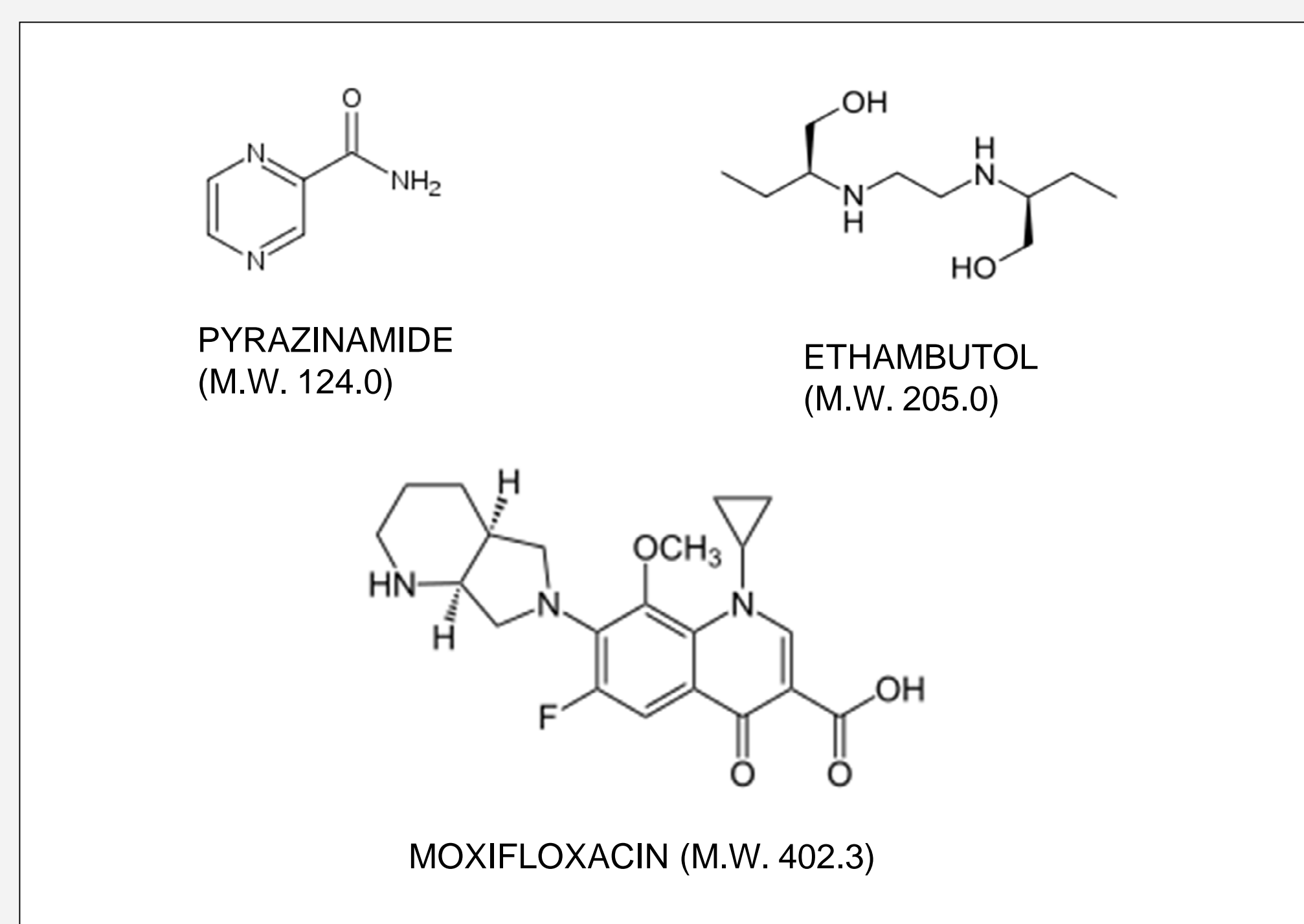


A Dried Blood Spot Method for Therapeutic Drug Monitoring of Ethambutol, Pyrazinamide, and Moxifloxacin to Optimize Tuberculosis Treatment in Children Using LC-MS/MS

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INTRODUCTION

Despite recent changes in the pediatric tuberculosis (TB) treatment guidelines published by the World Health Organization, there is limited data on the dose-exposure relationships of anti-TB drugs in the majority of the world's pediatric population. The ability to conduct comprehensive pharmacokinetic (PK) studies using traditional venipuncture is not feasible in remote, resource-constrained settings. This study was designed to validate a rapid and reproducible LC-MS/MS method for the measurement of clinically relevant concentrations of Ethambutol (ETH), Pyrazinamide (PZA), and Moxifloxacin (MOX) from dried blood spots (DBS). The availability of DBS-based assays would permit PK studies and therapeutic monitoring of children residing in low-income, high-burden countries to optimize the treatment in various TB subpopulations. The DBS format will be utilized for therapeutic drug monitoring (TDM) and will facilitate genetic analyses in combinational therapies to optimize treatment in children through a NIH funded project.



METHOD

ETH, PZA, and MOX were validated in DBS with K₂EDTA whole blood. ETH and MOX over a concentration range of 0.10 to 10 µg/mL and PZA over a concentration range of 1.00 to 100 µg/mL. Blood containing ETH, PZA, and MOX was spotted onto untreated DBS cards (Perkin Elmer 226) and 7 mm punches were used for analysis. Each punch was placed in extraction solvent (90/10 Methanol/Water, 300 µL) containing Ethambutol-d₄, Pyrazinamide-¹⁵N,₃, and Moxifloxacin-¹³C,₃ (I.S.) and sonicated for 60 minutes. A 100 µL aliquot from each extract was transferred to injection vials with 300 µL of 0.1% formic acid in deionized water.

ETH, PZA, and MOX were analyzed on a Luna Phenyl-Hexyl analytical column with gradient elution, using water/methanol/acetonitrile-based mobile phase solutions. The analytes were detected using an API 3000 LC-MS/MS systems under positive MRM mode.

Liquid Chromatography

HPLC : Shimadzu 10ADvp
Autosampler : Perkin Elmer 200
Column: Luna Phenyl-Hexyl (100.0 x 2.0 mm, 5µ)
MP A : 0.1% Formic acid in DI H₂O
MP B : ACN/MeOH/Formic Acid (40:60:0.1, v/v/v)

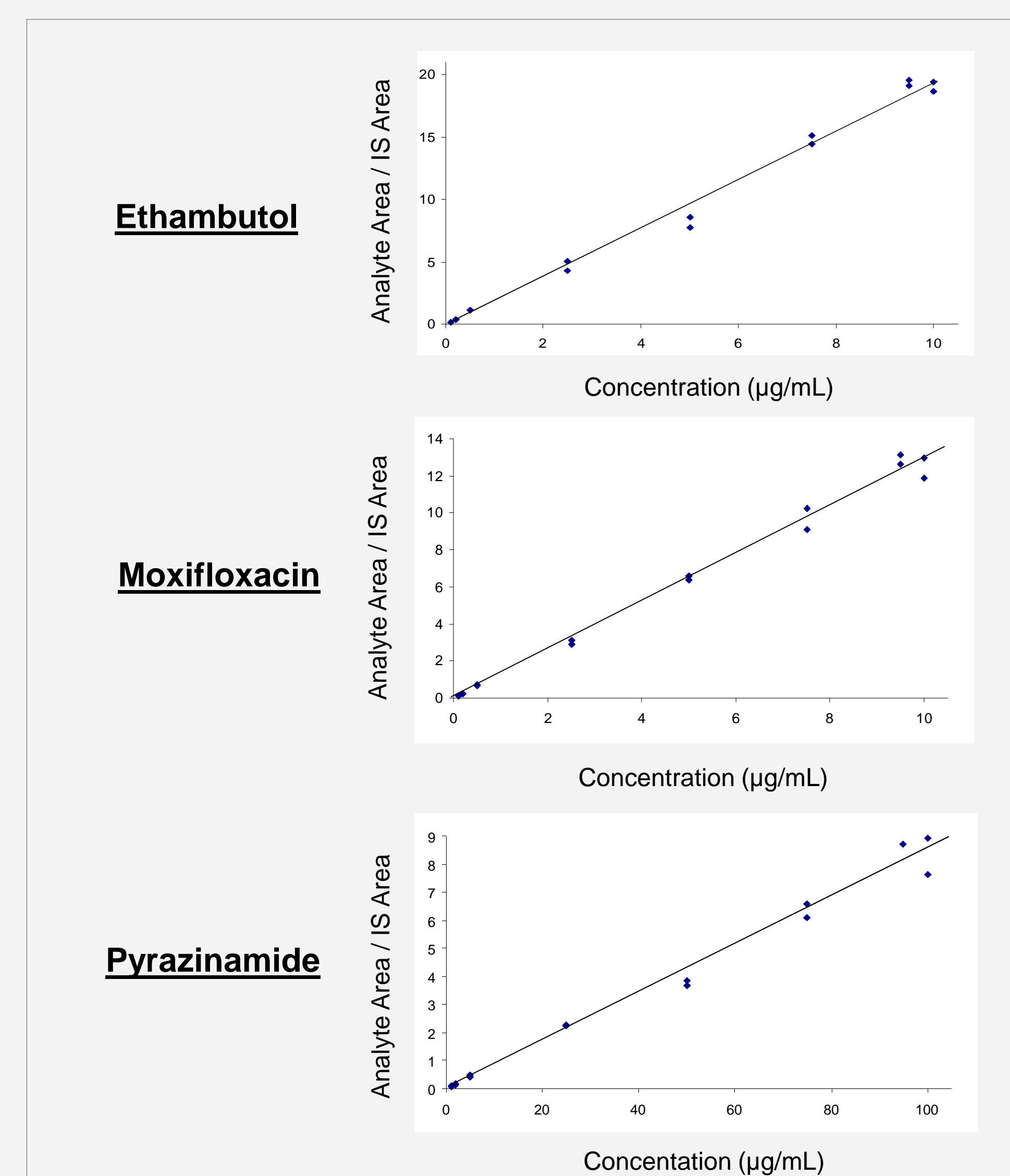
Mass Spectrometry

AB Sciex API 3000, Polarity : Positive
Scan type : Multiple Reaction Monitoring
Ethambutol: m/z 205.0 → 116.0
Ethambutol-d₄: m/z 209.0 → 120.0 (I.S.)
Pyrazinamide: m/z 124.0 → 79.0
Pyrazinamide-¹⁵N,₃: m/z 128.0 → 82.0 (I.S.)
Moxifloxacin: m/z 402.3 → 384.0
Moxifloxacin-¹³C,₃: m/z 406.3 → 388.0 (I.S.)

RESULTS

The responses were linear over a range of 0.10 to 10 µg/mL for ETH and MOX and 1.00 to 100 µg/mL for PZA with weighted linear regression (1/x). The correlation coefficient was 0.993 or better for all analytes (Figure 1).

Figure 1. Typical Calibration Curves for ETH, PZA, and MOX Using DBS Method



Figures 2 through 4 Show Typical Chromatograms at the Lower Limit of Quantitation (LLOQ) level for ETH, PZA, and MOX.

Figure 2. Chromatogram of an Extracted ETH LLOQ (0.10 µg/mL).

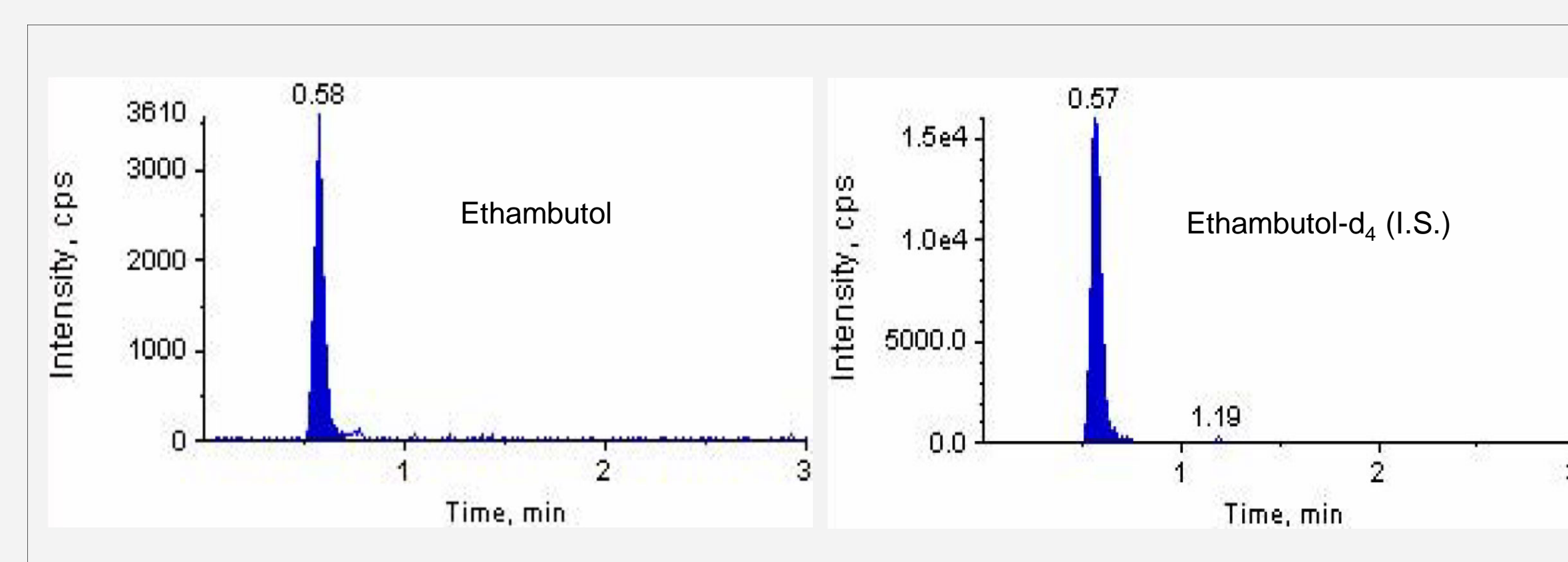


Figure 3. Chromatogram of an Extracted PZA LLOQ (1.00 µg/mL).

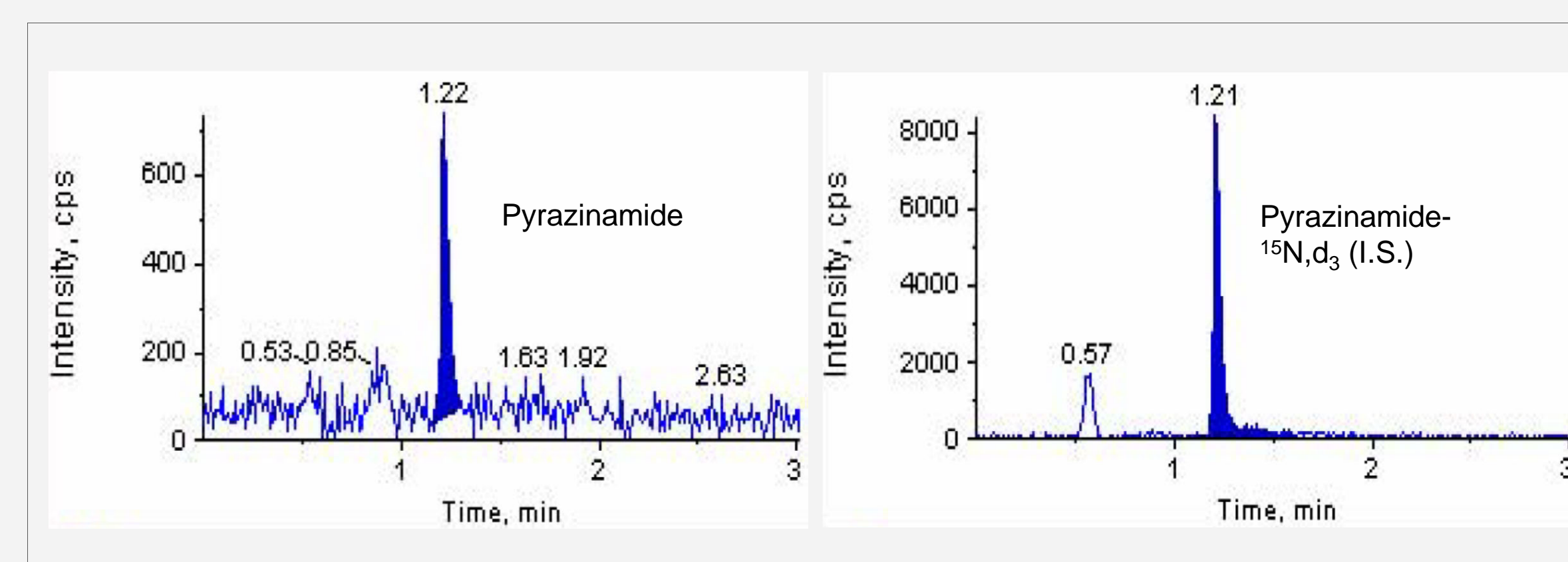
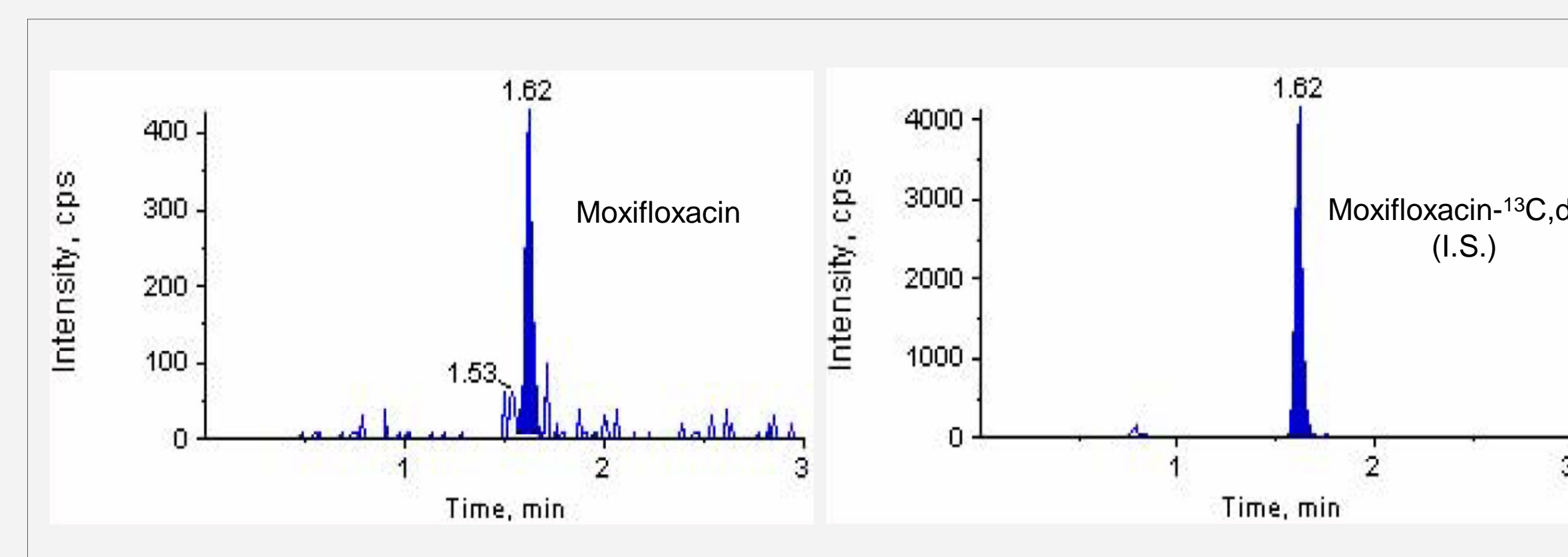


Figure 4. Chromatogram of an Extracted MOX LLOQ (0.10 µg/mL).



Stability of ETH, PZA, and MOX

The stability of each analyte in whole blood was investigated after being placed onto the DBS cards. Stability data was generated under storage conditions of 57 days at room temperature, 57 days at 30° C, and a Relative Humidity of 65%. In each instance ETH, PZA, and MOX demonstrated acceptable precision and accuracy meeting the FDA guidelines for all tested storage conditions.

Inter and Intra Assay Precision and Accuracy

Inter and Intra-assay precision and accuracy was evaluated for Ethambutol, Pyrazinamide, and Moxifloxacin. All analytes met the FDA guidelines for % bias and CV for a validated method with the results for the Inter- assay precision and accuracy presented in Table 1.

Matrix Effect Evaluation

Matrix effect was evaluated over seven different lots of K₂EDTA whole blood and the results are presented in Table 2.

Table 1. Inter-assay Precision and Accuracy Results for ETH, MOX, and PZA Quality Control Samples (n = 6 replicates per batch).

Analyte		0.10 µg/mL (LLOQ)	0.30 µg/mL (Low)	3.75 µg/mL (Mid)	8.75 µg/mL (High)
ETH	Mean	0.95	0.31	3.83	9.02
	n	18	18	18	18
	% CV	7.1	9.6	6.8	7.3
	% Bias	-5.0	3.3	2.2	3.1
MOX	Mean	0.11	0.31	3.93	9.32
	n	18	18	18	18
	% CV	7.3	8.3	3.7	6.8
	% Bias	10.0	4.0	4.7	6.6
PZA	Mean	1.10	3.01	38.5	90.3
	n	18	18	18	18
	% CV	14.6	9.5	8.3	10.3
	% Bias	10.0	0.3	2.7	3.3

Table 2. Matrix Effect for ETH, PZA, and MOX in Whole Blood on DBS Cards.

		Drug Area	I.S. Area	Area Ratio
ETH	mean	467698	66639	8.17
	n (lots)	7	7	7
	SD	35738	2484	0.36
	%CV	7.6	3.7	4.5
PZA	mean	72671	29597	3.35
	n	7	7	7
	SD	106	1884	0.22
	%CV	0.2	6.4	6.6
MOX	mean	43149	8149	7.23
	n	7	7	7
	SD	1825	418	0.06
	%CV	4.2	5.1	0.8

CONCLUSION

A reliable LC-MS/MS method has been developed for the measurement of clinically relevant concentrations of Ethambutol, Pyrazinamide, and Moxifloxacin using DBS. Coupled with our earlier development of Rifampin DBS analysis, these assays support pediatric PK assessment in settings where venipuncture is not practical without concern for sample integrity.

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