

Liquid Chromatographic Determination of Rifampin In Human Plasma, Bronchoalveolar Lavage Fluid, and Alveolar Cells

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Abstract

A technique is presented for the measurement of rifampin in humans by reversed-phase column chromatography and postcolumn photo irradiation. The assay employs diazepam as an internal standard and provides specific, rapid, and reliable determinations for drug concentration in plasma, bronchoalveolar lavage (BAL), and alveolar cells (AC). The preparation of plasma and AC samples utilizes a simple deproteinization step, whereas BAL supernatants require a solid-phase extraction. The assay produces sharp peaks with retention times of 6.2 and 15 min for rifampin and diazepam, respectively. The detection limits for rifampin were 0.5 µg/mL for plasma, 0.015 µg/mL for BAL supernatants, and 0.03 µg/mL for AC suspensions. The assay has excellent performance characteristics, making it suitable for pharmacological studies in humans, and it is being used to support a study of the intrapulmonary pharmacokinetics of rifampin.

Introduction

Rifampin has a primary role in the treatment of tuberculosis and is recommended with isoniazid, pyrazinamide, and ethambutol as initial therapy (1). Rifampin is a water- and lipid-soluble semisynthetic congener of rifamycin B that is well absorbed when taken orally. It has a plasma half-life of 3–4 h, and peak concentrations of 8–12 µg/mL are reached within 1–2 h of the recommended daily dose of 600 mg in adults (2).

A number of chromatographic assays for the determination of rifampin have been published (3–9). These techniques have been directed to the measurement of the drug and/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. These matrices require a more sensitive assay than blood or urine and

greater specificity due to the large amount of lidocaine present in BAL, which can interfere with the assay. This paper describes a reversed-phase high-performance liquid chromatographic (HPLC) method for the rapid, sensitive, and accurate determination of rifampin in human plasma, BAL fluid, and AC.

Experimental

HPLC method

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Rifampin (> 98.6% purity) (Figure 1) and diazepam (Figure 2) were purchased from

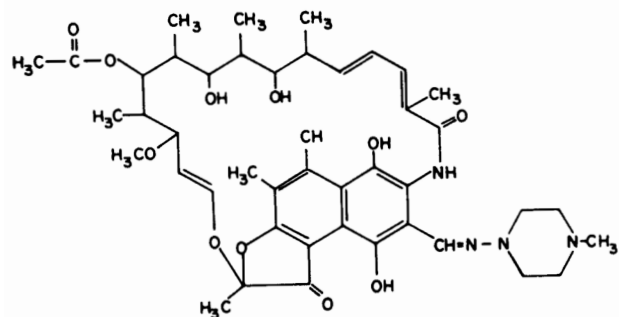


Figure 1. Molecular structure of rifampin¹⁶.

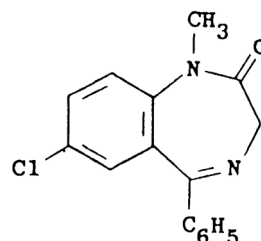


Figure 2. Molecular structure of diazepam¹⁷.

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Sigma Chemical Co. (St. Louis, MO). Rifampin and diazepam stock solutions were prepared in acetonitrile, to which 200 $\mu\text{g/mL}$ ascorbic acid was added as an antioxidant to stabilize rifampin, and the solutions were stored at -80°C (10). Ascorbic

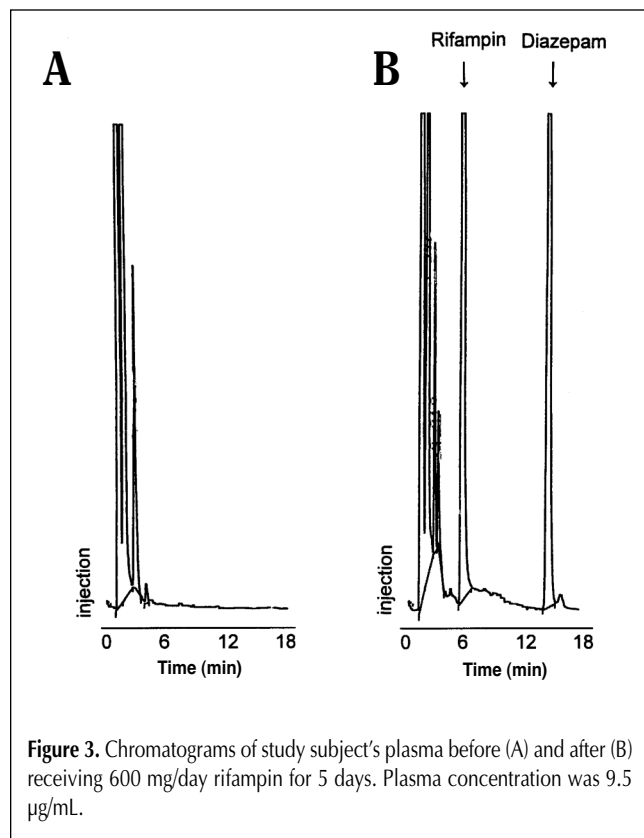


Figure 3. Chromatograms of study subject's plasma before (A) and after (B) receiving 600 mg/day rifampin for 5 days. Plasma concentration was 9.5 $\mu\text{g/mL}$.

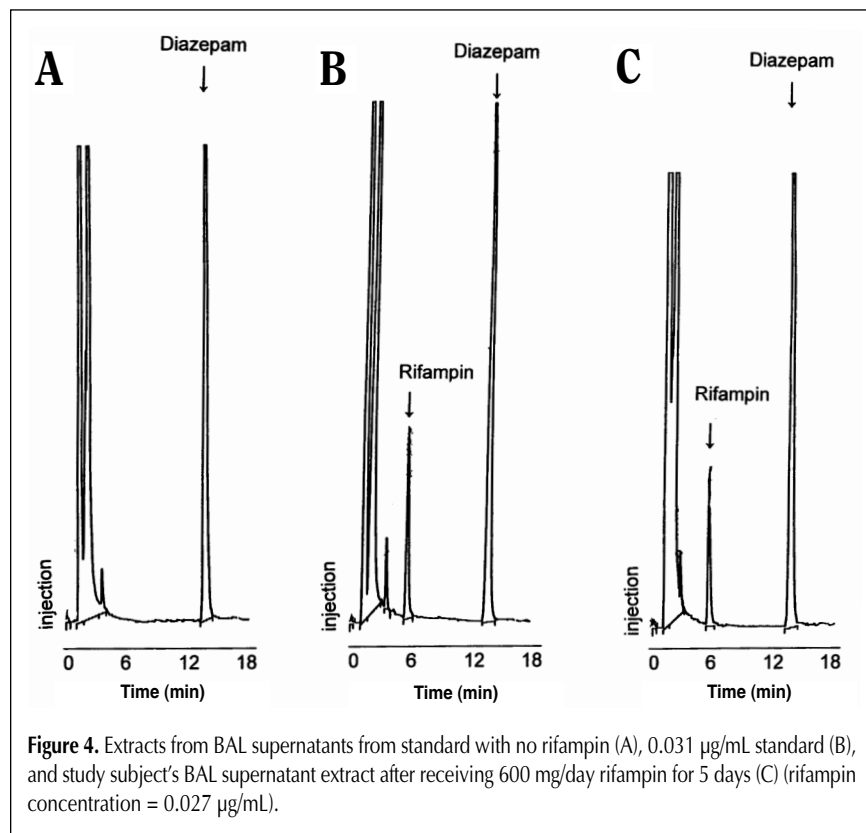


Figure 4. Extracts from BAL supernatants from standard with no rifampin (A), 0.031 $\mu\text{g/mL}$ standard (B), and study subject's BAL supernatant extract after receiving 600 mg/day rifampin for 5 days (C) (rifampin concentration = 0.027 $\mu\text{g/mL}$).

acid stock solution was prepared in deionized water. All methanol–ascorbic acid and acetonitrile–ascorbic acid solutions contained 200 $\mu\text{g/mL}$ of ascorbic acid. Diazepam was selected as an internal standard because its retention time produced good peak separation from rifampin and it fluoresces with photo irradiation.

Analyses were performed at room temperature using a Waters (Milford, MA) model 510 solvent delivery system, a Waters model 717 with autosampler, and a Shimadzu (Columbia, MD) RF535 fluorescence detector set at an excitation wavelength of 380 nm and emission wavelength of 490 nm. The mobile phase consisted of 36% acetonitrile in water, 0.2% phosphoric acid, and 0.5% hydrogen peroxide adjusted to pH 4.5 with sodium hydroxide, sonicated, and stirred (neither degassed nor recycled). The phosphoric acid buffers the mobile phase, and the hydrogen peroxide is added to enhance the efficiency of photo irradiation on rifampin and diazepam. Under these conditions, rifampin and diazepam are ionized, but they are still retained by the C_8 column, and the peaks are well separated. At a flow rate of 1 mL/min, the mobile phase was passed through a 5- μm Beckman Ultrasphere octyl column (15 cm \times 4.6-mm i.d.) followed by tubing (polytetrafluoroethylene teflon, 104 cm \times 0.5-mm i.d.) wrapped around a UVP (San Gabriel, CA) shortwave Pen-Ray lamp to irradiate the sample. This step was necessary because rifampin without irradiation did not fluoresce enough to be detected by fluorescence. Chromatograms were integrated on a Shimadzu Chromatopac CR601. The retention times for rifampin and diazepam were 6.2 and 15.0 min, respectively. Plasma, BAL supernatant, and AC samples were run for 20 min.

Standard curves were constructed by plotting rifampin/diazepam peak height ratios against the spiked concentration of rifampin and also weighted $1/y$ (y = the concentration of rifampin).

Preparation of plasma standards and samples

Rifampin stock solution (0.537 mg/mL) was diluted with the acetonitrile–ascorbic acid solution to a concentration of 0.054 mg/mL. To prepare the standard curve, 2, 4, 8, 20, and 40 μL of stock solution (0.054 mg/mL) and 6 and 8 μL of stock solution (0.537 mg/mL) were added to 0.2 mL plasma to yield concentrations of 0.54, 1.08, 2.15, 5.38, 10.75, 16.1, and 21.5 $\mu\text{g/mL}$ rifampin.

The internal standard solution was prepared from diazepam stock (1 mg/mL) solution diluted in acetonitrile to contain 0.037 $\mu\text{g/mL}$ diazepam. Two-tenths of a milliliter of spiked plasma was deproteinated by adding 0.4 mL internal standard solution, vortexing for 1 min, centrifugating at $3000 \times g$ for 10 min, decanting into a clean glass tube, evaporating to dryness under nitrogen at 37°C ,

and resuspending in 200 μL of methanol with ascorbic acid. Thirty-five microliters was injected into the column. Plasma protein precipitation with acetonitrile has been previously reported by Jamaluddin (4).

Plasma controls and samples were prepared the same way. The slope (mean \pm standard deviation), y -intercept, and linearity (r^2) for 10 standard curves run on separate days were 0.126 ± 0.026 , -0.020 ± 0.023 , and 0.9975 ± 0.002 , respectively.

Preparation of BAL supernatants and AC pellet standards and samples

Immediately after collection, a cell count and differential was performed on the BAL fluid. Then, a measured amount of BAL fluid was centrifuged, and the cells were immediately separated from the supernatant.

Standard curves were prepared by spiking 3.5-mL aliquots of pooled BAL supernatant with 1, 2, 4, 10, 20, 40, and 80 μL of 0.054 mg/mL stock solution to yield concentrations of 0.015, 0.03, 0.06, 0.15, 0.31, 0.61, and 1.22 $\mu\text{g}/\text{mL}$ of rifampin. Standards and controls were extracted in the same manner as the study subjects' samples as described later.

It was necessary to extract the BAL supernatants in the following manner in order to concentrate the specimen for greater sensitivity and remove interfering substances, primarily the large amount of lidocaine that is found in the BAL fluid. Immediately after centrifugation of a study subject's BAL fluid, a 3.0-mL aliquot of BAL supernatant was placed onto a Varian (Harbor City, CA) Bond Elut C_8 solid-phase extraction column that had been prewashed with 3 mL methanol and 3 mL water. The column was then washed with 2 mL of 10% methanol–ascorbic acid followed by 0.5 mL acetonitrile–ascorbic acid. The drug was eluted with 2 mL of eluting solution (2% ammonium hydroxide in methanol–ascorbic acid). For study subjects, this eluant was stored frozen at -80°C and batched for analysis. At the time of assay, 200 μL of diazepam (17.5 $\mu\text{g}/\text{mL}$ in acetonitrile–ascorbic acid) was added to the eluant as an internal standard. After vortexing, the eluant was evaporated to dryness and resuspended in 200 μL of methanol–ascorbic acid, and 25 μL was injected onto the column. Standards were spiked, eluted, and assayed on the same day that the assay was run.

AC pellets (containing a known number of cells calculated from the cell count that was performed on the BAL fluid prior to centrifugation) were immediately suspended in 2 mL of acetonitrile–ascorbic acid solution and kept frozen until assay. Two milliliters of acetonitrile–ascorbic acid solution was spiked with rifampin stock solution (0.054 $\mu\text{g}/\text{mL}$) to yield concentrations of 0.027, 0.054, 0.11, 0.19, 0.27, 0.40, 0.54, and 1.08 $\mu\text{g}/\text{mL}$ for the standard curve. The pellet samples were centrifuged for 10 min at $1000 \times g$ and decanted. Two hundred microliters of internal standard diazepam (17.5 $\mu\text{g}/\text{mL}$) was added to all samples.

Standards and pellets were evaporated under nitrogen at 37°C and resuspended in 200 μL of methanol–ascorbic acid solution. Twenty-five microliters were injected onto the column.

Preparation of controls for method validation

Two sets of stock solutions containing 0.054 and 0.537 mg/mL each were prepared; one was used for spiking standards, the other was used for spiking controls. Measured amounts of plasma were spiked at extra-low, low, medium, and high con-

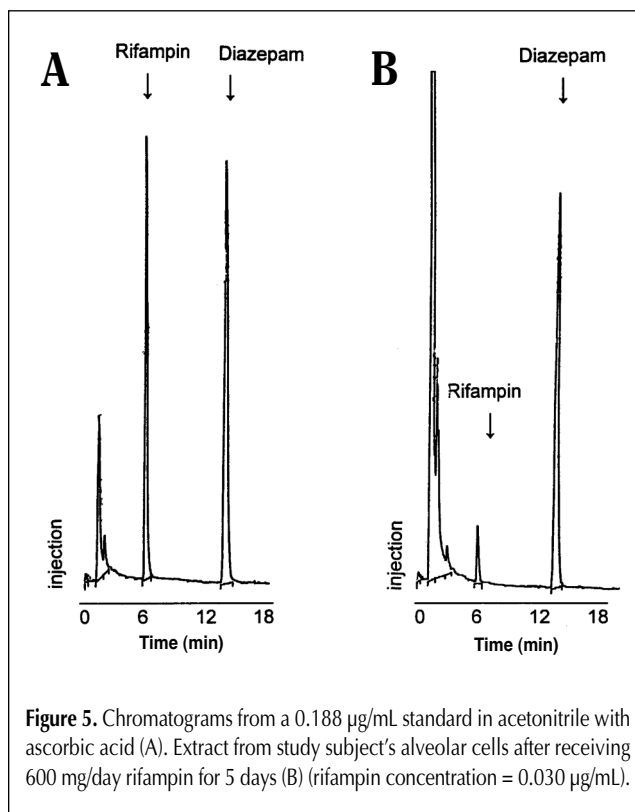


Figure 5. Chromatograms from a 0.188 $\mu\text{g}/\text{mL}$ standard in acetonitrile with ascorbic acid (A). Extract from study subject's alveolar cells after receiving 600 mg/day rifampin for 5 days (B) (rifampin concentration = 0.030 $\mu\text{g}/\text{mL}$).

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

Spiked concentration ($\mu\text{g}/\text{mL}$)	Measured concentration (mean \pm SD) $\mu\text{g}/\text{mL}$	Coefficient of variation (%)	Recovery* (%)	Accuracy [†] (%)
Intraday ($n = 6$)[‡]				
1.15	1.10 \pm 0.08	7.27	95.6	-4.35
4.31	4.09 \pm 0.15	3.67	94.9	-5.10
11.5	11.1 \pm 0.23	2.07	96.5	-3.48
17.2	17.4 \pm 0.58	3.33	101.2	1.16
Interday ($n = 12$)[§]				
1.15	1.16 \pm 0.07	6.03	100.9	0.87
4.31	4.08 \pm 0.15	3.68	94.7	-5.34
11.5	11.2 \pm 0.44	3.93	97.4	-2.61
17.2	17.6 \pm 0.43	2.44	102.3	2.33

* Measured/spiked \times 100% (13).
[†] (Measured - spiked)/spiked \times 100% (13).
[‡] Six separately spiked samples at each of 4 concentrations.
[§] On 6 different days, plasma spiked at 4 concentrations and analyzed in duplicate.

centrations; aliquoted; and frozen at -70°C for stability studies. Aliquots were analyzed in duplicate at ten different times over a period of 34 weeks. To assess interday reproducibility, standard curves with spiked controls were analyzed on six different days. Intraday reproducibility was assessed by ana-

lyzing 6 preparations of each of 4 concentrations on the same day. BAL supernatant and AC were analyzed at low and high concentrations for interday and intraday reproducibility. For stability assessment, the eluant of spiked BAL supernatant controls and controls spiked in acetonitrile–ascorbic acid for AC were aliquoted and frozen. The statistical analysis was performed using the PROPHET Computer Resource (10). Stability was assessed using weighted ($1/y$) linear regression. Linearity (r^2), 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 3 times the baseline noise level.

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

Spiked concentration ($\mu\text{g/mL}$)	Measured concentration (mean \pm SD) $\mu\text{g/mL}$	Coefficient of variation (%)	Recovery (%)	Accuracy (%)
Intraday ($n = 6$)[*]				
0.05 $\mu\text{g/mL}$	0.054 \pm 0.002	4.22	108	8.00
0.50 $\mu\text{g/mL}$	0.488 \pm 0.012	2.52	97.6	-2.40
Interday ($n = 15$)[†]				
0.05 $\mu\text{g/mL}$	0.052 \pm 0.004	7.78	104	4.00
0.50 $\mu\text{g/mL}$	0.511 \pm 0.023	4.46	102	2.20

^{*}Six separately spiked samples at low and high concentration.
[†]On 5 different days, BAL spiked at low and high concentrations and analyzed in triplicate.

Table III. Assay Precision, Recovery, and Accuracy for Rifampin Determination in AC

Spiked concentration ($\mu\text{g/mL}$)	Measured concentration (mean \pm SD) $\mu\text{g/mL}$	Coefficient of variation (%)	Recovery (%)	Accuracy (%)
Intraday ($n = 6$)[*]				
0.100	0.101 \pm 0.002	2.03	101	1.00
0.715	0.705 \pm 0.345	4.89	98.6	-1.40
Interday ($n = 15$)[†]				
0.100	0.102	5.61	102	2.00
0.715	0.726	4.55	102	1.54

^{*}Six separately spiked samples at low and high concentration.
[†]On 5 different days, acetonitrile spiked at low and high concentrations and analyzed in triplicate.

Table IV. Rifampin Concentrations in Plasma, ELF, and AC in Three Adult Volunteer Subjects

Compartment	Subject #1 concentration ($\mu\text{g/mL}$)	Subject #2 concentration ($\mu\text{g/mL}$)	Subject #3 concentration ($\mu\text{g/mL}$)
Plasma* 2 h	13.1	20.6	12.3
Plasma* 4 h	8.4	29.3**	15.7
ELF ^{†,‡}	2.7	2.0	2.4
AC [§]	10.4	13.4	14.2

* Blood drawn at 2 hr and 4 hr following the last of 5 daily doses of 600 mg of rifampin.

[†] Epithelial lining fluid obtained at the time of bronchoscopy. The amount of ELF collected in the BAL fluid was calculated from the ratio of urea concentration in the BAL and in serum, as previously reported (14).

[‡] Bronchoscopy and BAL performed 4 h after the last of 5 daily doses of 600 mg of rifampin.

[§] Alveolar cells obtained at the time of bronchoscopy. AC concentration is reported as micrograms per milliliter of cell volume, calculated as previously reported (15).

** This plasma was diluted 1:1 (v/v) with blank plasma and reanalyzed in order to measure the concentration within the range of the standard curve.

Results and Discussion

Chromatograms of rifampin and internal standard in plasma, BAL supernatant, and AC suspension are shown in Figures 3–5. The detection limits for rifampin were 0.5 $\mu\text{g/mL}$ for plasma, 0.015 $\mu\text{g/mL}$ for BAL supernatants, and 0.027 $\mu\text{g/mL}$ in AC suspensions. Results for assay precision, recovery, and accuracy assessments in plasma, BAL, and AC suspensions are summarized in Tables I–III. The mean (\pm standard deviation) coefficients of variation and ranges of the assay for intraday and interday determinations together for plasma, BAL supernatants, and AC were 4.05% \pm 1.76 (range 2.07–7.27%), 4.75% \pm 2.20 (range 2.52–7.78%), and 4.27% \pm 1.56 (range 2.03–5.61%), respectively.

The mean (\pm standard deviation) recoveries and ranges of the assay for intraday and interday determinations together in plasma, BAL supernatants, and AC were 97.9% \pm 3.07 (range 94.7–102.3%), 102.9% \pm 4.33 (range 97.6–108%), and 100.9% \pm 1.60 (range 98.6–102%), respectively. The accuracy ranges for all determinations in plasma, BAL supernatants, and AC were -5.34 to 2.33%, -2.40 to 8.00%, and -1.40 to 2.00%, respectively.

The stability of rifampin in plasma was studied at 1.15, 4.31, 11.5, and 17.2 $\mu\text{g/mL}$. There was no significant drug degradation at any of the four concentrations during the 241 days of observation. There also was no significant drug degradation in BAL supernatant stored for 106 days or AC stored for 42 days at -70°C .

Table IV summarizes the concentrations of rifampin in plasma, BAL supernatant, and

AC in 3 subjects. Bronchoscopy and BAL were performed, and blood was drawn at 4 h following the last dose of a 5-day course of 600 mg/day of rifampin.

Conclusion

We have developed a reversed-phase HPLC assay that provides specific, rapid, and reliable determinations for rifampin in plasma, BAL, and AC. The method is currently being used to support Phase One studies of the pulmonary pharmacokinetics of rifampin in patients with tuberculosis and in normal volunteers.

Preparation of plasma and alveolar cell samples requires a simple deproteinization step, assuring the development of sharp peaks. Ascorbic acid is added to stabilize rifampin, and the stability data indicate that no significant drug degradation occurs in plasma, BAL eluant, or AC suspended in acetonitrile-ascorbic acid stored at -70°C for 6 weeks. The linearity of the standard curve in the range described is excellent. Assay precision is high for plasma, BAL, and AC. 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.

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