High-Performance Liquid Chromatographic Determination of Pyrazinamide In Human Plasma, Bronchoalveolar Lavage Fluid, and Alveolar Cells

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Abstract

A technique is presented for the measurement of pyrazinamide in human plasma, bronchoalveolar lavage, and alveolar cells by reversed-phase column chromatography. The assay utilizes acetazolamide as an internal standard, ultraviolet detection at 268 nm, and an acetonitrile-based mobile phase. Preparation of plasma samples requires a simple deproteinization step, resulting in the development of sharp peaks with retention times of 8.4 and 17 minutes for pyrazinamide and acetazolamide, respectively. Bronchoalveolar lavage and alveolar cell suspensions require an acid extraction with ethyl acetate, evaporation to dryness, and reconstitution. This method provides specific, rapid, and reliable determinations of drug concentrations and therefore is suitable for pharmacological studies, particularly those that are designed to quantitate the intrapulmonary concentrations of pyrazinamide.

Introduction

Pyrazinamide is a water-soluble synthetic congener of nicotinamide that is well absorbed when taken orally (1). It has an important role in the treatment of tuberculosis and is recommended as one of four drugs for primary therapy (2). Pyrazinamide has a plasma half-life of 10 h and is metabolized to pyrazinoic acid and other metabolites by the liver. Renal clearance is low (< 2 mL/min), and urinary elimination accounts for less than 4% of the ingested dose (1,3,4).

A number of chromatographic assays for the determination of pyrazinamide have been published (5–9). These techniques have been directed to the measurement of the drug or its metabolites in plasma, serum, or urine. An assay that can be used to measure the drug concentrations in bronchoalveolar lavage (BAL) or alveolar cells (AC) has not been reported. This paper describes a reversed-phase high-performance liquid chromatographic (HPLC) method for the rapid, sensitive, and accurate determination of pyrazinamide in human plasma, BAL fluid, and AC. This assay is more sensitive than those previously published (0.005 µg/mL versus 0.2 µg/mL) and extracts the large amount of lidocaine present in the bronchoalveolar lavage fluid that interferes with the assay.

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Experimental

HPLC method

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Pyrazinamide (> 99% purity, Figure 1) and acetazolamide (Figure 2) were purchased from Sigma Chemical (St. Louis, MO). Pyrazinamide stock solutions were prepared in deionized water and stored at –80°C. Acetazolamide solutions were prepared in 50% aqueous methanol and refrigerated.

Acetazolamide was selected as the internal standard, because its chemical properties are similar to those of pyrazinamide. Both compounds are amides and acids. They were extractable from the matrices and well-separated on this HPLC system.

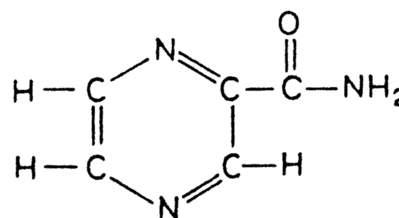

Figure 1. Structure of pyrazinamide.

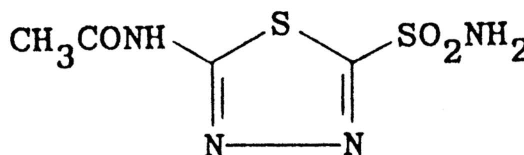

Figure 2. Structure of acetazolamide.

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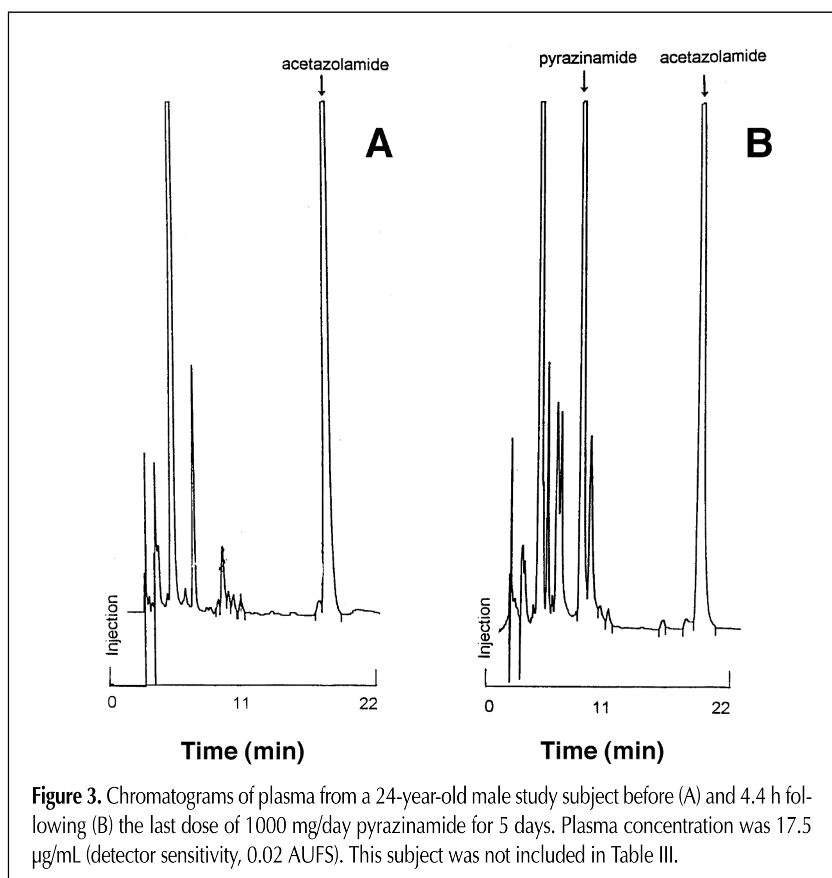

Figure 3. Chromatograms of plasma from a 24-year-old male study subject before (A) and 4.4 h following (B) the last dose of 1000 mg/day pyrazinamide for 5 days. Plasma concentration was 17.5 μg/mL (detector sensitivity, 0.02 AUFS). This subject was not included in Table III.

Analyses were performed at room temperature using a Shimadzu (Columbia, MD) LC-10AD solvent delivery system, a Waters (Milford, MA) model 717 Plus autosampler, and an ABI (Perkin Elmer, Norwalk, CO) Spectroflow 783 absorbance detector set at 268 nm with a sensitivity of 0.02 absorbance units full scale (AUFS) for plasma and 0.002 AUFS for BAL and AC suspension. The mobile phase consisted of 2.0% acetonitrile in 0.02M KH_2PO_4, adjusted to pH 2.6 with phosphoric acid, degassed through a Millipore (Milford, MA) filter (0.22 μm), recycled to complete one run, and then discarded. Chromatography was performed with a 25-cm × 4.6-mm Axxiom (Richard Scientific, Novato, CA) ODS column (5-μm particle size) at a flow rate of 1.0 mL/min. Under these acidic conditions, both compounds are in nonionic states and are well partitioned by the ODS column. Chromatograms were integrated on a Shimadzu Chromatopac CR601. The retention times for pyrazinamide and acetazolamide were 8.4 and 17.0 min, respectively. Plasma, BAL supernatants, and AC samples were run for 45 min because of late peaks that interfered with the next injection. Phosphate buffered saline (PBS) standards were run for 33 min.

Figure 4. Chromatograms from 0.100 μg/mL standard in PBS (A), extract of alveolar cells from a 41-year-old female study subject in the bronchoscopy group of 4 h following the last dose of 1000 mg/day pyrazinamide for 5 days (pyrazinamide concentration, 0.029 μg/mL) (B), and extract of the same patient's BAL supernatant (pyrazinamide concentration, 0.345 μg/mL; detector sensitivity, 0.002 AUFS) (C). This subject was not included in Table III.

Preparation of plasma standards and samples

100- and 10-ng/µL working stock solutions of pyrazinamide were used to prepare the standard curves. These solutions were added to 0.2 mL of plasma (pooled) to yield the following concentrations for the standard curve: 0.5, 1.0, 2.5, 5.0, 10, 20, 40, 80 µg/mL. An internal standard solution was prepared from acetazolamide stock (1 mg/mL) solution by diluting to 10 ng/µL in acetonitrile. Spiked plasma (0.2 mL) was deproteinated by adding 0.4 mL internal standard solution, vortexing for 30 s, centrifuging at 3000 × g in a Marathon 21K centrifuge (Fisher Scientific, Pittsburgh, PA) for 10 min, decanting into a clean glass tube, and evaporating to approximately 200 µL. After the addition of 200 µL of mobile phase and vortexing, 20 µL was injected onto the column. Plasma controls and samples were prepared the same way.

The standard curve was constructed by plotting the peak-height ratios of pzrazinamide to acetazolamide against the spiked concentration of pyrazinamide (weighted as $1/y$ where y equals the concentration of pyrazinamide). The slope (mean ± standard deviation), y-intercept, and linearity (r^2) for 6 standard curves run on separate days were 0.10 ± 0.02, –0.01 ± 0.01, and 0.999 ± 0.000 (n = 6), respectively.

Preparation of BAL supernatants and alveolar cell pellet standards and samples

Bronchial lavage fluid (30 mL) was centrifuged at 400 × g for 5 min, and the cells separated from the supernatant immediately after collection. The cells were resuspended in PBS to yield a tenfold concentration of the original lavage fluid centrifuged. To lyse the cells, the suspension was sonicated for 2 min at 50-µm amplitude on a Fisher 550 Sonic Dismembrator.

PBS was chosen as the matrix for standard curves and controls utilized to measure pzrazinamide concentrations in both BAL and AC suspensions. It was ascertained that the slopes, y-intercepts, and linearity of these matrices were similar. Furthermore, the cells are resuspended in PBS, and BAL fluid is primarily normal saline. PBS (0.5 mL) was spiked with stock solutions containing 1 ng/µL and 10 ng/µL of pyrazinamide to yield the following concentrations for the standard curve: 0.005, 0.007, 0.010, 0.020, 0.050, 0.100, 0.200, 0.500 µg/mL. The slope (mean ± SD), y-intercept, and linearity (r^2) for 6 standard curves run on separate days were 0.131 ± 0.02, 0.0007 ± 0.0006, and 0.9997 ± 0.0002 (n = 6), respectively.

The drug was extracted as follows: 0.5 mL of spiked PBS, 0.5 mL of lysed cell suspension, or 0.5 mL of diluted BAL supernatant were pipetted into screwcap glass tubes (because of high concentrations in the BAL supernatants, 25 µL of BAL supernatant was added to 475 µL of PBS). Twenty microliters of internal standard solution (10 ng/µL acetazolamide in acetonitrile) and 50 µL of 50% hydrochloric acid were then added (resulting pH 0.8). After vortexing for 2 min, 4.0 mL of ethyl acetate was added, vortexed for 2 min, and then centrifuged for 10 min at 1000 × g (Marathon 21K centrifuge). The organic phase was transferred to a clean tube, and the aqueous phase left in the screwcap tube was extracted again in the same manner. The resulting 8 mL of solvent was pooled, evaporated to dryness under nitrogen, and then reconstituted in 0.2 mL of mobile phase. Forty microliters was injected into the column.

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 1.08, 4.32, 26.9, and 53.8 µg/mL; aliquoted; and frozen at –80°C for stability studies. Aliquots were

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

Spiked concentration (µg/mL)	Measured concentration (mean ± SD) (µg/mL)	Coefficient of variation (%)	Recovery* (%)	Accuracy† (%)
Intraday‡ (n=6)				
4.32	4.44 ± 0.25	5.69	102.8	2.78
26.9	28.54 ± 0.81	2.83	106.1	6.10
53.8	58.57 ± 1.89	3.22	108.9	8.87
Interday§ (n=6)				
1.08	1.09 ± 0.11	10.41	100.9	0.93
4.32	4.24 ± 0.31	7.40	98.1	–1.85
26.9	25.64 ± 1.54	5.99	95.3	–4.68
53.8	52.17 ± 2.67	5.11	97.0	–3.03

* Measured/spiked × 100% (3).
† Measured-spiked/spiked × 100% (6).
‡ Six separately spiked samples at each of three concentrations.
§ On six different days, plasma spiked at four concentrations and analyzed in duplicate.

Table II. Assay Precision, Recovery, and Accuracy for Pyrazinamide Determination in PBS*

Spiked concentration (µg/mL)	Measured concentration (mean ± SD, µg/mL)	Coefficient of variation (%)	Recovery (%)	Accuracy (%)
Intraday† (n=6)				
0.025	0.026 ± 0.0004	1.58	104	4.00
0.250	0.264 ± 0.046	1.74	106	5.60
Interday‡ (n=5)				
0.025	0.025 ± 0.001	2.35	100	0.00
0.250	0.257 ± 0.009	3.56	103	2.80

* Phosphate buffered saline is a surrogate matrix for BAL and AC (see methods).
† Six separately spiked samples at each of two concentrations.
‡ On five different days, PBS spiked at two concentrations and analyzed in duplicate.

analyzed in duplicate weekly over a period of 4 weeks, then at 3, 4, and 11 months. To assess interday reproducibility, standard curves with spiked controls were analyzed on 6 different days. Intraday reproducibility was assessed by analyzing 6 preparations of each of 3 concentrations on the same day.

Controls in PBS were spiked at 0.025- and 0.25-µg/mL concentrations, aliquoted, and frozen at –80°C for stability studies. Stability and reproducibility studies were carried out the same as for plasma.

The statistical analysis was performed using the PROPHET Computer Resource (10). Linearity, precision (coefficient of variation), recovery (relation of test result to the true concentration) (11), and percentage accuracy (12) were calculated. The detection limit was defined as the smallest peak height that was three times the baseline noise level.

Results and Discussion

Sample chromatograms of plasma, PBS, BAL supernatant, and AC suspension are shown in Figures 3 and 4. The detection limits for pyrazinamide were 0.5 µg/mL for plasma and 0.005 µg/mL for BAL supernatants and AC suspensions. Results for assay precision, recovery, and accuracy assessments in plasma and PBS are summarized in Tables I and II. The mean coefficients of variation for all plasma and PBS assays were 5.81% (range, 2.83–10.41%) and 2.31% (range, 1.58–3.56%), respectively. For both plasma and PBS, there were no statistically significant differences among the means for each concentration when comparing intra- and interday precision assessment ($p > 0.05$, Student's *t*-test).

The mean recoveries of the assay for all determinations in plasma and PBS were 101.3% (range, 95.3–108.9%) and 103.1% (range, 100–105.6%), respectively. The accuracy ranges for all determinations in plasma and PBS were –4.68 to 8.87% and 0.00 to 5.6%, respectively.

The results of weekly determinations of pyrazinamide in spiked plasma and PBS stored at –80°C revealed no significant degradation of the drug for 6 weeks. Spiked PBS controls were analyzed after storage for 1 year at –80°C. No significant degradation of the drug was detected.

Table III summarizes the concentrations of pyrazinamide in plasma, BAL supernatant, and AC in two HIV positive and two HIV negative subjects with normal renal and hepatic function. Blood was drawn, and the bronchoscopy with BAL was performed at 4 h following the last dose of a 5-day course of 1000 mg/day pyrazinamide administered orally in a dose of 1000 mg/day.

We have developed a reversed-phase HPLC assay that provides specific, rapid, and reliable determinations for pyrazinamide in plasma, BAL, and AC. The preparation of plasma specimens requires a simple deproteinization step. PBS can be utilized as a surrogate for BAL fluid and AC lysate, thus solving the problem of the scarcity of large amounts of these specimens required to standardize and validate the method. The BAL and AC lysate require an acid extraction of the drug. These procedures assure the development of sharp peaks without interference.

The stability study indicates that no significant drug degradation occurs in plasma or PBS stored at –80°C for 6 weeks or PBS stored for 1 year. The linearity of the standard curve in the range described is excellent. Assay precision is high for plasma, BAL, PBS, and AC. The performance characteristics of this assay are comparable to those previously published (5–9) but with greater sensitivity, therefore making the method suitable for clinical and pharmacological investigations, particularly those that are designed to quantitate the intrapulmonary concentration of drugs.

Table III. Pyrazinamide Concentrations in Plasma, Epithelial Lining Fluid (ELF), and AC in 4 Subjects Who Received 1 g/day Pyrazinamide for 5 Days

	Subject 1	Subject 2	Subject 3	Subject 4	
Dose (mg/kg)	14.2	15.5	10.6	19.2	
Gender	male	female	male	female	
HIV status	negative	negative	positive	positive	
	Pyrazinamide concentration (µg/mL)				
	Subject 1	**Subject 2**	**Subject 3**	**Subject 4**	
Plasma (2 h)*	19.6	22.5	19.1	38.2	
Plasma (4 h)*	13.7	17.6	14.6	30.2	
ELF† (4 h)*	311		493.1	447.1	421.5
AC‡ (4 h)*	8.7	31.3	24.4	11.3	

* Hours elapsed since the last of 5 doses.
† The amount of ELF collected in the BAL fluid was calculated from the urea concentration in BAL and serum as previously reported (13).
‡ The concentration in AC is reported as micrograms per milliliter of cell volume and was calculated as previously reported (14).

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