A High-Pressure Liquid Chromatographic–Tandem Mass Spectrometric Method for the Determination of Ethambutol in Human Plasma, Bronchoalveolar Lavage Fluid, and Alveolar Cells

John E. Conte, Jr.[1,2,3,*], Emil Lin[4], Yeping Zhao[4], and **Elisabeth Zurlinden[1]**

[1]Department of Epidemiology and Biostatistics, Infectious Diseases Research Laboratory, [2]Department of Medicine, [3]Department of Microbiology and Immunology, and [4]Department of Biopharmaceutical Sciences, University of California, San Francisco, 350 Parnassus Avenue, Suite 507, San Francisco, CA 94117

Abstract

A technique is presented for the specific and sensitive determination of ethambutol concentrations in plasma, bronchoalveolar lavage (BAL), and alveolar cells (AC) using a high-pressure liquid chromatographic (HPLC)–tandem mass spectrometric (MS–MS) method. The preparation of samples requires a deproteinization step with acetonitrile. The retention times for ethambutol, neostigmine bromide, and propranolol are 2.0, 1.4, and 1.1 min, respectively, with a total run time of 2.8 min. The detection limits for ethambutol are 0.05 µg/mL for plasma and 0.005 µg/mL for the BAL supernatants and AC suspensions. The assay has excellent performance characteristics and has been used to support a study of the intrapulmonary pharmacokinetics of ethambutol in human subjects.

Introduction

Ethambutol has a primary role in the treatment of tuberculosis and is recommended with isoniazid, rifampin, and pyrazinamide as initial therapy (1). Ethambutol is rapidly absorbed and has a bioavailability of 7% after oral administration (2,3). Under fasting conditions, the maximum concentration (mean ± standard deviation, SD) of the drug in serum is 4.5 ± 1.0 µg/mL and the time to maximum concentration is 2.5 ± 0.9 h (2). The minimum inhibitory concentration of ethambutol for *M. tuberculosis* ranges from 0.5 to 2 µg/mL in broth media (4). A microbiological assay (detection limit of 0.4 µg/mL) using *M. smegmatis* as the test organism for determining ethambutol in serum has been reported (5). A gas chromatographic (GC)–mass spectrometric (MS) method has been used for the determination of ethambutol in tablets (6). A GC–liquid chromatographic (LC) assay with improved performance characteristics (detection limit of 0.1 µg/mL in plasma) has been used to study the pharmacokinetics of ethambutol in humans (3,7–9) and rabbits (10), and a high-pressure liquid chromatographic (HPLC) method for the determination of ethambutol in plasma (detection limit of 10 ng/mL) and urine (detection limit of 10 µg/mL) has been described (11).

We report the use of a sensitive HPLC–tandem mass spectrometric (MS–MS) technique to measure ethambutol in human plasma, bronchoalveolar fluid (BAL) (detection limit of 0.05 µg/mL), alveolar cells (AC) (detection limit of 0.005 µg/mL), and plasma (detection limit of 0.05 µg/mL). Compared with other methods, the technique has the advantages of increased sensitivity and a capability to analyze small sample volumes. The specificity of HPLC–MS–MS detection greatly minimizes the risk of interference from other substances. This is especially important when analyzing specimens from patients such as those with AIDS who are taking numerous concomitant medications. It currently is being used to support a phase-one study of the intrapulmonary pharmacokinetics of ethionamide in normal subjects and subjects with AIDS.

Experimental

Chemicals

All solvents and chemicals were HPLC grade except ammonium acetate, which was certified. A 1.0-mg/mL solution of ethambutol HCl (Lederle Laboratories, Wayne, NJ) was made in 50% methanol and stored refrigerated. This solution was further diluted to produce working stock solutions of 0.1, 1.0, and 10 µg/mL of ethambutol. Stock solutions of 1.0 mg/mL neostigmine bromide (Aldrich Chemical Co., Milwaukee, WI) and propranolol (USP Reference, Rockville, MD) were prepared in 50% methanol. Neostigmine bromide and propranolol were then diluted to a concentration of 0.050 µg/mL in acetonitrile and used as the internal standard for plasma, and propranolol was diluted to 0.300 µg/mL and used as the internal standard for BAL and AC.

* Author to whom correspondence should be addressed.

Instrumental

Chromatography

The mobile phase (containing 80% acetonitrile, 4mM ammonium acetate, and 0.10% trifluoroacetic acid) was run through a hypersil silica column (50- × 4.6-mm i.d., 5-µm particle size) at a flow rate of 0.8 mL/min using a Shimadzu (Columbia, MD) LC-10 AD pump. Extracts from samples were injected onto the system with a Waters (Milford, MA) Intelligent Sample Processor 717 Plus. The retention times for ethambutol, neostigmine, and propranolol were 2.0, 1.4, and 1.1 min, respectively, with a total run time of 2.8 min.

MS

We used two different MS systems during the development and validation of this assay to explore different types of MS equipment. Neostigmine bromide was the internal standard used for the plasma and BAL that were assayed on the PE Sciex API III (PerkinElmer, Foster City, CA), whereas propranolol was used as the internal standard for the assays in plasma, BAL, and ACs performed on the Micromass (Manchester, U.K.) Quattro LC. Peak detection and area determinations for some plasma and BAL were

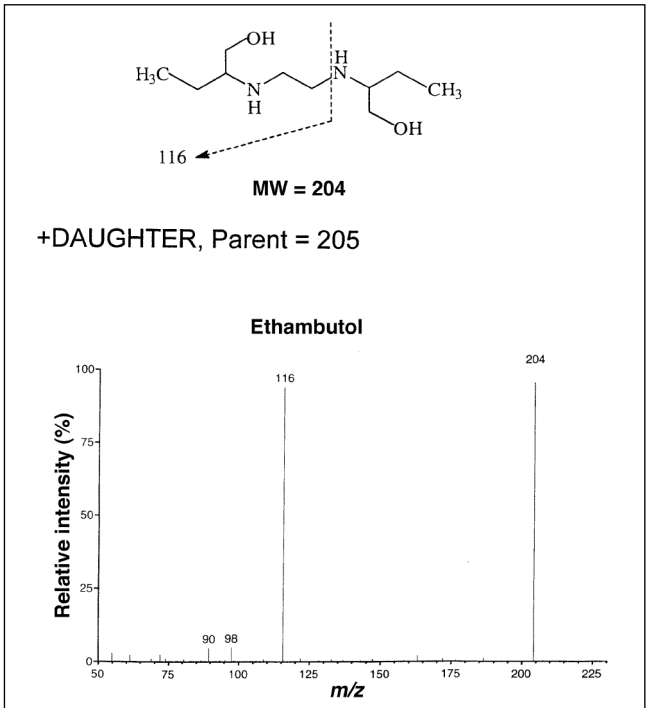

Figure 1. Daughter ion spectra and chemical structures of ethambutol using the Sciex APCI mode.

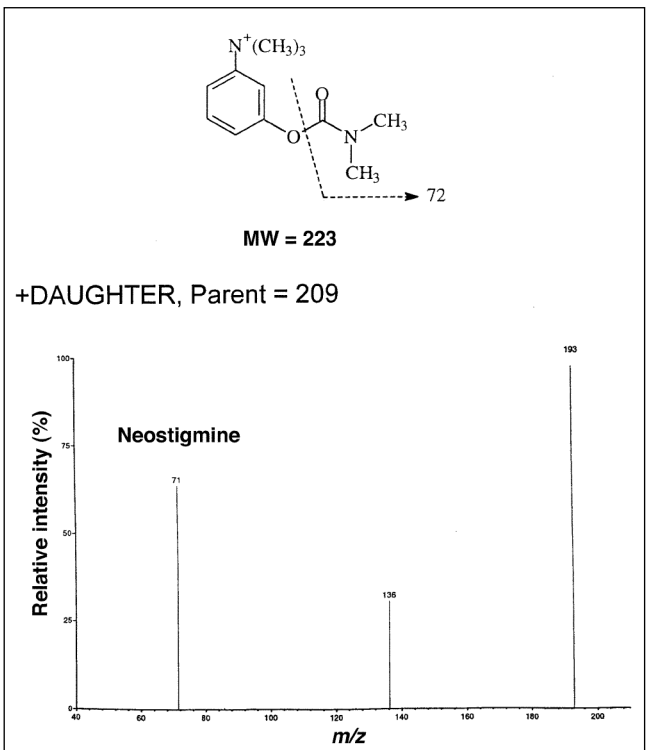

Figure 2. Daughter ion spectra and chemical structures of neostigmine (the internal standard) using the Sciex APCI mode.

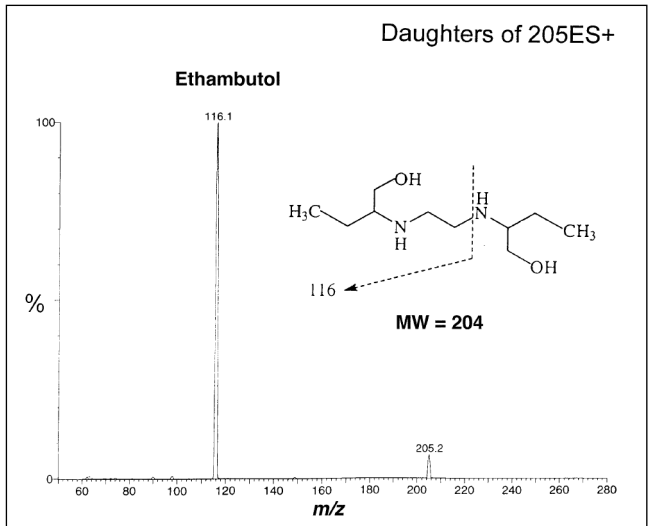

Figure 3. Daughter ion spectra and chemical structures of ethambutol using the Micromass Quattro LC electrospray mode.

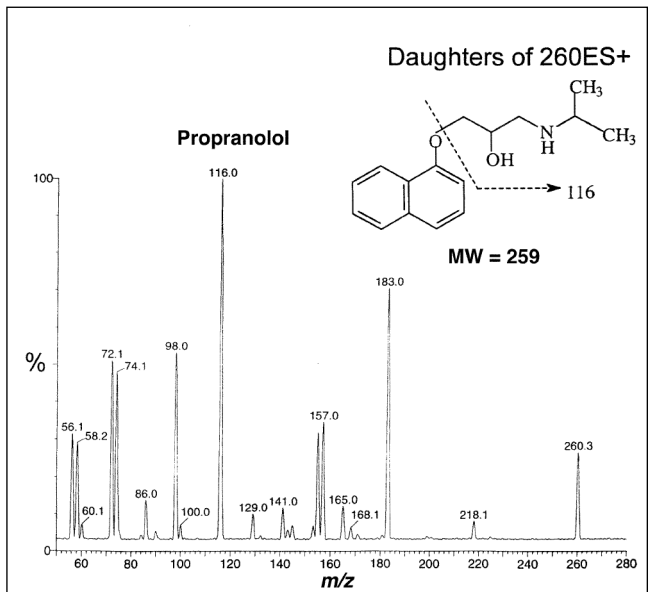

Figure 4. Daughter ion spectra and chemical structures of propranolol (the internal standard) using the Micromass Quattro LC electrospray mode.

carried out with a PE Sciex API III.

The MS used the following settings and conditions. The multiple reaction monitor scanning mode was set at *m/z* 205–116 for ethambutol and *m/z* 209–71 for neostigmine (Figures 1 and 2). Atmospheric pressure chemical ionization (APCI)–positive ionization was used. The sample inlet used a heated nebulizer at 450°C. The discharge current was +3 µA. The gas curtain flow was 1.2 L/min (N_2 = 99.999%). The nebulizer pressure was 551.4 kPa. The collision gas consisted of a 9.99% nitrogen–90.01% argon mixture (set at 250 × 10^{12} molecules/cm^2). Peak detection for the ACs and some plasma and BAL specimens was carried out on a Micromass Quattro LC. For these specimens the reaction channel was *m/z* 205.35–116.10 for ethambutol and *m/z* 260.18–115.95 for propranolol (Figures 3 and 4). Electrospray–positive ionization with a flow rate of 0.2 mL (5-to-1 split ratio of 1.0 mL/min) to the Micromass system was used. The sample inlet used a heated nebulizer. The sample cone was set to 25 V for ethambutol and 35 V for propranolol. The energy collision was set to 15.0 eV for both ethambutol and propranolol. A Macintosh Quadra 800 computer (Apple Computers, Cupertino, CA) was used for peak integration and analysis.

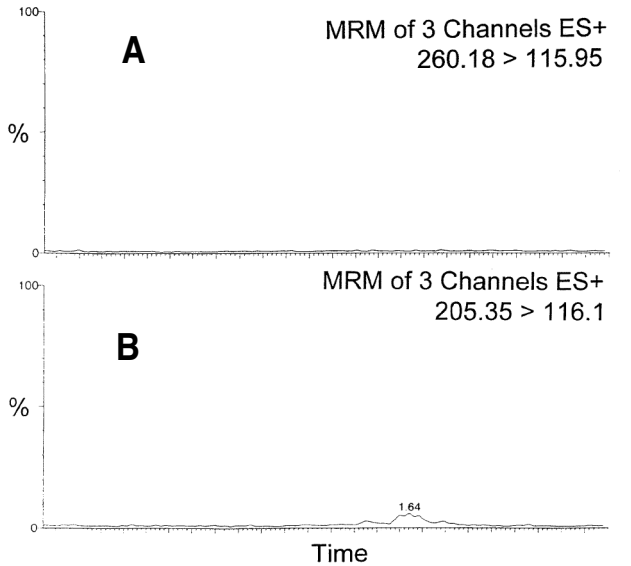

Figure 5. Chromatograms of blank plasma: (A) the internal standard and (B) ethambutol.

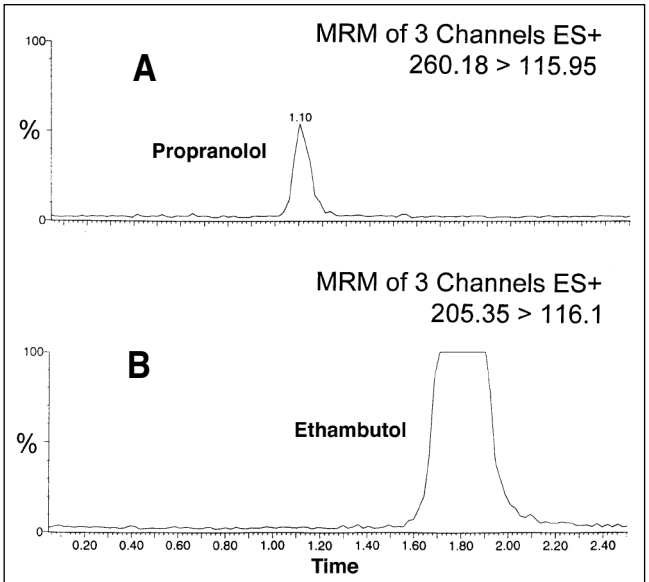

Figure 6. Chromatograms of a study subject's plasma obtained 4 h after the fifth dose of 15 mg/kg ethambutol administered once a day: (A) the internal standard and (B) ethambutol. The ethambutol concentration was 0.734 µg/mL.

Sample preparation

Standard curves

Plasma standard curves were prepared by adding appropriate volumes of ethambutol working stock solutions into 0.2 mL of blank plasma to yield the concentrations of 0.05, 0.10, 0.20, 0.40, 0.80, 1.2, 1.6, and 2.4 µg/mL of ethambutol. The standards for BAL supernatants were spiked to yield concentrations of 0.005, 0.010, 0.020, 0.040, 0.080, 0.160, 0.320, and 0.640 µg/mL of ethambutol. The AC suspension standards were spiked to yield concentrations of 0.005, 0.010, 0.020, 0.040, 0.100, 0.400, 0.800, 1.600, and 2.000 µg/mL ethambutol. Standard curves were constructed by plotting a 1/y weighted least-squares linear regression of ethambutol to the internal standard peak-area ratios versus the spiked concentration of ethambutol.

Preparation of plasma standards and samples

In order to ensure consistency of recovery, 200 µL of acetonitrile containing 0.050 µg/mL neostigmine or propranolol as the internal standard was added to 0.2 mL plasma standards and samples. After vortexing, an additional 0.2 mL of the internal standard solution was added. After vortexing and then centrifuging for 5 min at 1800 × g, the solvent phase was transferred to a 400-µL microfuge tube, and 2.0 µL were injected onto the HPLC system.

Preparation of BAL supernatants and AC pellet standards and samples

A cell count and differential was performed on the BAL lavage fluid, then a 30-mL aliquot was centrifuged at 400 × g for 5 min and the supernatant immediately separated from the cells. BAL supernatant standards and samples were prepared by adding 0.5 mL of the internal standard solution (0.015 µg/mL neostigmine or 0.150 µg/mL propranolol) to 0.25 mL of the sample, vortexing, and then centrifuging for 5 min at 1800 × g. The solvent phase was transferred to a 400-µL microfuge tube, and 2.0 µL were injected onto the HPLC system.

ACs were resuspended volumetrically in deionized water and sonicated for 2 min on a Fisher 550 dismembrator (Fisher Scientific, Santa Clara, CA) to lyse the cells. A 250-µL volume of the internal standard (0.300 µg/mL propranolol) was added to 250 µL of an AC cell suspension and vortexed. A 250-µL volume of acetonitrile was added and mixed by vortexing. Following centrifugation for 5 min at 1800 × g, 2 µL of the solvent phase was injected onto the HPLC system.

Preparation of controls for method validation

Two sets of stock solutions were prepared; one was used for spiking standards and the other for spiking controls. Measured amounts of plasma were spiked at 0.15, 0.4, 0.8, and 1.4 µg/mL; aliquoted; and frozen at –70°C for stability studies. Aliquots were

analyzed in duplicate weekly over a period of six weeks. In order to assess interday reproducibility, standard curves with controls spiked at concentrations of 0.1, 0.3, 1.2, and 2.4 µg/mL were analyzed on five different days. Intraday reproducibility was assessed by analyzing six preparations of each of the four concentrations on the same day. The validation for BAL supernatants was carried out in the same time frames as for plasma, with controls spiked at concentrations of 0.015, 0.04, 0.16, and 0.24 µg/mL. The validation for ACs was performed at concentrations of 0.010, 0.40, and 1.60 µg/mL.

Statistics

The statistical analysis was performed using the PROPHET Computer Resource (12). Linearity (r^2), precision (coefficient of variation, CV), recovery (relation of test result to the true concentration) (13), and percentage accuracy (14) were calculated. The detection limit was defined as the lowest point of the standard curve. Drug concentrations in epithelial lining fluid (ELF) were calculated using the urea diffusion method, and AC concentrations were calculated using cell counts in alveolar fluid as we have previously reported (15–17).

Results and Discussion

Linearity, assay precision, recovery, and accuracy assessments

HPLC–MS–MS chromatograms of ethambutol and the internal standard in plasma, BAL supernatant, and AC suspension are shown in Figures 5–10. The detection limits for ethambutol were 0.05 µg/mL for plasma and 0.005 µg/mL for the BAL supernatants

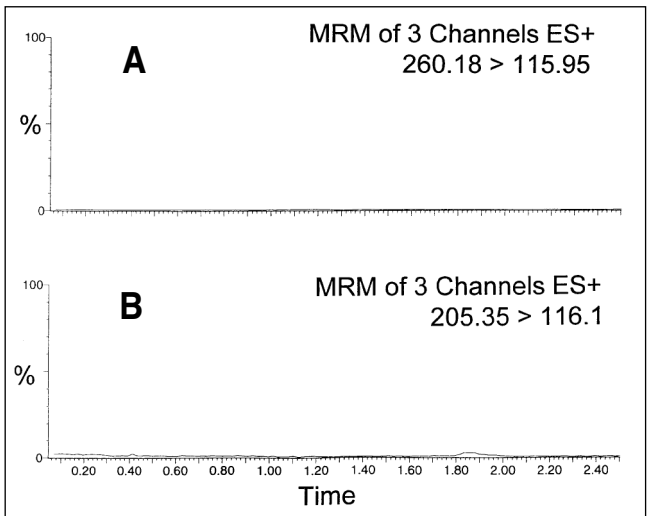

Figure 7. Chromatograms of blank BAL supernatant: (A) the internal standard and (B) ethambutol.

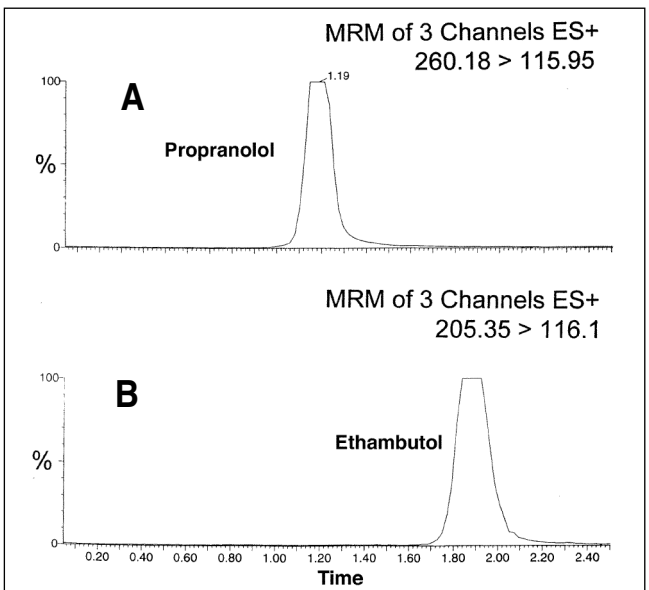

Figure 8. Chromatograms of a study subject's BAL supernatant obtained 4 h after the fifth dose of 15 mg/kg ethambutol administered once a day: (A) the internal standard and (B) ethambutol. The ethambutol concentration was 0.053 µg/mL.

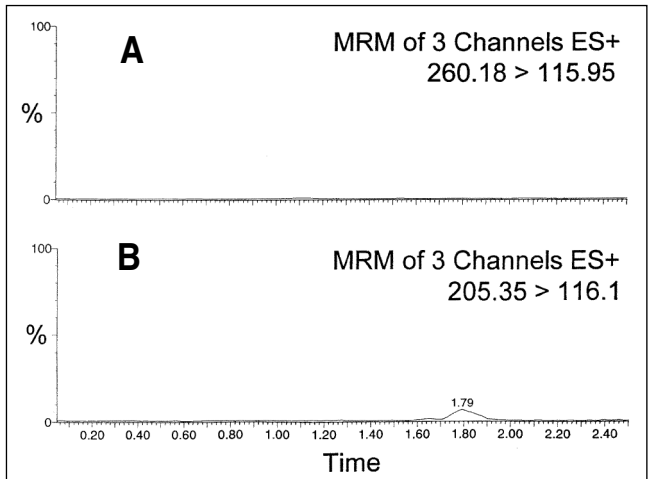

Figure 9. Chromatograms of blank AC suspension: (A) the internal standard and (B) ethambutol.

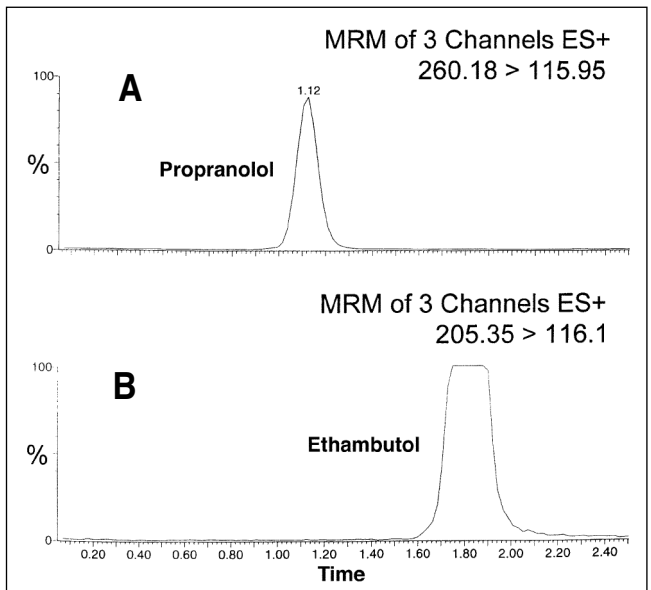

Figure 10. Chromatograms of a study subject's AC suspension obtained 4 h after the fifth dose of 15 mg/kg ethambutol administered once a day: (A) the internal standard and (B) ethambutol. The ethambutol concentration was 0.316 µg/mL.

and AC suspensions. The detection limit referred to the lowest point of the standard curve and was at least five times the noise level. The mean ± SD of the r^2 from 24 standard curves (8 in plasma, 8 in BAL, and 8 in ACs) was 0.9941 ± 0.0060. Results for the assay precision, recovery, and accuracy assessments in the plasma, BAL, and AC suspensions are summarized in Tables I–III.

CV

The mean (± SD) CVs and the ranges of the assay for intraday and interday determinations together for plasma, BAL supernatants, and ACs were 7.81% ± 2.02% (ranging from 3.9% to 10.14%), 6.46% ± 3.69% (ranging from 1.42% to 11.42%), and 12.67% ± 4.59% (ranging from 6.0% to 20.0%), respectively (Tables I–III).

The mean (± SD) recoveries and the ranges of the assays for intraday and interday determinations together in plasma, BAL supernatants, and ACs were 105.91% ± 7.73% (ranging from 93.3% to 119.0%), 95.94% ± 10.43% (ranging from 80.0% to 106.88%), and 105.48% ± 3.60% (ranging from 100.00% to 110.00%), respectively (Tables I–III). The accuracy ranges for all of the determinations in plasma, BAL supernatants, and ACs were –6.67% to 19.0%, –20.0% to 6.88%, and 0.0% to 10.0%, respectively (Tables I–III).

Table I. Assay Precision, Recovery, and Accuracy for Ethambutol Determination in Plasma

Spiked concentration (µg/mL)	Measured concentration (mean ± SD) (µg/mL)	CV (%)	Recovery* (%)	Accuracy† (%)
Intraday‡ (*n* = 6)				
2.4	2.24 ± 0.227	10.1	93.33	–6.67
1.2	1.30 ± 0.111	8.6	108.33	8.33
0.3	0.32 ± 0.012	6.1	106.67	6.67
0.1	0.12 ± 0.011	9.2	119.00	19.00
Interday§ (*n* = 10)				
2.4	2.35 ± 0.168	7.2	97.92	–2.08
1.2	1.26 ± 0.114	9.1	105.00	5.00
0.3	0.33 ± 0.013	3.9	110.00	10.00
0.1	0.107 ± 0.009	8.3	107.00	7.00

* Measured/spiked × 100%.
† (Measured – spiked)/spiked × 100%.
‡ Six separately spiked samples at each of four concentrations.
§ Plasma spiked at four concentrations and analyzed in duplicate on five different days.

Table II. Assay Precision, Recovery, and Accuracy for Ethambutol Determination in BAL Supernatant

Spiked concentration (µg/mL)	Measured concentration (mean ± SD) (µg/mL)	CV (%)	Recovery* (%)	Accuracy† (%)
Intraday‡ (*n* = 6)				
0.240	0.248 ± 0.004	1.4	103.33	3.33
0.160	0.171 ± 0.004	2.2	106.88	6.88
0.040	0.040 ± 0.002	6.2	100.00	0.00
0.015	0.012 ± 0.001	4.4	80.00	–20.0
Interday§ (*n* = 12)				
0.240	0.238 ± 0.023	9.9	99.17	–0.83
0.160	0.165 ± 0.011	6.4	103.13	3.13
0.040	0.038 ± 0.004	11.4	95.00	–5.0
0.015	0.012 ± 0.001	9.8	80.00	–20.0

* Measured/spiked × 100%.
† (Measured – spiked)/spiked × 100%.
‡ Six separately spiked samples at each of four concentrations.
§ Plasma spiked at four concentrations and analyzed in duplicate on six different days.

Stability

The results of repeated determinations of ethambutol in spiked plasma, BAL supernatants, and ACs stored at –70°C revealed no significant degradation of the drug. These determinations were performed over a period of 4 mo for plasma, 7 weeks for BAL

Table III. Assay Precision, Recovery, and Accuracy for Ethambutol Determination in Alveolar Cells

Spiked concentration (µg/mL)	Measured concentration (mean ± SD) (µg/mL)	CV (%)	Recovery* (%)	Accuracy† (%)
Intraday‡ (*n* = 6)				
1.600	1.643 ± 0.099	6.0	102.69	2.69
0.400	0.423 ± 0.053	12.6	105.75	5.75
0.010	0.010 ± 0.001	14.5	100.00	0.00
Interday§ (*n* = 10)				
1.600	1.707 ± 0.207	12.1	106.69	6.69
0.400	0.431 ± 0.047	10.8	107.75	7.75
0.010	0.011 ± 0.002	20.0	110.00	10.00

* Measured/spiked × 100%.
† (Measure – spiked)/spiked × 100%.
‡ Six separately spiked samples at each of three concentrations.
§ Plasma spiked at three concentrations and analyzed in duplicate on five different days.

Table IV. Ethambutol Concentrations* in Plasma, ELF, and AC in Five Adult Volunteer Subjects

Sample	Subject #1	Subject #2	Subject #3	Subject #4	Subject #5
Plasma (2 h after fifth dose†)	3.41	1.79	1.15	1.75	0.86
Plasma (4 h after fifth dose)	4.99	1.15	2.11	1.90	2.39
ELF‡ (4 h after fifth dose)	3.61	1.14	3.05	1.80	2.51
AC§ (4 h after fifth dose)	64.82	18.92	59.98	108.9	35.42

* All concentrations are given in micrograms per milliliter.
† A single oral daily dose of 15 mg/kg was given for 5 days.
‡ The amount of ELF collected in the BAL fluid was calculated from the urea concentration in BAL and serum, as previously reported (15–17).
§ The concentration of ethambutol in ACs is given as micrograms per milliliter of cell volume and was calculated as previously reported (15–17).

supernatant, and 9 mo for ACs (data not shown). The mean (± SD) CV of the stability studies at four concentrations in plasma and BAL supernatant were 8.38% and 7.36%, respectively. Repeat analyses of BAL pellets from four study subjects resulted in a mean (± SD) CV of 0.10%.

Patient data

The concentrations of ethambutol in plasma, BAL supernatant, and ACs in five of forty subjects who participated in an NIH-supported study of the intrapulmonary pharmacokinetics of ethambutol are summarized in Table IV. Bronchoscopy and BAL were performed, and blood was drawn at 4 h following the last dose of a 5-day course of 15 mg/kg ethambutol. Blood samples were also obtained 2 h after the last dose. From this preliminary analysis, it can be seen that ethambutol concentrations in plasma ranged from 0.86 to 3.41 µg/mL at 2 h and 1.15 to 4.99 µg/mL at 4 h after the last dose was administered. The concentrations in ELF ranged from 1.14 to 3.61 µg/mL and in AC ranged from 18.92 to 108.9 µg/mL.

Conclusion

We have developed a sensitive HPLC–MS–MS assay that provides specific, rapid, and reliable determinations for ethambutol in small volumes of plasma, BAL, and AC. The preparation of plasma, BAL supernatant, and AC samples requires a deproteinization step. The stability data indicated that no significant drug degradation occurred in plasma, BAL supernatant, or ACs stored at –70°C over a period of 4 mo, 6 weeks, and 9 mo, respectively. The linearity of the standard curve in the range described was excellent. Assay precision was high for plasma, BAL, and ACs. The performance characteristics of this assay make the method suitable for clinical and pharmacological studies, particularly those that are designed to quantitate the intrapulmonary concentration of drugs.

This method is currently being used to support phase-one studies of the pulmonary pharmacokinetics of ethambutol in patients with tuberculosis and normal volunteers. In this preliminary analysis, ethambutol concentrations in plasma and ELF appear to be similar (i.e., the drug diffuses passively from plasma into ELF). Ethambutol concentrations are considerably greater in the AC than in plasma or ELF, indicating that ethambutol is concentrated in ACs. This finding may be of importance in the treatment of tuberculosis, which is an intracellular infection. A complete analysis of this pharmacokinetic study will be published elsewhere.

Acknowledgments

This work was carried out with funds provided by NIH Grant #AI36054 and NIH Grant #5 MO1 RR-00079 (General Clinical Research Center) at the University of California, San Francisco.

The authors would like to thank Ganfeng Wong for assay development, Margareta Andersson for performing the assays, and Eve Benton for manuscript preparation.

References

1. J.B. Bass, Jr., L.S. Farer, P.C. Hopewell, R. O'Brien, R.F. Jacobs, F. Ruben, D.E. Snider, Jr., and G. Thornton. Treatment of tuberculosis and tuberculosis infection in adults and children. American Thoracic Society and The Centers for Disease Control and Prevention. *Am. J. Respir. Crit. Care Med.* **149:** 1359–74 (1994).
2. C.A. Peloquin, A.E. Bulpitt, G.S. Jaresko, R.W. Jeliffe, J.M. Childs, and D.E. Nix. Pharmacokinetics of ethambutol under fasting conditions, with food, and with antacids. *Antimicrob. Agents Chemother.* **43:** 568–72 (1999).
3. C.S. Lee, J.G. Gambertoglio, D.C. Brater, and L.Z. Benet. Kinetics of oral ethambutol in the normal subject. *Clin. Pharmacol. Ther.* **22:** 615–21 (1977).
4. C.N. Lee and L.B. Heifets. Determination of minimal inhibitory concentrations of antituberculosis drugs by radiometric and conventional methods. *Am. Rev. Respir. Dis.* **136:** 346–52 (1987).
5. P.R. Gangadharam and E.R. Candler. Microbiological assay of ethambutol. *J. Antimicrob. Chemother.* **3:** 57–63 (1977).
6. T.L. Ng. A gas chromatographic/mass spectrometric study of the trimethylsilylation of ethambutol and a tablets assay method based on the trimethylsilyl derivative. *J. Chromatogr. Sci.* **20:** 479–82 (1982).
7. C.S. Lee and L.Z. Benet. Gas–liquid chromatographic determination of ethambutol in plasma and urine of man and monkey. *J. Chromatogr.* **128:** 188–92 (1976).
8. C.S. Lee and L.Z. Benet. Micro and macro GLC determination of ethambutol in biological fluids. *J. Pharm. Sci.* **67:** 470–73 (1978).
9. C.S. Lee, D.C. Brater, J.G. Gambertoglio, and L.Z. Benet. Disposition kinetics of ethambutol in man. *J. Pharmacokinet. Biopharm.* **8:** 335–46 (1980).
10. M.M. Chen, C.S. Lee, and J.H. Perrin. Absorption and disposition of ethambutol in rabbits. *J. Pharm. Sci.* **73:** 1053–55 (1984).
11. M. Breda, P. Marrari, E. Pianezzola, and B.M. Strolin. Determination of ethambutol in human plasma and urine by high-performance liquid chromatography with fluorescene detection. *J. Chromatogr. A* **729:** 301–307 (1996).
12. *Prophet.* Abtech Corporation, Charlottesville, VA, 1999.
13. S.A. Signs, T.M. File, and J.S. Tan. *Antimicrob. Agents Chemother.* **26:** 652–55 (1984).
14. R.V. Smith and J.T. Stewart. *Textbook of Biopharmaceutic Analysis.* Lea and Febiger, Philadelphia, PA, pp. 79–94, 1981.
15. J.E. Conte, Jr., J.A. Golden, S. Duncan, E. McKenna, E. Lin, and E. Zurlinden. Single-dose intrapulmonary pharmacokinetics of azithromycin, clarithromycin, ciprofloxacin, and cefuroxime in volunteer subjects. *Antimicrob. Agents Chemother.* **40:** 1617–22 (1996).
16. J.E. Conte, Jr., J.A. Golden, S. Duncan, E. McKenna, and E. Zurlinden. Intrapulmonary pharmacokinetics of clarithromycin and of erythromycin. *Antimicrob. Agents Chemother.* **39:** 334–38 (1995).
17. J.E. Conte, Jr., J.A. Golden, S. Duncan, E. McKenna, and E. Zurlinden. Intrapulmonary concentrations of pyrazinamide. *Antimicrob. Agents Chemother.* **43:** 1329–33 (1999).

Manuscript accepted December 7, 2001.