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High-performance liquid chromatographic–tandem mass spectrometric method for the determination of ethionamide in human plasma, bronchoalveolar lavage fluid and alveolar cells

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Abstract

We have developed and validated an accurate, sensitive, and rapid high-performance liquid chromatographic–tandem mass spectrometric method (HPLC–MS–MS) for the determination of ethionamide in plasma, bronchoalveolar fluid (BAL) and alveolar cells (AC). The retention times for ethionamide, clemastine fumarate (internal standard for plasma), promethazine (internal standard for plasma) and propranolol (internal standard for BAL and AC) were approximately 2.62, 1.21, 2.14, and 2.22 min, respectively, with a total run time of 3.2 min. Ethionamide detection for plasma was carried out on a PE Sciex API III (Perkin-Elmer, Foster City, CA, USA). BAL and cell pellets and some plasma specimens were analyzed on a Micromass Quattro LC (Micromass Co., Manchester, UK). The detection limits for ethionamide were 0.05 μg/ml for plasma, and 0.005 μg/ml for BAL supernatants and alveolar cell suspensions.

Keywords: Ethionamide

1. Introduction

Ethionamide is an important second-line drug in the treatment of tuberculosis. It has bacteriostatic and bacteriocidal activity against *M. tuberculosis*, including multiple-drug resistant strains (MDRTB) [1–3]. Ethionamide is 2-ethylthioisonicotinamide; it is structurally related to isoniazid, an important first-line antituberculous agent. Peak plasma concentrations (C_{max}) occur at approximately 2 h following drug administration and are 0.6–1.9 μg/ml following an oral dose of 250 mg [4,5] and 2.2 μg/ml following an oral dose of 500 mg [6]. The elimination half-life ($T_{1/2}$) is 2–3 h [5,6]. Approximately 1% of the dose can be recovered in urine as the primary metabolite, ethionamide sulphoxide, and a number of other metabolites have been identified [5]. Ethionamide sulphoxide has antimycobacterial ac-

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tivity; however, the contribution of the metabolites to the clinical effectiveness of ethionamide has not been studied and is unknown.

Earlier studies used a bioassay [4,7] while more recent investigations have used HPLC [8–10]. The HPLC techniques have been specific and sensitive and can detect 0.5 μg/ml [6] or 0.05 μg/ml [8] of ethionamide in plasma.

We have developed a new HPLC–MS–MS procedure to measure drug concentrations in plasma, bronchoalveolar fluid (BAL) and alveolar cells (AC). Compared with other methods, the technique has the advantages of increased sensitivity (0.05 μg/ml in plasma and 0.005 μg/ml in BAL) 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 is being used to support a phase one study of the intrapulmonary pharmacokinetics of ethionamide in normal subjects and subjects with AIDS.

2. Experimental

2.1. Chemicals

All solvents and chemicals were HPLC-grade except ammonium acetate which was certified. A 1.0 mg/ml solution of ethionamide (Sigma, St. Louis, MO, USA) 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 ethionamide. Clemastine fumarate (Watson Laboratories, Corona, CA, USA), promethazine HCl (Sigma) and propranolol (USP Reference, Rockville, MD, USA) stock solutions, 1.0 mg/ml, were prepared in 50% methanol then diluted to the appropriate concentrations for each assay in 100% acetonitrile for use as internal standards.

2.2. Instrumental

2.2.1. Chromatography

Mobile phase containing 90% acetonitrile and 0.06% trifluoroacetic acid was run through a hypersil silica column (50 mm×4.6 mm I.D., particle size 5 μm at a flow-rate of 1.0 ml/min) utilizing a Shimadzu LC-10 AD pump (Shimadzu, Columbia, MD, USA). Extracts from samples were injected onto the system with a Waters intelligent sample processor 717 Plus (Waters, Milford, MA, USA). The retention times for ethionamide, clemastine fumarate, promethazine and propranolol were approximately 2.62, 1.21, 2.14 and 2.22 min, respectively, with a total run time of 3.2 min.

2.2.2. Mass spectrometry

We utilized two different mass spectrometry systems during the development and validation of this assay to explore different types of mass spectrometric equipment. Clemastine fumarate was used as internal standard for plasma assays which were run on the PE Sciex API III HPLC–MS–MS system. Promethazine hydrochloride was used as internal standard for plasma assay, and propranolol was used for BAL supernatant and alveolar cell pellets which were run on the Micromass Quattro LC. Peak detection and area determinations for plasma samples were made with a PE Sciex API III (Perkin-Elmer, Foster City, CA, USA). The mass spectrometry utilized the following settings and conditions. The multiple reaction monitor scanning mode was set at 167–139 m/z for ethionamide and 244–215 m/z for clemastine fumarate (Fig. 1); atmospheric pressure chemical ionization (APCI)/positive ionization; the sample inlet used a heated nebulizer at 450°C; the discharge current was set to +3 μA; the gas curtain flow-rate 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 and 90.01% argon mixture (set at $250 \cdot 10^{12}$ molecules/cm^2). Peak integration and analysis was performed on a Macintosh Quadra 800 computer. BAL and cell pellets and some plasma specimens were analyzed on a Micromass Quattro LC (Micromass Co., Manchester, UK). For these specimens the reaction channel was 166.77–106.79 m/z for ethionamide, 260.18–115.95 m/z for propranolol, and 285.7–86.04 m/z for promethazine (internal standard for plasma) (Fig. 2); electrospray/positive ionization was used with a flow-rate of 0.2 ml/min (5:1 ratio split of 1.0 ml/min) to the Micromass system; the sample inlet utilized a heated nebulizer; the sample cone was set to 35 V; the

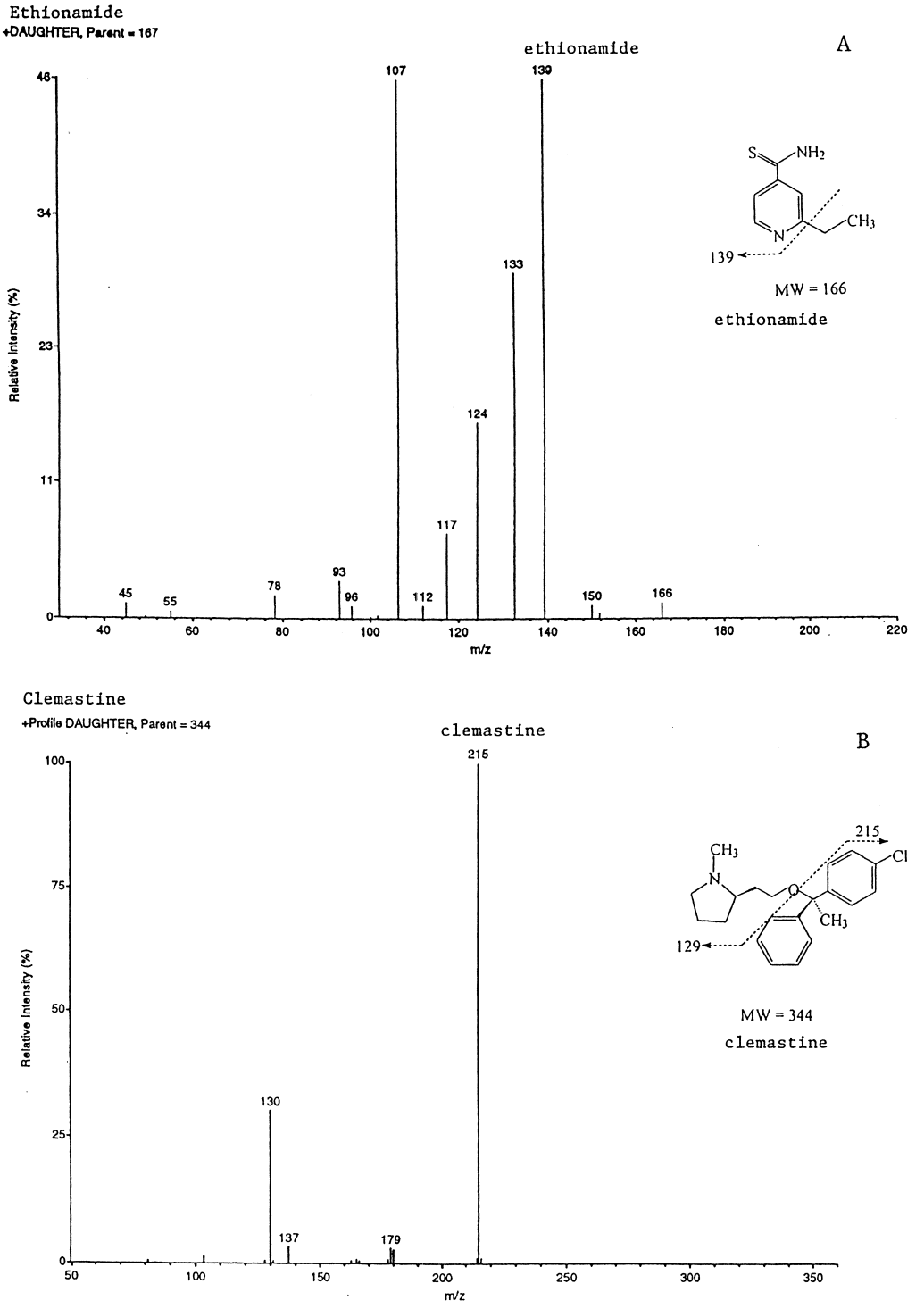

Fig. 1. Daughter ion spectra and chemical structures of (a) ethionamide, (b) clemastine (IS), using Sciex APCI mode.

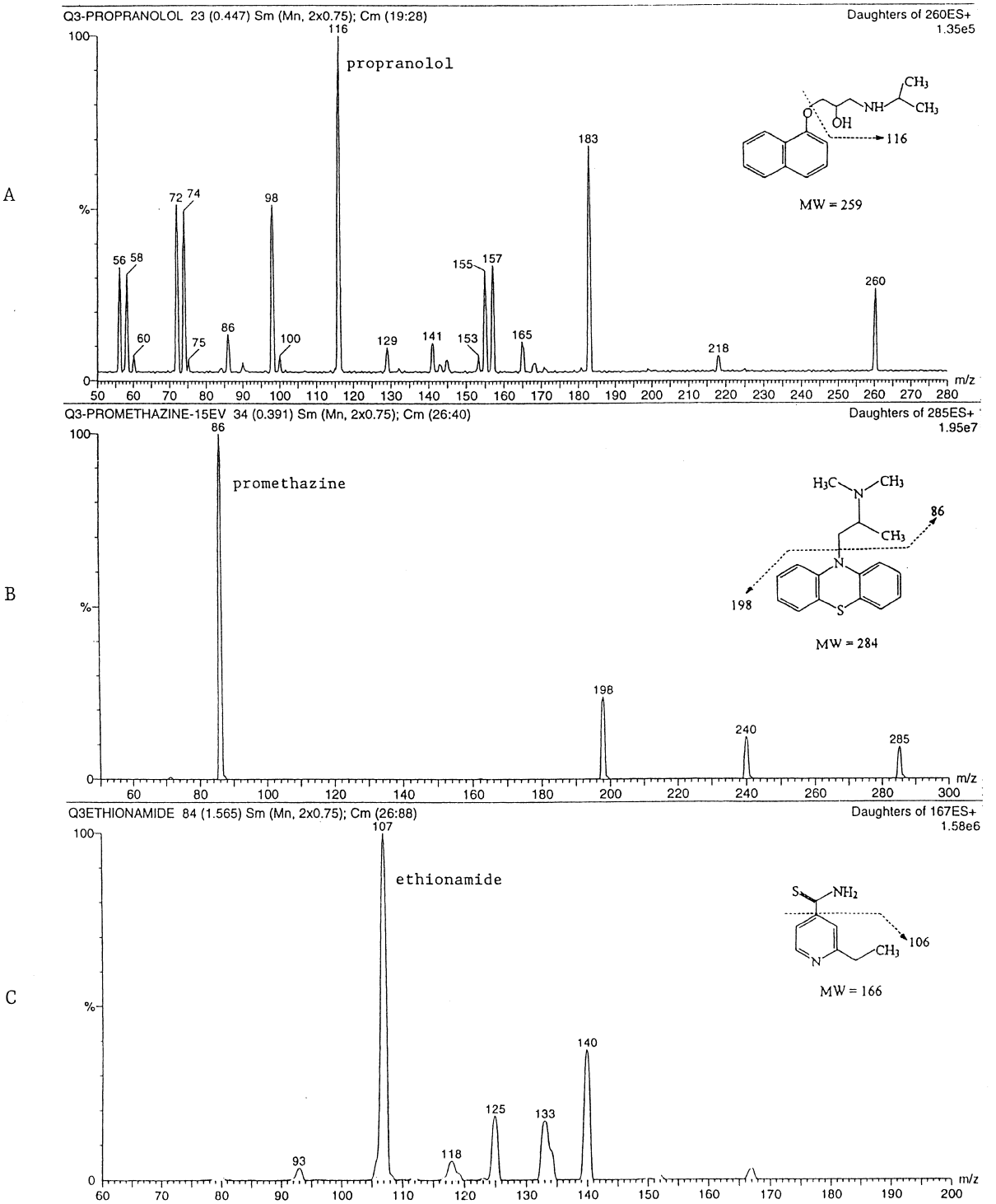

Fig. 2. Daughter ion spectra and chemical structures of (a) propranolol (IS), (b) promethazine (IS), (c) ethionamide, using Micromass Quatro LC electrospray mode.

collision energy was set to 25.0 eV (ethionamide), 15 eV (propranolol), and 28 eV (promethazine); the collision gas consisted of 100% argon with the gas cell set at 2.0e−3 mbar.

2.3. Sample preparation

2.3.1. Standard curves

Plasma standard curves were prepared by adding appropriate volumes of ethionamide working stock solutions to 0.2 ml of blank plasma to yield the following concentrations: 0.05, 0.10, 0.20, 0.40, 0.80, 1.2, 1.6 and 2.4 μg/ml of ethionamide. For BAL supernatants and alveolar cell suspensions, 0.2 ml aliquots were spiked to yield the following concentrations: 0.005, 0.010, 0.020, 0.040, 0.080, 0.160, 0.320 and 0.640 μg/ml of ethionamide. Standard curves were constructed by $1/y$ weighted least squares linear regression of ethionamide to internal standard (IS) peak area ratios versus the spiked concentration of ethionamide.

2.3.2. Preparation of plasma standards and samples

Two hundred microliters of acetonitrile containing internal standard was twice added to 0.200 ml of plasma standards and samples to ensure the consistency of recovery. After vortexing and centrifuging for 5 min at 1800 *g*, the solvent phase was transferred to a 400 μl microfuge tube. Twelve microliters were injected onto the HPLC column.

2.3.3. Preparation of BAL supernatants and alveolar cell 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 0.200 ml of human plasma to 0.200 ml of BAL supernatant (standards were then spiked with ethionamide) and 0.5 ml of internal standard solution (150 ng/ml propranolol in CH_3CN). After vortexing, another 0.5 ml of internal standard solution was added. The preparations were then centrifuged 5 min at 1800 *g* and the solvent phase was transferred to a 400 μl microfuge tube. Twelve microliters were injected onto the HPLC system.

Alveolar cells were suspended in a measured volume of deionized water and the cells lysed on a Fisher 550 dismembrator. Standard curves and controls were prepared in a suspension of monocyte enriched human leukocytes (Biological Specialty Corporation, Colmar, PA, USA) which were further purified through ficoll-hypaque separation and sonicated. The samples were prepared as for BAL supernatants, but without the addition of human plasma.

2.3.4. Preparation of controls for method validation

Two sets of stock solutions were prepared, one used for spiking standards, 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 6 weeks. To assess inter-day reproducibility, standard curves with spiked controls were analyzed on 5 different days. Intra-day reproducibility was assessed by analyzing six preparations of each of four concentrations on the same day. Validation for BAL supernatants was carried out in the same time frames as for plasma, with controls spiked at the following concentrations: 0.320, 0.040, and 0.010 μg/ml. Controls for the cell pellets were spiked at 1.6, 0.400 and 0.010 μg/ml.

2.4. Statistics

The statistical analysis was performed using the PROPHET Computer Resource [11]. Linearity (r^2), precision (coefficient of variation), recovery (relation of test result to the true concentration) [12] and percentage accuracy [13] were calculated. Detection limit was defined as the lowest point of the standard curve. Drug concentrations in epithelial lining fluid were calculated using the urea diffusion method and alveolar cell concentrations were calculated using cell counts in alveolar fluid as we have previously reported [14–16].

3. Results

3.1. Linearity, assay precision, recovery and accuracy assessments

HPLC–MS–MS chromatograms of ethionamide and internal standard in plasma, BAL supernatant, and alveolar cell suspension are shown in Figs. 3–5. The detection limits for ethionamide were 0.05 μg/ml for plasma, and 0.005 μg/ml for BAL supernatants and alveolar cell suspensions. The mean±standard deviation (SD) of the r^2 from 19 standard curves (five in plasma, six in BAL supernatants, and eight in BAL pellets) was 0.9938±0.0084. Results for assay precision, recovery and accuracy assessments in plasma, BAL,

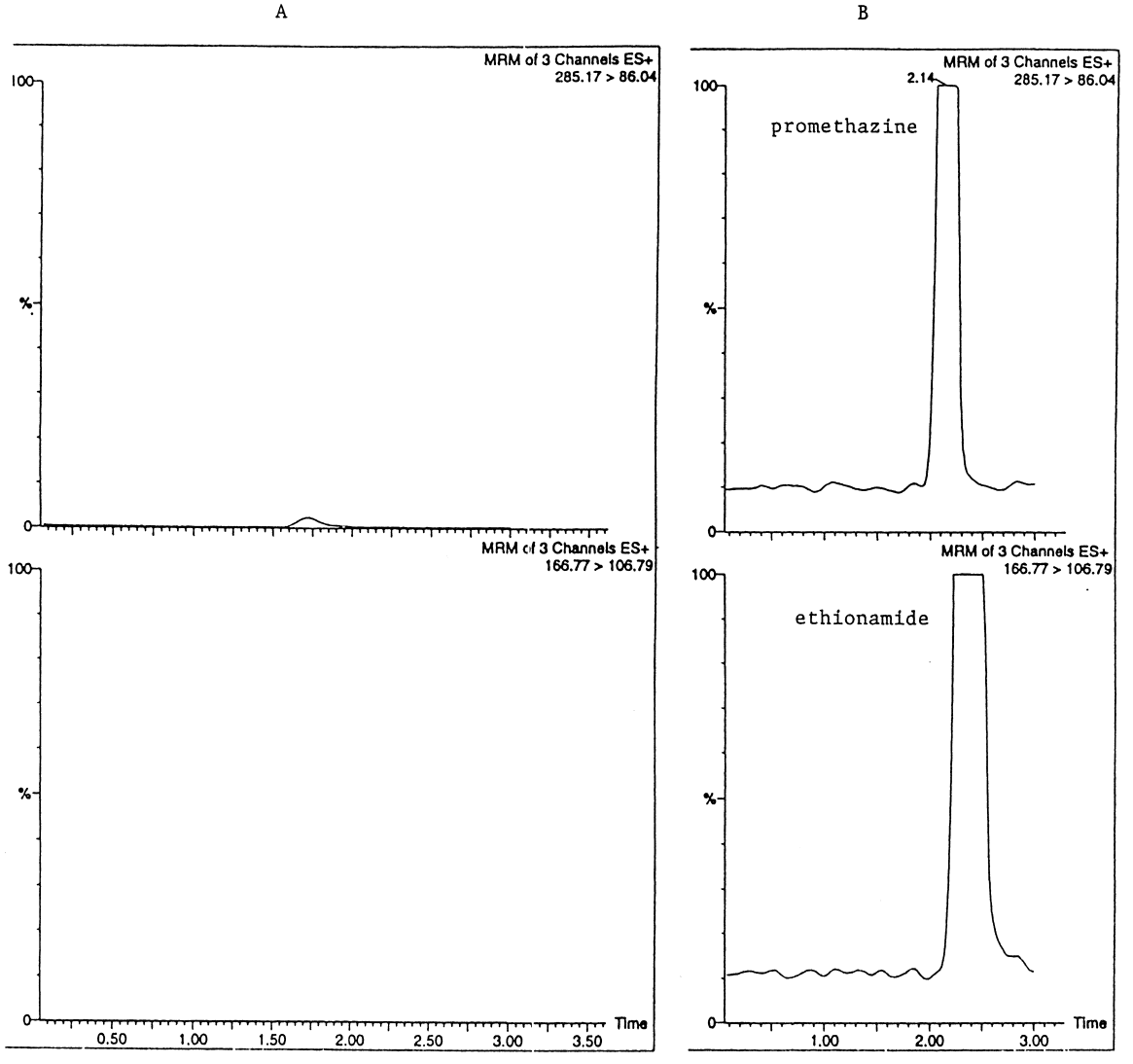

Fig. 3. Chromatograms of (a) blank plasma, (b) study subject's plasma obtained 4 h after the ninth dose of ethionamide of 250 mg, administered every 12 h. The ethionamide concentration is 0.250 μg/ml. In each graphic, the internal standard and ethionamide are presented in the upper and lower panels, respectively.

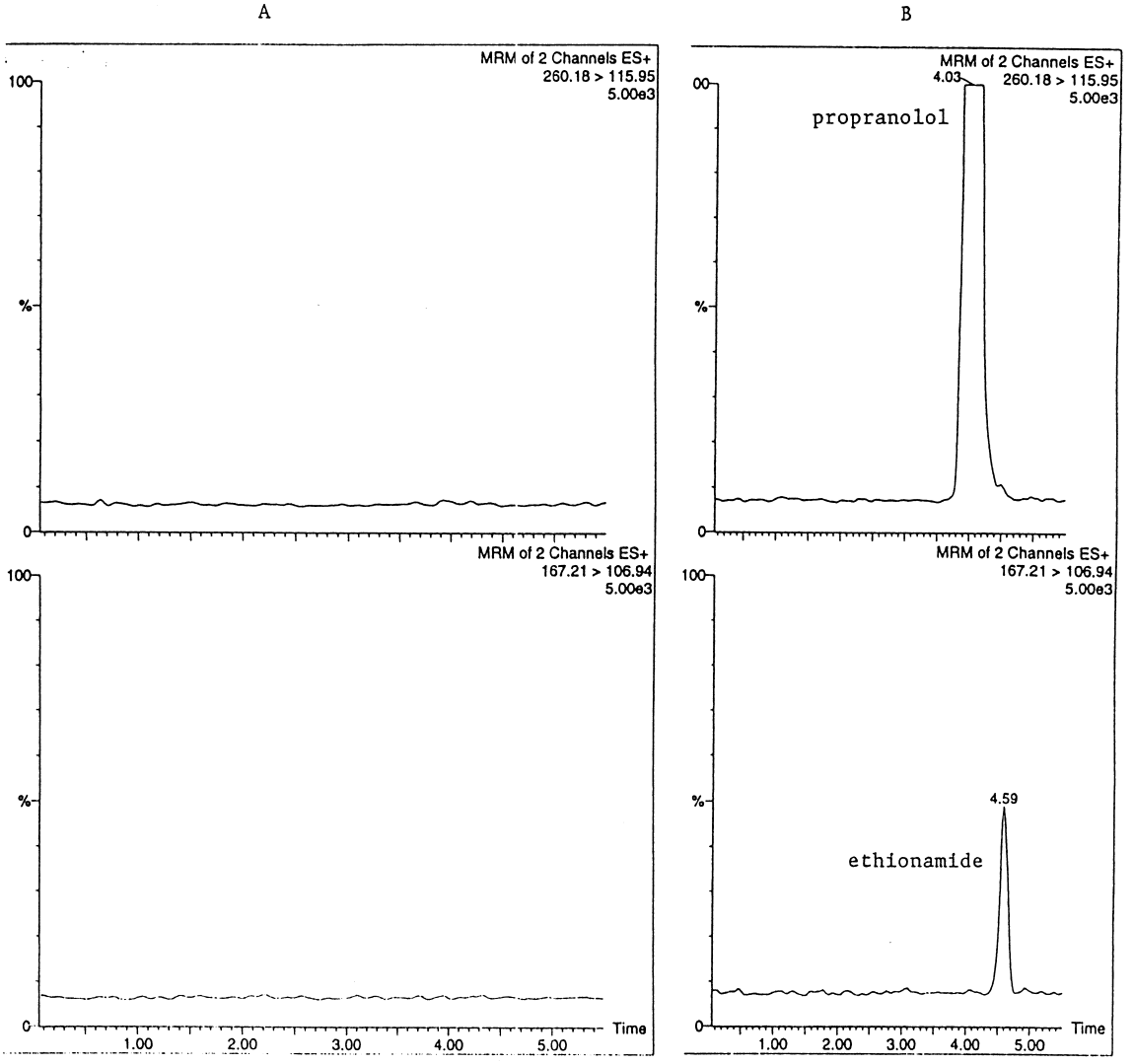

Fig. 4. Chromatograms of (a) blank BAL supernatant, (b) study subject's BAL supernatant obtained 4 h after the ninth dose of ethionamide of 250 mg, administered every 12 h. The ethionamide concentration is 0.060 μg/ml. In each graphic, the internal standard and ethionamide are presented in the upper and lower panels, respectively.

and alveolar cell suspensions are summarized in Tables 1–3.

3.2. Coefficients of variation

The mean (±SD) coefficients of variation and ranges of the assay for intra-day and inter-day determinations together, for plasma, BAL supernatants and alveolar cells were 9.66±2.67% (range 5.0–12.47%), 7.65±4.78% (range 0.09–11.8%) and 7.73±3.94% (range 3.40–13.64%), respectively (Tables 1–3).

The mean (±SD) recoveries and ranges of the assay for intra-day and inter-day determinations together, in plasma, BAL supernatants and alveolar

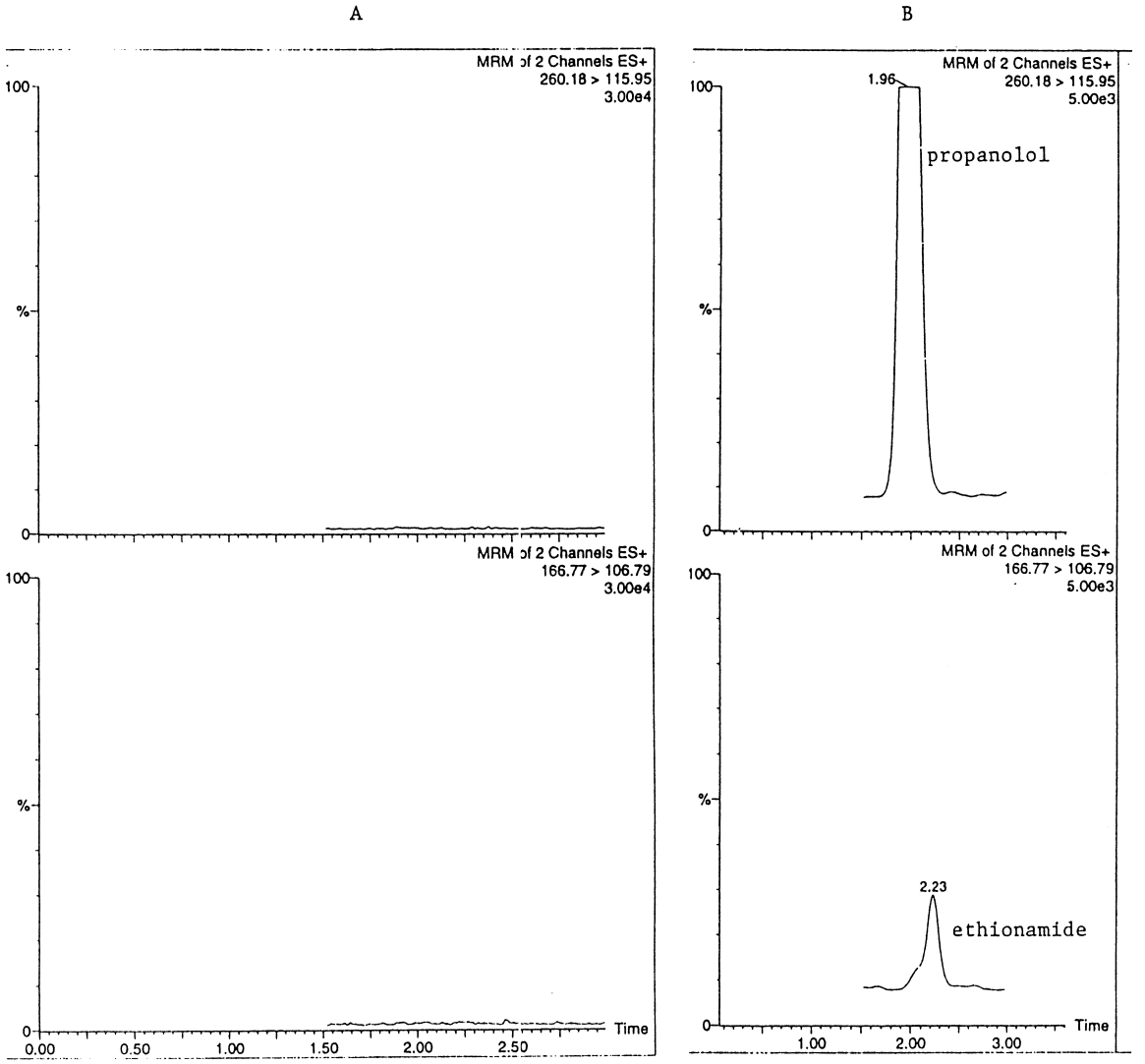

Fig. 5. Chromatograms of (a) blank AC suspension, (b) study subject's AC suspension obtained 4 h after the ninth dose of ethionamide of 250 mg, administered every 12 h. The ethionamide concentration is 0.026 μg/ml. In each graphic, the internal standard and ethionamide are presented in the upper and lower panels, respectively.

cells were 101.3±4.06% (range 92–104.2%), 100.8±3.87% (range 95.3–107.2%) and 102.8±7.33% (range 94.0–111.1%), respectively (Tables 1–3). The accuracy ranges for all determinations in plasma, BAL supernatants and alveolar cells were −8.0 to 5.0%, −4.71 to 7.2% and −6.0 to 11.1%, respectively (Tables 1–3).

3.3. Stability

The results of weekly determinations of ethionamide in spiked plasma, BAL supernatants and alveolar cells stored at −70°C revealed no significant degradation of the drug for 6 weeks (data not shown).

Table 1
Assay precision, recovery and accuracy for ethionamide determination in plasma[a] [intra-day ($n = 6$), inter-day ($n = 10$) assay precision, recovery and accuracy for ethionamide determination by HPLC–MS–MS at four concentrations in plasma[a]]

Spiked concentration (μg/ml)	Measured concentration (mean±SD) (μg/ml)	Coefficient of variation (%)	Recovery[b] (%)	Accuracy[c] (%)
Intra-day[d] ($n = 6$)				
2.4	2.45±0.218	8.91	102.1	2.08
1.2	1.24±0.062	5.00	103.3	3.33
0.3	0.28±0.031	11.30	92.0	−8.0
0.1	0.11±0.013	12.11	105.0	5.0
Inter-day[e] ($n = 10$)				
2.4	2.40±0.219	9.13	100.0	0.00
1.2	1.25±0.156	12.47	104.2	4.17
0.3	0.31±0.021	6.94	101.7	1.67
0.1	0.10±0.012	11.45	102.0	2.00

[a] Analyzed on PE Sciex API III.
[b] Measured/spiked·100%.
[c] Measured−spiked)/spiked·100%.
[d] Six separately spiked samples at each of four concentrations.
[e] On 5 different days, plasma spiked at four concentrations and analyzed in duplicate.

Table 4 summarizes the concentrations of ethionamide in plasma, BAL supernatant and alveolar cells in five of 40 subjects who participated in an NIH-supported study of intrapulmonary pharmacokinetics of ethionamide. Ten healthy men, 10 healthy women, 10 men with AIDS, and 10 women with AIDS were studied. Bronchoscopy and BAL were performed and blood was drawn at 4 h follow-

Table 2
Assay precision, recovery and accuracy for ethionamide determination in BAL supernatant[a] [intra-day ($n = 4$), inter-day ($n = 10$) assay precision, recovery and accuracy for ethionamide determination by HPLC–MS–MS at three concentrations in BAL supernatant[a]]

Spiked concentration (μg/ml)	Measured concentration (mean±SD) (μg/ml)	Coefficient of variation (%)	Recovery[b] (%)	Accuracy[c] (%)
Intra-day[d] ($n = 4$)				
0.320	0.305±0.014	4.6	95.29	−4.71
0.040	0.040±0.003	6.5	100.34	0.34
0.010	0.011±0.001	11.2	107.16	7.16
Inter-day[e] ($n = 10$)				
0.320	0.318±0.038	11.8	99.31	−0.69
0.040	0.040±0.005	11.7	100.5	0.5
0.010	0.010±0.001	0.09	102	2.0

[a] Performed on Micromass Quattro LC.
[b] Measured/spiked·100%.
[c] (Measured−spiked)/spiked·100%.
[d] Four separately spiked samples at each of three concentrations.
[e] On 5 different days, plasma spiked at three concentrations and analyzed in duplicate.

Table 3
Assay precision, recovery and accuracy for ethionamide determination in alveolar cells[a] [intra-day ($n = 6$), inter-day ($n = 10$) assay precision, recovery and accuracy for ethionamide determination by HPLC–MS–MS at three concentrations in alveolar cells[a]]

Spiked concentration (μg/ml)	Measured concentration (mean±SD) (μg/ml)	Coefficient of variation (%)	Recovery[b] (%)	Accuracy[c] (%)
Intra-day[d] ($n = 6$)				
1.600	1.515±0.052	3.4	94.7	−5.3
0.400	0.431±0.023	5.4	107.7	7.7
0.010	0.011±0.002	13.6	108.2	8.2
Inter-day[e] ($n = 10$)				
1.600	1.504±0.126	8.4	94.0	−6.0
0.400	0.404±0.020	4.8	101.0	1.0
0.010	0.011±0.001	10.8	111.1	11.1

[a] Performed on Micromass Quattro LC.
[b] Measured/spiked·100%.
[c] (Measured − spiked)/spiked·100%.
[d] Six separately spiked samples at each of three concentrations.
[e] On 5 different days, plasma spiked at three concentrations and analyzed in duplicate.

ing the last dose of a nine dose oral regimen of 250 mg of ethionamide administered every 12 h. Blood samples were also obtained 2 h after the last dose. Plasma concentrations ranged from 0.32 to 1.36 μg/ml at 2 h and from 0.43 to 1.08 μg/ml at 4 h after the last dose. Epithelial lining fluid (ELF) and alveolar cell (AC) concentrations ranged from 2.53 to 16.27 and 0 to 0.30 μg/ml, respectively. Thus in these five subjects there was a considerable concentration of ethionamide in ELF and in one subject ethionamide was not detectable in alveolar cells.

4. Discussion

We have developed a HPLC–MS–MS assay using either APCI or electrospray mode that provides specific, rapid, and reliable determinations for ethionamide in plasma, BAL and AC. The method is more sensitive than reported HPLC techniques and can detect 0.005 μg/ml of ethionamide in BAL or AC. The assay is currently being used to support a study of the pulmonary pharmacokinetics of ethionamide in patients with AIDS and in normal

Table 4
Ethionamide concentrations[a] in plasma, ELF and AC in five adult volunteer subjects [ethionamide concentrations[a] determined by HPLC–MS–MS in plasma, epithelial lining fluid (ELF) and alveolar cells (AC) in five adult volunteer subjects]

Sample	Subject				
	1	2	3	4	5
Plasma					
2 h post ninth dose[b]	0.32	1.19	1.22	1.25	1.36
4 h post ninth dose	0.43	1.08	0.97	0.47	0.96
ELF — 4 h post ninth dose	2.53	16.27	4.75	4.80	4.86
AC — 4 h post ninth dose	0	0.91	1.09	0.00	0.30

[a] All concentrations are given in μg/ml.
[b] The dose given was 250 mg every 12 h for nine doses (see Results).

volunteers. Preparation of plasma, BAL supernatant and AC samples requires deproteinization steps to ensure high recovery of samples. The stability data indicate that no significant drug degradation occurs in plasma, BAL supernatant or AC stored at −70°C for 6 weeks. The linearity of the standard curve, in the range described, is excellent. The method has a high assay precision and increased sensitivity for the detection of ethionamide in BAL and AC. The method was designed specifically to detect ethionamide molecules and may avoid interfering peaks from the sulphoxide metabolite or other metabolites.

Minimum inhibitory concentrations (MIC) for sensitive strains of *M. tuberculosis*, tested with the BACTEC method have been reported to be 0.25 to 0.50 μg/ml [2] and 0.3 to 1.2 μg/ml [17]. For clinical purposes, breakpoints of ≤1.25, 2.5, 5.0 and >5.0 μg/ml have been recommended for the categories of susceptible, moderately susceptible, moderately resistant, and resistant, respectively [18]. In these five subjects, ethionamide was measurable in plasma at concentrations that approximate the reported MICs for *M. tuberculosis* and the drug was concentrated in the ELF. Drug was also measurable in three of five subjects in AC at concentrations below (one subject) or approximating (two subjects) the reported MICs for *M. tuberculosis*. In two subjects, drug was not detectable in AC. These observations are preliminary and further investigation is needed to define the intrapulmonary pharmacokinetics of ethionamide.

In conclusion, we have described a new HPLC–MS–MS assay that is suitable for clinical and pharmacologic studies, particularly those that are designed to quantify the intrapulmonary concentration of drugs.

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