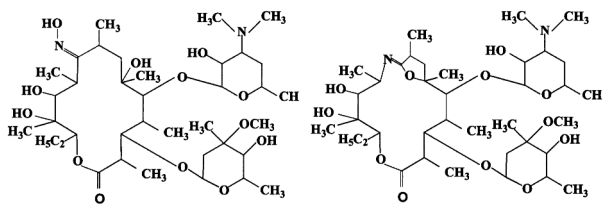
Liquid chromatographic (LC) methods using alkyl phenyl or polymer-coated alumina or Nucleosil C18 stationary phases have been reported for the analysis of AZT in biological samples (4–6). These methods used coulometric and amperometric detections because of the poor UV absorbance of this drug. An LC method using Hypersil ODS has also been described for the determination of AZT in serum using atmospheric pressure chemical ionization–mass spectrometry (MS) as the detection mode (7). Supelcosil LC-18 reversed phase and mobile phase containing acetonitrile (ACN)–2-propanol–pH 9.0 to 9.5 0.002M ammonium phosphate (60:25:15) with UV detection at 215 nm has also been reported (8). With this method, baseline separation was not achieved between EA and EA imino ether (EAIE) nor between EAIE and EA oxime (EAOX) in the spiked sample. Also, the separation of other minor impurities from the main component (AZT) was not described. All of these cited methods suffer from peak tailing of the main component. The United States Pharmacopeia prescribes an LC method with electrochemical detection (which uses an alumina-based hydrocarboneous column (15-cm × 4.6-mm i.d.) and a mobile phase with an apparent pH of 11.0) for the assay of AZT (9). This method shows significant peak tailing for the main component and the apolar components. From our observations, UV detection was found to be more sensitive.

It was decided, therefore, to develop a simple, sensitive, and



	R_1	R_2	Clad
Azithromycin (AZT)	CH_3	CH_3	+
Azathromycin (Azaerythromycin A) (AZAEA)	H	CH_3	+
N-demethylazithromycin (NdMeAZT)	CH_3	H	+
Decladinosylazithromycin (dclAZT)	CH_3	CH_3	-

Clad = Cladinose sugar.



Erythromycin A oxime (EAOX) Erythromycin A imino ether (EAIE)

Figure 1. Structures of AZT and related substances.

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selective LC method using UV detection and a novel stationary phase based on silica gel. XTerra RP18 was chosen because it also had shown good results in terms of selectivity and efficiency in the analysis of erythromycin and troleandomycin (10,11).

XTerra packing material is based on silica gel into which methyl groups are incorporated to partly replace silanol groups. Its organosilane substituent contains a polar carbamate group that protects the free surface silanol groups from interference with basic components. It is claimed to have an extended pH range and be thermally stable as compared with conventional silica-based packings.

This study describes an LC method capable of separating AZT from its known related substances and from several unknown minor impurities under isocratic conditions with XTerra RP18 as a stationary phase and UV detection.

Experimental

Reagents and samples

ACN, HPLC-grade S was obtained from Biosolve Limited (Valkenswaard, The Netherlands). A 0.2M solution of dipotassium hydrogen phosphate (Acros Organics, Geel, Belgium) was brought to the required pH by adding 0.2M phosphoric acid (Merck, Darmstadt, Germany) before bringing it to volume. Deionized water was distilled from a glass apparatus.

AZT bulk samples and impurity reference substances dclAZT, EAOX, EAIE, azaerythromycin A (AZAEA), and *N*-demethylazithromycin (NdMeAZT) were obtained from the European Pharmacopoeia Laboratory (Strasbourg, France).

LC instrumentation and chromatographic conditions

The LC apparatus consisted of a SpectraSystem P1000XR quaternary pump, an Autosampler SpectraSeries AS100 equipped with a 100-µL loop, a variable-wavelength Spectra 100 UV–vis detector set at 215 nm (Thermo Separation Products, Fremont, CA), and a Hewlett-Packard (Avondale, PA) integrator Model HP 3396 Series II. An XTerra RP18 5 µm (25-cm × 4.6-mm i.d.) (Waters, Milford, MA) was used as a stationary phase at 70°C. The temperature was maintained by a Polyscience 9501 digital temperature controller (Polyscience, Niles, IL) in a water bath. ACN–pH 6.5 0.2M potassium phosphate–water (35:10:55, v/v/v) delivered at a flow rate of 1.0 mL/min was used as the mobile phase. The mixture was purged by helium to degas.

Sample preparation

For identification purposes, the impurity reference substances were each dissolved separately at a concentration of 0.2 mg/mL, except for EAOX and EAIE (0.05 mg/mL). A test mixture containing a bulk sample at a concentration of 4.0 mg/mL and all the impurities at the concentration mentioned previously was also prepared. A bulk sample solution (4.0 mg/mL) was also prepared. ACN–water (3:7, v/v) was used as the dissolution solvent.

Results and Discussion

Method development

Preliminary studies using ACN–pH 7.0 0.2M K_2HPO_4–water (35:5:60, v/v/v) as a mobile phase on an XTerra column at 65°C gave good separation but too long of a retention of the main component (50 min) and poor peak symmetry. This mobile phase was adopted from earlier work done on the separation of erythromycin (10). In order to improve the separation and peak symmetry, the chromatographic variables buffer concentration, buffer pH, temperature, and ACN (%) were investigated.

The buffer concentration was investigated in the range of 2.5% to 15.0% (v/v) of pH 7.0 0.2M K_2HPO_4 with a fixed ACN content (35%, v/v) and temperature (60°C). A buffer concentration of 15% gave high sensitivity but poor selectivity because of fast elution, whereas a lower buffer concentration (2.5%) resulted in good selectivity, poor peak symmetry for the main component, and longer analysis time (over 2 h). A 10% buffer concentration was therefore chosen as the best compromise because it afforded optimal separation for all the components with better peak symmetry for the main component (< 2.0).

The effect of pH on the selectivity was investigated in the range of pH 6.0 to 7.5 with a fixed content of 0.2M K_2HPO_4 (10%, v/v) and ACN (35%, v/v) and with a fixed temperature (60°C). At pH 6.0 the separation of the main component from the surrounding impurities was good. The elution was too fast, with AZT being eluted at less than 10 min and EAOX and unknown component II after 10 min. The separation of early eluted components such as dclAZT, AZAEA, NdMeAZT, EAIE, and unknown component I was less favorable (especially NdMeAZT and EAIE showed partial coelution), but the separation remained good for components eluted after AZT. An increase in pH by 0.5 units led to a longer retention of all of the components except for EAOX and unknown component II,

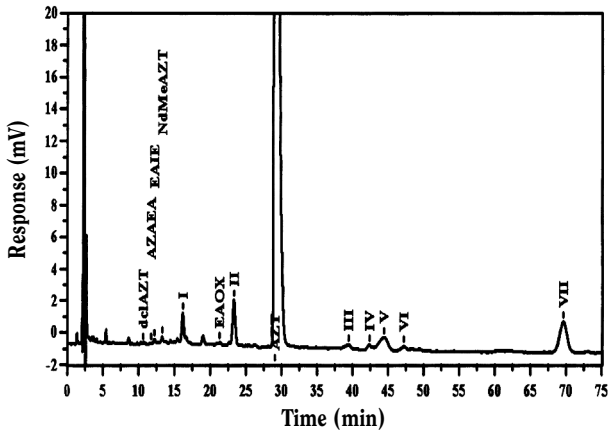

Figure 2. Typical chromatogram of an AZT bulk sample (concentration = 4.0 mg/mL) obtained under optimal chromatographic conditions. The column used was an XTerra RP18 5 µm (25-cm × 4.6-mm i.d. at 70°C. The mobile phase was ACN–pH 6.5 0.2M K2HPO4–water (35:10:55, v/v/v). The flow rate was 1.0 mL/min, the volume injected 100 µL (total mass injected = 400 µg), and the UV detection was set at 215 nm. Components I–VII are unknown.

which were less affected. Probably these two components are structurally more closely related to each other than to AZT. At pH 7.5 the separation of all of the components was good but the elution was too slow, with the main component (AZT) being eluted after 70 min and showing peak tailing. A buffer of pH 6.5 was chosen as a compromise for further experimental work because the separation of the components was sufficient and AZT showed less peak tailing.

The temperature and ACN content of the mobile phase were optimized using DryLab software (LC Resources, Berlin, Germany). Temperature was examined in the range of 50°C to 70°C using ACN (30–40%, v/v)–pH 6.5 0.2M K_2HPO_4 (10%, v/v)–water (60–50%, v/v) as a mobile phase. The retention times and the surface areas of the different components were recorded according to the specified requirements of DryLab. It was observed that an increase in temperature led to an increase in retention times. This chromatographic retention behavior of macrolides has also been reported for erythromycin and spiramycin (10,12,13). It appears that high temperature results in poorer solvation. As a consequence, the hydrophobicity of these molecules increases and the solubility of these molecules in aqueous solutions is also reduced at higher temperatures. A combination of temperature (70°C) and ACN (35%) gave good separation for all of the components. Therefore, these conditions were chosen for further examination in a robustness study. Figure 2 shows a typical chromatogram of an AZT bulk sample. It is clear that the impurities of the known identity were of minor importance. Expressed in terms of peak normalization, the known impurities were present up to ≤ 0.1%, whereas the unknowns were up to 3% (VII).

For reasons of easy transferability and reproducibility it was chosen to develop an isocratic method, although it is clear that it is possible to apply gradient elution between components VI and VII. This will speed up the elution of VII, and it will also need an equilibration time before the next analysis, which will reduce the gain in time.

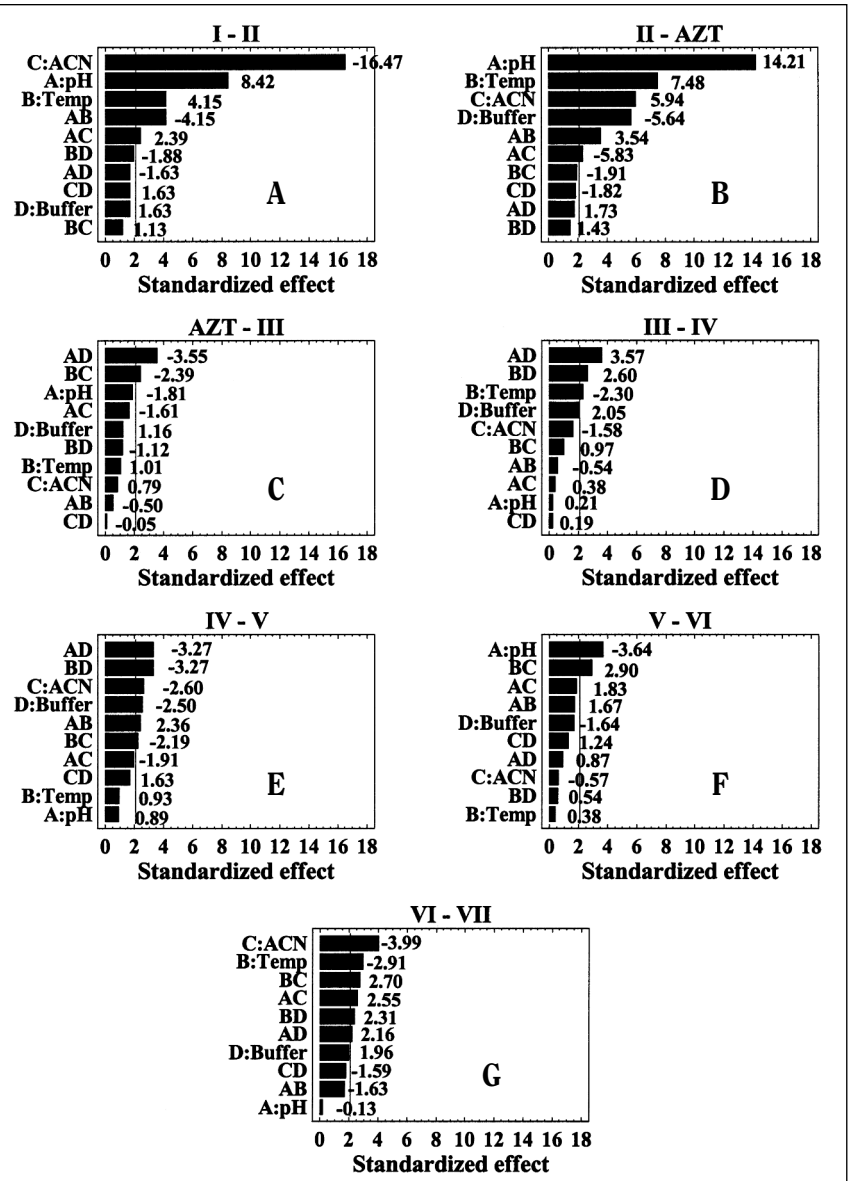

Figure 3. Standardized Pareto charts representing the estimated effects of parameters A (mobile phase pH), B (column temperature, °C), C (concentration of ACN in %), and D (phosphate buffer concentration in %) and parameter interactions on the selectivity between (A) I–II, (B) II–AZT, (C) AZT–III, (D) III–IV, (E) IV–V, (F) V–VI, and (G) VI–VII. Components I–VII are unknown.

Table I. Robustness Study: Nominal Values Corresponding with Low, Central, and Upper Levels

Chromatographic variable	Low value	Central value	High value
A (pH)	6.25	6.5	6.75
B (temperature, °C)	65	70	75
C (%ACN)	33	35	37
D (%phosphate buffer)	8	10	12

Robustness

Robustness is an important aspect of method validation. The influence of small changes in the operating conditions (variables) of the analytical procedure is evaluated on measured or calculated responses. The changes introduced when performing a robustness test reflect the changes that can occur when a method is transferred between different laboratories.

A 4-factorial design experiment was used to evaluate the robustness of the method with the help of the statistical graphic software system STATGRAPHICS plus, standard edition Version 4.1 (Manugistics, Rockville, MD). The chromatographic variables investigated were the concentration

of organic modifier in the mobile phase, the pH of the mobile phase, the column temperature, and the concentration of phosphate buffer. The values of the variables in the design are listed in Table I. For these four variables (2^4 different experiments) one central level experiment plus duplicate experiments were performed and 34 chromatograms were obtained. The measured response variables were the retention times of the different AZT components. For each experiment, the average result of two analyses was used for further calculations. The estimated effects of the four variables with their second-order interactions on the selectivity between AZT and the most important impurities were studied. Thus, the selectivity was examined between pairs I–II, II–AZT, AZT–III, III–IV, IV–V, V–VI, and VI–VII. Results are presented on the standardized Pareto charts in Figure 3. The bars are displayed in order of the size of the effects, with the largest effects on top. The charts include a vertical line at the critical t-value for a level of significance (α) of 0.05. Effects for which the bars are smaller than the critical t-value are considered as not significant.

The charts show that the parameters examined mainly influenced the selectivities I–II and II–AZT. An increase in ACN concentration (%) decreased the selectivity I–II but increased the selectivity II–AZT. The selectivities I–II and II–AZT were positively influenced by an increase in temperature or pH. An increase in buffer concentration had only a moderate negative effect on some selectivities. Chromatographic parameter interactions showed, at most, a moderate effect on selectivity.

In view of the fact that the concentration of ACN (%) and temperature mainly influenced the selectivity, response surface plots were constructed using these two parameters. Figure 4 shows how the retention times (min) corresponding with I, II, AZT, III, IV, V, VI, and VII change with respect to the concentration of ACN (%) and column temperature (°C). The figures show that in the range examined, there was not only no overlapping but also that in the more extreme conditions several separations became much poorer. Therefore, the important method parameters (such as temperature and organic modifier content) must be watched carefully when applying this method.

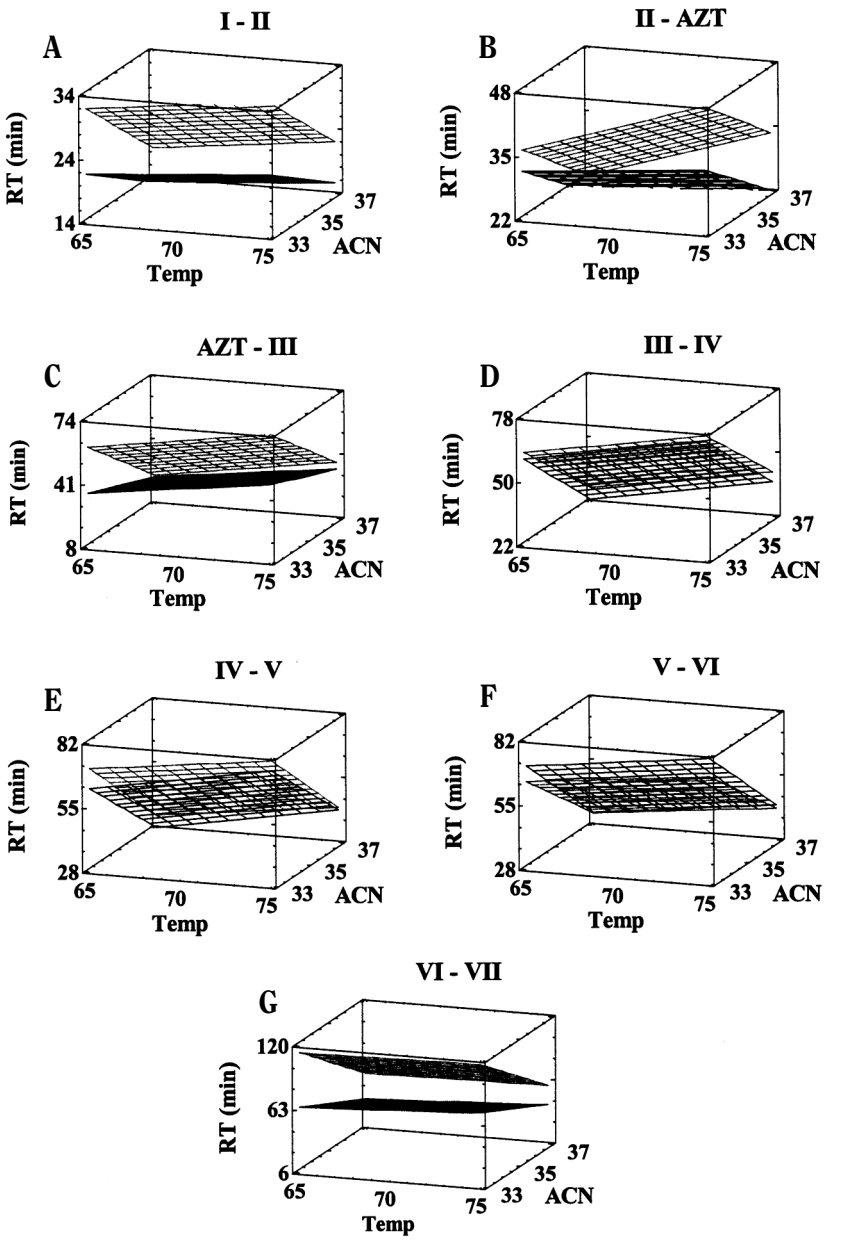

Figure 4. Estimated response plots for (A) I–II, (B) II–AZT, (C) AZT–III, (D) III–IV, (E) IV–V, (F) V–VI, and (G) VI–VII (lower planes and upper planes correspond with the first and second components respectively in each pair of selectivity) constructed with the retention times as a function of temperature and ACN concentration (%).

Repeatability, linearity, and detection limits

The repeatability of the method was assessed using six replicate injections of a 4.0-mg/mL solution of AZT. The relative standard deviation (RSD) of the peak area of the main component was 0.2%. The calibration curve obtained by replicate (n = 3) analysis of a series of analyte concentrations corresponding with 0.1%, 1%, 25%, 50%, 75%, 100%, and 125% of the nominal content of 4.0 mg/mL in the sample solution (400 µg on column for an injection volume of 100 µL) was subjected to linear regression analysis (y = 10872236x – 434346, r = 0.998, and $S_{y,x}$ = 873695, where y = peak area, x = concentration in %, r = correlation coefficient, and $S_{y,x}$ = standard error of estimate. The limit of quantitation (LOQ) for AZT with a signal-to-noise ratio of 10 was 0.09% (n = 6, RSD = 15%). The limit of detection, with a signal-to-noise ratio of 3, was 0.03% and was calculated against a 400-µg injection.

Conclusion

This isocratic LC method using UV detection is suitable for the separation of AZT, its synthesis intermediates and acid degradation product, and seven other unknown impurities. The method shows good selectivity, repeatability, and linearity. The introduction of UV detection allows the use of simpler equipment, compared with electrochemical detection. The sensitivity is sufficient because the LOQ is below 0.1%. Given the importance of the unknown impurities, LC–MS studies will be carried out to further identify these unknowns. A potential impurity of AZT formed from erythromycin B, which is a fermentation impurity of EA, (AZT B) was obtained from the European Pharmacopoeia Laboratory after the end of this study. It was shown to correspond with the unknown IV.

Acknowledgments

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