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Specialists in Ultra-Durable, High Efficiency HPLC Phases

Technical Bulletin #239

Recommendations for the Application of a Quantitative Assay for Azithromycin Using a ZirChrom®-PBD Column

HPLC Column

- A 15 cm x 4.6 mm i.d. ZirChrom®-PBD (part # ZR03-1546) column is recommended for this application.
- The ZirChrom®-PBD column is nearly identical in chromatographic selectivity to the L-29 (Alumina-PBD) column.
- The use of a guard column is strongly recommended to extend column life and enhance column performance (Holder part # 850-00, Insert part # ZR03-G40).

Mobile Phase

- The use of highly basic buffers with ZirChrom®-PBD is recommended to enhance the performance of the assay.
- The recommended mobile phase is 29/71 Acetonitrile/14mM Potassium phosphate, pH 11, at a flow rate of 1.0 ml/min.

Detection Parameters

- Electrochemical detection may be used in aperometric mode with dual glassy carbon electrodes.
- Electrode 1 should be set at +.070V, electrode 2 at +0.82V.
- The background current should be set at 85 nA.

Column Loading Capacity

Using a 15 cm x 4.6 mm i.d. column, the following column capacities are indicated by a 10% reduction in k'.

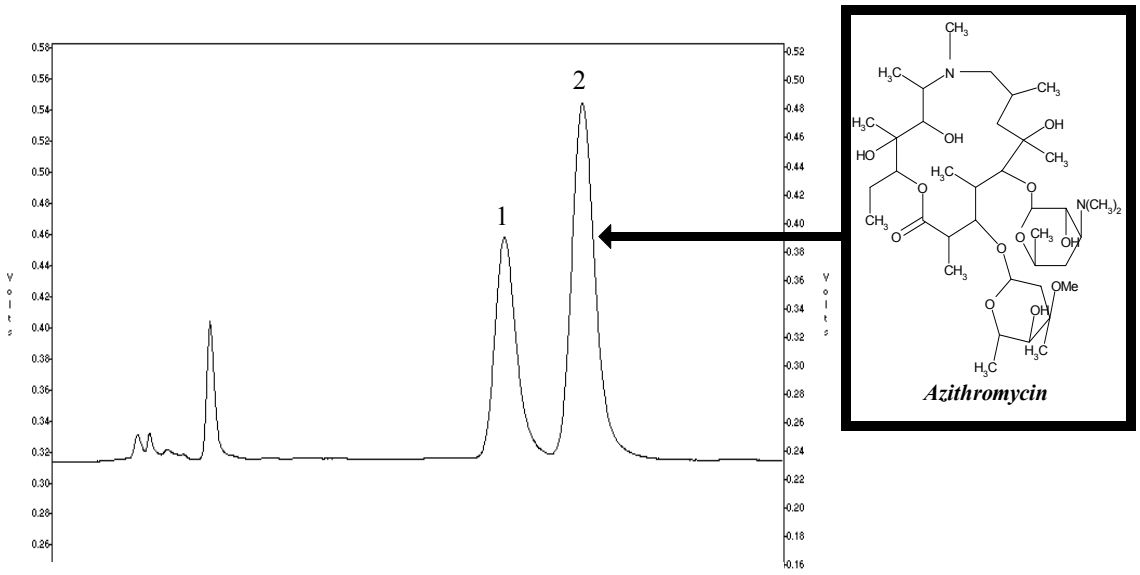
- Azithromycin — 2.0 mg/ml
- Azaerythromycin — 1.6 mg/ml

Analyte Detection Limits

Using the electrochemical detection scheme given above, the minimum detection limits are given below.

- Azithromycin — 4.7 ng/ml
- Azaerythromycin — 8.5 ng/ml

An analysis of Azithromycin and its analog Azaerythromycin is shown below.



LC Conditions: Column, ZirChrom®-PBD, 150 x 4.6 mm i.d.; Flow Rate, 1.0 ml/min.; Mobile Phase, 29/71 acetonitrile/14 mM potassium phosphate monobasic at pH 11; Column Temperature = ambient; Detector, amperometric electrochemical; Injection volume, 50 microliters; Sample concentration, 5 micrograms/ml. Solutes: 1 = azaerythromycin, 2 = azithromycin.

Analytical Performance

- The ZirChrom®-PBD column gives plate counts of better than 2000 plates/column, with asymmetry factors of 1.37 and 1.45 for azithromycin and azaerythromycin respectively.
- Calibration curves constructed using this analysis show excellent linearity over a concentration range of 0.0 to 10.0 micrograms/ml.
- This level of performance is similar to what is reported for L-29 (Alumina-PBD).
- Studies of column life at pH 11 for ZirChrom-PBD and Alumina-PBD have shown that ZirChrom®-PBD is stable for more than 10,000 column volumes of operation, while the Alumina-PBD fails after 2500 column volumes of use.
- ZirChrom columns exhibit a high degree of reprodubibility from column to column, making them an excellent choice for the application of the Azithromycin assay.

ZirChrom column are available worldwide. For on-line ordering and for a listing of distributors, please visit our website at http://www.zirchrom.com.

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Analysis of Azithromycin using ZirChrom®-PBD

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Technical Bulletin # 311

The ZirChrom®-PBD column has been designated by the USP as L49 and can be used for the analysis of azithromycin. The retention time of azithromycin is very sensitive to small changes in mobile phase composition and in the surface chemistry of the ZirChrom®-PBD column. This application note lists helpful method suggestions to ensure good peak shape and consistent results.

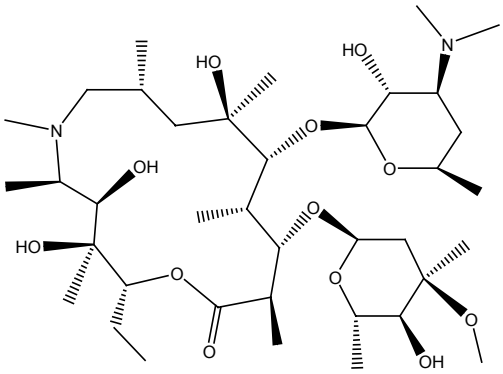

Figure 1: Structure of azithromycin.

Introduction

Azithromycin is a macrolide antibiotic that interferes with the growth of bacterial cells. Macrolides have activity against many gram-positive bacteria (excluding enterococci and methicillin-resistant *Staphylococcus aureus*), and have variable activity against respiratory gram-negative pathogens, *Mycobacterium avium* infections, gonorrhea, and *Chlamydia* infections (2). Azithromycin is used to treat bacterial infections in many different parts of the body but most often used to treat respiratory infections in children and adults.

The high pH necessary for the analysis of azithromycin prohibits the use of traditional silica based substrates and necessitates the use of the pH stable zirconia-based ZirChrom®-PBD. Azithromycin and its impurities lack good chromaphores and thus any analytical study of purity must be made using an electrochemical detector. For the purpose of this study we have injected a very concentrated sample and detected in the UV to avoid any issues inherent with that method of detection.

Experimental

USP standard solution of azithromycin was injected at 30ºC using a ZirChrom®-PBD column (see figure 2). The separation conditions were as follows:

Column: ZirChrom®-PBD, 150 mm x 4.6 mm i.d. (Part Number: ZR03-1546)
Mobile Phase: 5.8 g monobasic potassium phosphate in 2130 mL of water, added to 870 mL of acetonitrile adjusted to pH 11.0 with potassium hydroxide
Temperature: 30 ºC with Metalox™ 200-C column heater
Flow Rate: 1 ml/min.
Injection Vol.: 5 µl, 1 mg/mL
Pressure Drop: 195 bar
Detection: UV at 215 nm

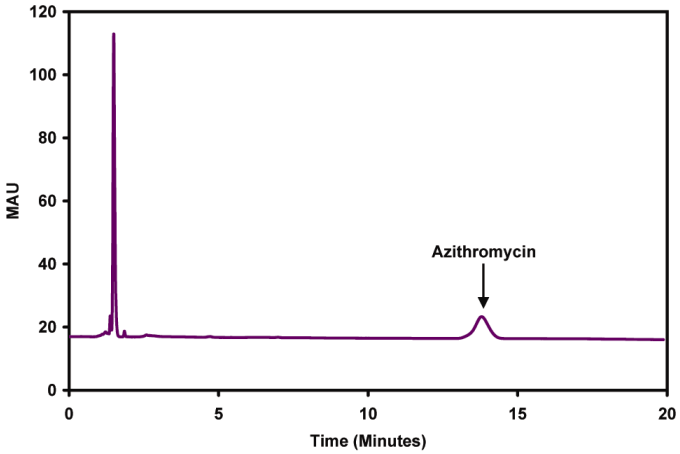

Figure 2: USP standard azithromycin.

Several method conditions were found to affect both the retention time and peak shape of the analyte:

1. Over-degassing of the mobile phase: The retention time of azithromycin is sensitive to both the concentration of phosphate and the percentage of organic, thus it is critical that these remain constant. We recommend degassing the aqueous and organic portions of the mobile phase separately then combining to adjust pH. This prevents loss of organic and changes in phosphate concentration. We also recommend a short flush of the column with pure water before equilibrating the column in the mobile phase.
2. Mobile phase left in column without flow: This mobile phase CANNOT be left in the column for any length of time without flow. The phosphate in the mobile phase will precipitate out into the column without flow and when flow is resumed it will dissolve back into the mobile phase leaving voids and greatly deteriorated peak shapes.
3. Uncontrolled column temperature: Placing the column in a thermostatted environment prevents retention fluctuations due to changes in ambient temperature and drafts.
4. Changes in pH: Small changes in pH can cause significant changes in retention time. It is very important to adjust up to but not over pH 11.0 $\pm$ 0.1 as indicated in the USP method. The pH will jump sharply for a period during adjustment and the potassium hydroxide must be added drop wise to prevent over correction.
5. Injection before complete equilibration: The column takes approximately 4-5 hours at 1 ml/min to fully equilibrate in the mobile phase buffer. Equilibration was defined as the stabilized retention time of azithromycin (see Figure 3). As this equilibration time is often equal to or less than the equilibration time of the electrochemical detector, it does not usually pose a significant issue.
6. Use of other mobile phase components and conditions: The use of different mobile phase components (such as ammonium phosphate or dibasic potassium phosphate) and pH conditions will change both the column surface chemistry and its interactions with the analyte. These changes can greatly effect equilibration and retention times.

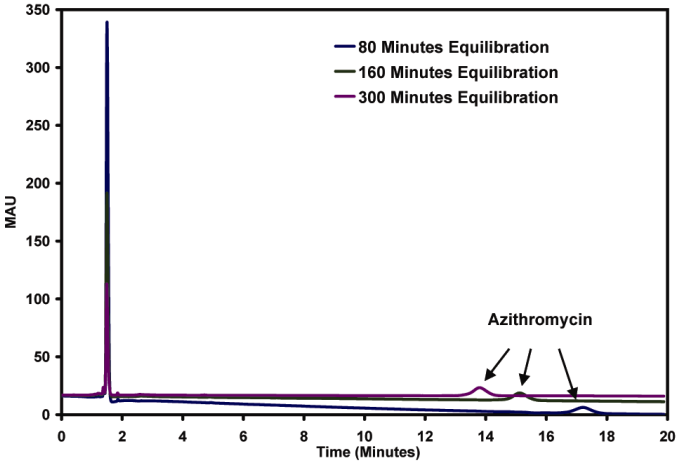

Figure 3: Affect of column exposure to USP mobile phase (equilibration) on the retention time of azithromycin.

Please contact ZirChrom technical support at 1-866-STABLE-1 or support@zirchrom.com with any additional questions or concerns.

ZirChrom phases offer unique selectivity, high efficiency, and excellent chemical and thermal stability.

References

(1) USP, USP Monograph: Azithromycin (2005).

(2) http://www.pfizer.com/download/uspi_zithromax.pdf

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