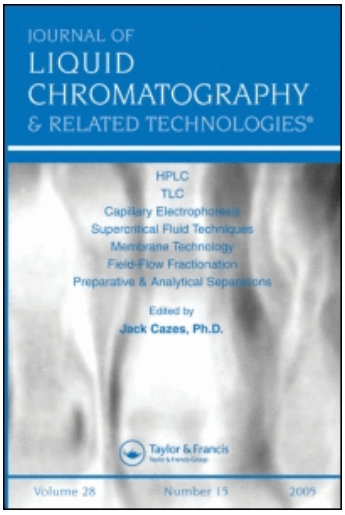

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Wigman, L. S. , Darrington, R. T. and Young, J. W.(1998) 'An Accurate Procedure for the Analysis of Azithromycin in Ferret Feces Using Solid Phase Extraction and HPLC', Journal of Liquid Chromatography & Related Technologies, 21: 6, 819 — 828
To link to this Article: DOI: 10.1080/10826079808000511
URL: http://dx.doi.org/10.1080/10826079808000511

J. LIQ. CHROM. & REL. TECHNOL., 21(6), 819–828 (1998)

AN ACCURATE PROCEDURE FOR THE ANALYSIS OF AZITHROMYCIN IN FERRET FECES USING SOLID PHASE EXTRACTION AND HPLC

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ABSTRACT

Azithromycin has become one of the most widely prescribed macrolide antibiotics due to the pharmacokinetic profile and broad spectrum of activity. A method for determining the level of azithromycin in ferret feces involving drying, methylene chloride extraction, solid phase extraction (SPE) clean-up, and HPLC analysis was developed. The method was linear from 0.8 to 3.2 mg/mL with a mean recovery of 97% (0.6% RSD for 50 mg spiked simulated feces). The analysis was developed to quantitate approximately 50 mg of azithromycin in approximately 0.6g of dried feces.

The HPLC method uses a pH 7.5 phosphate buffered mobile phase with a conventional C_{18} column and UV detection at 205 nm. The HPLC system is robust and column life is at least 1 month (~ 200 injections).

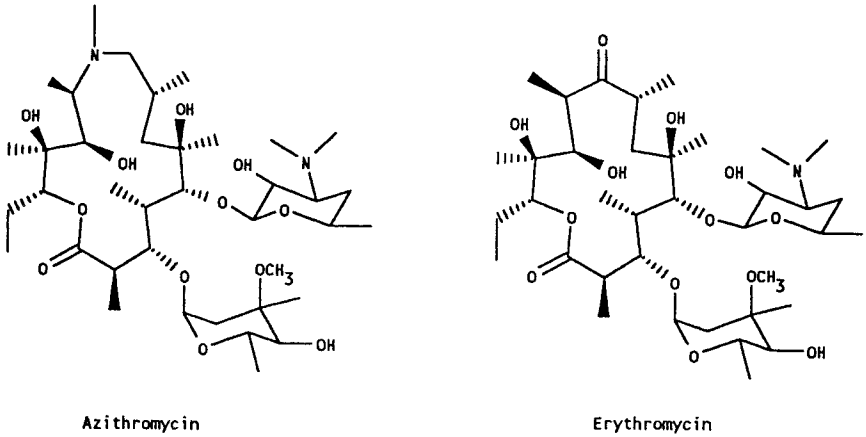

Figure 1. Structures of Azithromycin and Erythromycin.

INTRODUCTION

Azithromycin is a broad spectrum macrolide antibiotic (Figure 1) similar in structure to erythromycin. However, azithromycin has been shown to have a better pharmacokinetic profile and broader spectrum of activity than erythromycin. Thus, azithromycin has become one of the most widely prescribed macrolide antibiotics. Recent investigations of *in vivo* release characteristics in Ferrets required the determination of azithromycin levels being excreted. Ferrets were being investigated as a digestive system model similar to the one described by Naylor.[1] The determination included a solid phase extraction method coupled with a quantitative HPLC analysis.

Quantitative analysis of azithromycin has historically been challenging, requiring γ-bonded alumina columns, elevated pH and electrochemical detection.[2] Similar difficulties have been encountered for analysis of erythromycin and other macrolide antibiotics which required extremes of pH or temperature to improve peak shape and resolution.[3-6] These difficulties are largely due to the basic amine(s) (pKa 8.8 for erythromycin)[7] which contribute to the poor peak shape at elevated pH and the low k' (capacity factor) at reduced pH. The poor peak shape at elevated pH is most likely due to amine interaction with residual silanols.[8-10] The poor retention at low pH is due to ionization of the analyte (secondary equilibra)[11-14] where the ionized form is poorly retained.

A novel mobile phase composition using high organic levels at slightly basic pH was developed for the analysis of dirithromycin using conventional C_{18} columns.[15] Modifications to this HPLC system were the basis of the method described in this paper. Resolution of potential metabolites from azithromycin are adequate for accurate analysis.

The liquid extraction and SPE clean-up steps leave the sample in a solution suitable for direct HPLC analysis, with no interfering peaks. Average recovery and precision were good: 97% recovery (0.6% RSD for 50 mg spiked simulated feces).

EXPERIMENTAL

Standard Preparation

Standards of azithromycin dihydrate (obtained from Pfizer Central Research, Groton, CT) were prepared in mobile phase containing 44:29:27 (v/v) acetonitrile (HPLC Grade): methanol (HPLC Grade): (0.05M phosphate buffer pH = 7.5). The phosphate buffer was prepared form Milli-Q water®, (Millipore corporation, Bedford MA), potassium phosphate dibasic, anhydrous (reagent grade) and phosphoric acid (reagent grade).

The working standard was prepared to contain 1 mg/mL of azithromycin (anhydrous). Linearity samples were prepared at the following concentrations 0.8, 1.2, 1.6, 2.4, and 3.2 mg/mL.

Sample Preparation

Drying and liquid extraction

Ferret feces samples (obtained from Pfizer Central Research, Groton, CT) were dried in a vacuum desiccator over anhydrous calcium sulfate (W.A. Hammond Drierite Co., Stock #23001, Xenia, OH) at room temperature. Moisture levels and sample weights were determined by difference. The samples were transferred to a 50 cc HDPE centrifuge tube and extracted for 30 minutes with 15 mL of methylene chloride (reagent grade). Extraction was facilitated by shaking at 350 RPM (Cole-Parmer, model #51502 reciprocating shaker, Chicago, IL).

Recovery was checked using spiked samples prepared as follows: A 50 mg enteric coated azithromycin tablet (obtained from Pfizer Central Research, Groton, CT) was crushed in glassine paper and added to a 50 cc HDPE centrifuge tube. A 0.6g dry sample of tryptic soy broth (DIFCO Labs, Catalog #0370-17-3, Detroit, MI) was then added to simulate feces. The same procedures used for samples were used for these spikes beyond this point.

Clean-up

The clean-up was performed as follows using a 24 port vacuum manifold (J.T. Baker, Phillipsburgh, NJ): Activate a Baker 500mg spe-Silica gel cartridge (J.T. Baker, P/N 7086-06, Phillipsburgh, NJ) with 2ml of methylene chloride. Pipette 5 mL of sample extract onto the cartridge. Wash with 20 mL of methylene chloride. Elute with 8 mL of 70:30 water/methanol (containing 0.10% by volume trifluoroacetic acid). Both the methanol and trifluoroacetic acid were reagent grade. Dilute the eluted sample to 10 mL with mobile phase, mix well, and assay by HPLC.

Chromatographic System

A model 1090 (Hewlett-Packard, Avondale, PA) HPLC with autosampler, column oven and diode array detector or a model 501 HPLC pump, model 717 autosampler, model 1122 column oven and a model 486 UV/VIS detector (Waters, Milford, MA) were used. A Beckman Ultrasphere ODS, 4.6 x 250 mm, 5μm column (Beckman Instruments, P/N 235329, Fullerton, CA) was installed in the column oven and held at 40°C. The isocratic mobile phase was described in the standard preparation section and run at 2 mL/minute. A 25 μL aliquot was injected and peaks were detected by UV at 205nm.

RESULTS AND DISCUSSION

Method Development

HPLC system

The HPLC system was based upon conditions described in Reference 15 and optimized for azithromycin. An initial experiment using a 37% pH 7.5 phosphate buffer, 19% methanol, 44% acetonitrile mobile phase flowing at 2 mL/minute with a Beckman Ultrasphere ODS, 4.6 x 250 mm, 5 microns column was reasonably good for azithromycin retention (18 minutes), and

Table 1

SPE Recovery Optimization

Eluent*	% Recovery of Azithromycin
7:3 water:methanol (0.1% v/v trifluoroacetic acid)	101.5
HPLC mobile phase	90.4
1:1 tetrahydrofuran:water	54.1
1:1 acetonitrile:methanol	53.4
1:1 acetonitrile:water	<5
7:3 water:methanol	<5

*8mL of eluent were used

resolution of related impurities was acceptable, however, azithromycin peak shape was poor (tailing factor = 2.3).[16] Increasing the methanol content by 10% greatly improved the peak shape (tailing factor = 1.6),[16] without significantly changing the resolution of related impurities. Azithromycin retention time was decreased to approximately 12 minutes. Slight variations in the mobile phase pH (from 6.5 to 7.5) had little effect on retention time or peak shape.

SPE Clean-Up

The clean-up step was developed by determining the recovery of azithromycin from silica gel SPE cartridges (2 mL of 40mg/mL dissolved in 1:1 hexane:methylene chloride). No other SPE cartridges were evaluated. Azithromycin was not detected in the break-through volume or in the wash volume. Optimization of recovery is reported in Table 1.

Recovery and Reproducibility

Recovery was determined using a crushed 50 mg tablet. The same formulation was used both to dose the ferrets (1 tablet per animal) and to determine recovery. Ferret feces were simulated by using an equivalent weight (dry) of tryptic soy broth. Tryptic soy broth was used to simulate ferret feces, since, some commercial ferret feeds contains high levels of soy meal, the tryptic soy broth emulates the digestive process, and authentic control samples were not available. The crushed tablet and tryptic soy broth were mixed and run

through the liquid extraction, clean-up and HPLC procedures. A Recovery of 97% was determined for each of two separate spike samples. Injection precision for duplicate injections was found to be 0.5% and 0.6% RSD for each spike sample respectively. The recovery and reproducibility of azithromycin were very good considering the complexity of the matrix and the number of steps required for analysis including: liquid extraction, clean-up by SPE, and HPLC analysis. No internal standard was evaluated due to the high recovery.

Linearity, Precision, Performance, and Robustness

Linearity was determined by serial dilution of a standard stock solution covering the concentration range from 0.8 to 3.2 mg/mL (48% to 192% of target). The line equation parameters are as follows: slope = 1009.9, y-intercept = -0.0145, R^2 = 0.9999. The intercept was not significant since it represents less than 0.01% of the lowest area evaluated. Closeness of fit was excellent as shown by the correlation coefficient. Based on these results a single point standard (1.0 mg/mL) was used for analysis.

Analysis precision was tested by making 5 replicate injections of a working standard at 1.0 mg/mL of azithromycin. A 0.6% RSD was calculated for these replicate injections. This was the same level of precision determined for duplicate injections of the recovery spikes.

Performance of the HPLC system was good with 6000 theoretical plates calculated for azithromycin using the USP tangent method[16] or 3400 plates calculated as in reference 17. Peak shape, as expected for strongly basic compounds, was not ideal and the tailing factor was determined to be 1.6 using the USP[16] method. Tailing did not interfere with integration, accuracy or precision. Retention time varied by less than 0.3 minutes for samples and standards covering the linear range.

Method robustness was good considering the high mobile phase pH and silica based column. Typical columns have been used for at least 1 month and ~ 200 injections. Column fronting began to appear as the column aged and columns should be removed from service at this point. Over 10 columns have been used by at least 4 analysts in two laboratories without adverse incidents.

Selectivity

Although the blank results were obtained using "simulated" feces, no interfering peaks were observed in the blank chromatogram or in the sample or

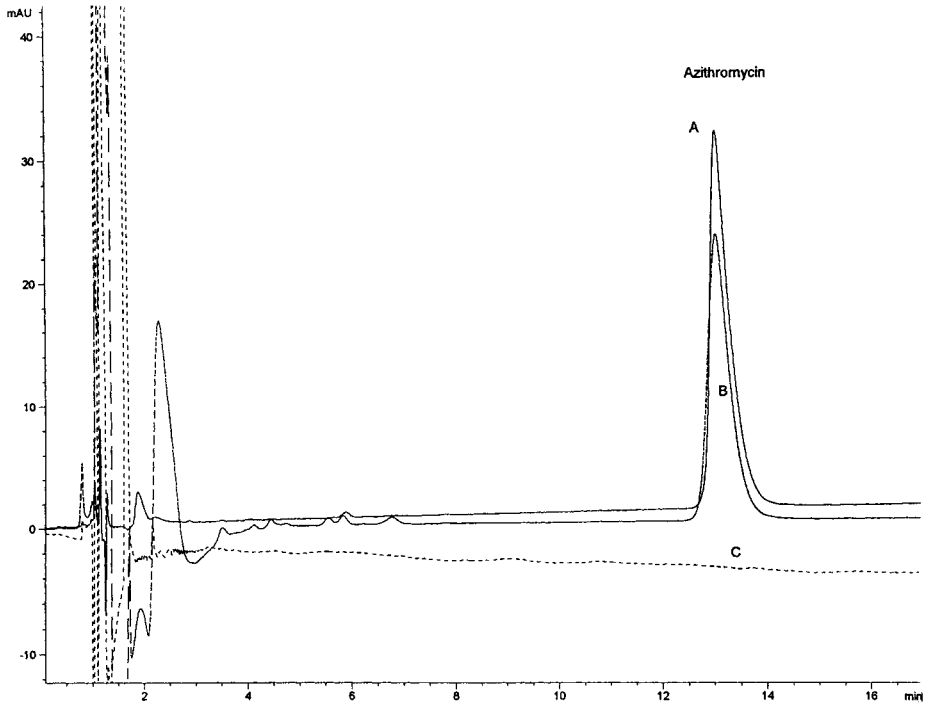

Figure 2. Chromatogram of (A) 1.0 mg/mL azithromycin standard, (B) ferret feces sample containing 22.1 mg of azithromycin, (C) simulated feces blank sample. Each sample was chromatographed using the following conditions: Column-Beckman Ultrasphere ODS, 4.6 x 250 mm, 5 microns, Column Temperature- 40°C, Detection wavelength- 205 nm Flow Rate- 2.0 mL/min., Injection volume 25 µL, Mobile Phase- 27:29:44 (v/v) pH 7.5, 0.05 M Phosphate buffer: Methanol:Acetonitrile.

Table 2

Potential Azithromycin Metabolites

Potential Metabolite	Relative Retention Time*
N-Desmethylazithromycin	0.274
Descladinoseazithromycin	0.431
Erythromycin A	0.559
Azithromycin	1.00

*Calculated relative to azithromycin (retention time 12.17 minutes).

Table 3

Ferret Feces Sample Results

Sample	Azithromycin Found (mg)	Dry Sample Weight (g)	% Moisture
1	6.8	0.595	76.0
2	22.1	0.200	79.7
3	24.4	0.649	76.8
4	not detected	0.341	76.7
5	26.7	0.090	77.5

standard chromatograms (Figure 2). Thus, the combination of sample drying, selective liquid extraction, SPE clean-up and HPLC analysis appear to be suitable in terms of eliminating matrix interference. Resolution of potential azithromycin metabolites is sufficient for accurate analysis of azithromycin as shown in Table 2.

Sample Analysis and Discussion

A total of 5 ferret feces samples were analyzed as obtained from 4 animals each dosed with a single 33 mg azithromycin tablet. The samples were obtained at various times post dose. Results of the study are contained in Table 3 and demonstrate that a significant amount of azithromycin was excreted in feces.

CONCLUSIONS

An accurate (97% mean recovery) method for the determination of azithromycin in ferret feces has been developed. The method involves sample drying, liquid extraction, SPE clean-up and HPLC analysis. Method selectivity, sensitivity and precision are suitable for the analysis of ferret feces samples collected after dosing with 33 mg azithromycin tablets.

Use of the ferret as a digestive system model with azithromycin was not successful due to the rapid digestive cycle. A high percentage of the oral dose was excreted in feces.

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Received July 10, 1997
Accepted August 6, 1997
Manuscript 4527