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# Determination of rifabutin in human plasma by high-performance liquid chromatography with ultaviolet detection

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**Abstract**

A simple, specific and sensitive high-performance liquid chromatographic (HPLC) method was developed for the determination of rifabutin in human plasma. Rifabutin and sulindac (internal standard) are extracted from human plasma using a $C_8$ Bond Elut extraction column. Methanol (1 ml) is used to elute the compounds. The methanol is dried down under nitrogen and reconstituted in 250 $\mu$l of mobile phase. Separation is achieved by HPLC on a Zorbax Rx $C_8$ column with a mobile phase composed of 0.05 *M* potassium dihydrogen phosphate and 0.05 *M* sodium acetate at pH 4.0–acetonitrile (53:47, v/v). Detection is by ultraviolet absorbance at 275 nm. The retention times of rifabutin and internal standard were approximately 10.8 and 6.9 min, respectively. The assay is linear over the concentration range of 5–600 ng/ml. The quantitation limit was 5 ng/ml. Both intra-day and inter-day accuracy and precision data showed good reproducibility.

*Keywords:* Rifabutin

## 1. Introduction

Rifabutin, (4-deoxo-3,4-[2-*spiro*-(N-isobutyl-4-piperidyl)-2,5-dihydro-1H-imidazo]-rifamycin-S), is a semisynthetic derivative of rifampicin S that has shown broad-spectrum antibacterial activity against gram-positive and gram-negative organisms, including mycobacteria. Rifabutin was found to have activity against *Mycobacterium avium-intracellulare* isolated from patients with AIDS [1,2]. The major metabolite of rifabutin is LM-565, the 25-desacetyl derivative, which also exhibits antibacterial activity. Rifabutin is undergoing phase III evaluation for the prophylaxis and treatment of *M. avium* complex infection in patients with AIDS. Therefore, a specific method is needed to study the interaction of rifabutin with other potential AIDS drugs. High-performance liquid chromatography (HPLC) methods have been developed for the quantitation of rifabutin and its desacetyl metabolite in human biological fluids [3], [4]. These methods employed liquid–liquid extraction which were very complicated and time consuming. In addition, these methods required a long HPLC run time of 25 min or more. Other methods have been developed for the related antibiotic, rifampin, and its 25-desacetyl metabolite [5–12].

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A simple, sensitive and specific method using solid-phase extraction is described in this paper.

## 2. Experimental

### 2.1. Materials

Rifabutin and internal standard (sulindac) were obtained from Adria Laboratory (Columbus, OH, USA) and USP (Rockville, MD, USA), respectively. Atevirdine (U-87,201) and its metabolite (U-89,255); and delavirdine (U-90,152) and its metabolite (U-96,183) were provided by The Upjohn Company. Heparinized human plasma was donated by Corning Hazleton Inc. employees. Acetonitrile, HPLC grade, was obtained from Fisher (Fairlawn, NJ, USA). Acetic acid (analytical reagent grade), sodium acetate trihydrate and potassium dihydrogen phosphate (anhydrous) were obtained from EM Science (Gibbstown, NJ, USA). Methanol, HPLC grade, was obtained from Burdick and Jackson (Muskegon, MI, USA). Deionized water was processed through a Milli-Q water purification system, Millipore Corporation. The Bond Elut $C_8$ (1 ml) extraction cartridge was obtained from Varian (Harbor City, CA, USA).

### 2.2. Chromatographic systems

The HPLC system consisted of an SSI 222C micro pump (State College, PA, USA), a Perkin-Elmer ISS 100 autoinjector (Norwalk, CT, USA), and ABI/Kratos spectroflow 783 UV detector (San Jose, CA, USA) at 275 nm. The analytical column was a Zorbax RX $C_8$, 250×4.6 mm I.D., 5-$\mu$m particle size (Mac-Mod, Chadds Ford, PA, USA) protected by a Brownlee RP-8 pre-column (15×3.2 mm I.D., 7-$\mu$m particle size, ABI, San Jose, CA, USA). The mobile phase was 0.05 *M* potassium dihydrogen phosphate and 0.05 *M* sodium acetate (pH adjusted to 4.0 with acetic acid)–acetonitrile (53:47, v/v) with a flow-rate of 1 ml/min at ambient temperature. Sodium acetate was needed as an additive in the mobile phase to reduce tailing. Data collection and calculations were conducted using an HP1000 Model A900 computer with a 3350A Laboratory Automation System (Hewlett-Packard, Palo Alto, PA, USA).

### 2.3. Preparation of standard solutions

A stock standard solution of rifabutin (100 $\mu$g/ml) was prepared by dissolving 5.139 mg of rifabutin in 50 ml of methanol. The factor to correct purity was 0.973. A stock solution of internal standard (100 $\mu$g/ml) was prepared by dissolving 10 mg of sulindac in 100 ml of water. Working solutions of rifabutin (0.1 to 12 $\mu$g/ml) were prepared by diluting the stock solution with methanol. The internal standard working solution (2 $\mu$g/ml) was prepared by diluting the stock solution with water. The rifabutin standard solutions and the internal standard solutions were stored at 5°C. These solutions were stable for at least two months. All rifabutin standard solutions were protected from light to minimize photodegradation.

### 2.4. Quality control samples

Pooled quality control samples (controls) were prepared to determine the precision and accuracy of the method, and to evaluate the stability of samples. Over-curve control pools were also prepared to evaluate parallelism when specimens required analysis at partial volume.

Plasma control pools (20, 80, and 400 ng/ml) were prepared by diluting 400 $\mu$l of 10 $\mu$g/ml, 1600 $\mu$l of 10 $\mu$g/ml, and 800 $\mu$l of 100 $\mu$g/ml rifabutin, respectively, to a 200-ml volume, using blank human plasma. An over-curve control (1200 ng/ml) was prepared by diluting 600 $\mu$l of 100 $\mu$g/ml to a 50-ml volume with blank human plasma.

All control pools were aliquoted into 4-ml polypropylene vials and stored at approximately −20°C.

### 2.5. Sample preparation

Calibration standards were prepared by adding 50 $\mu$l of rifabutin working solutions to 1 ml of blank human plasma. Clinical specimens and controls were prepared by aliquoting 1 ml into glass tubes. Calibration standards, clinical specimens and controls were processed by adding 25 $\mu$l of internal standard into glass tubes, mixed by vortexing briefly and centrifuged at approximately 630 rpm for 5 min before loading onto the Bond Elut cartridges.

Each $C_8$ Bond Elut extraction column was con-

ditioned with 1 ml of methanol, followed by 1 ml of water. The plasma mixture was loaded onto the column and the column was washed with 1 ml of water. The analytes were eluted with 1 ml of methanol into 10×75-mm tubes. The methanol was dried down under nitrogen and the specimen reconstituted in 250 $\mu$l of mobile phase. Aliquots of 100 $\mu$l were injected onto the HPLC system.

### 2.6. Validation

Duplicate calibration curves were analyzed on each of three days. One reagent blank (water substituted for plasma), blank plasma, control zero (blank plasma spiked with internal standard) and triplicate controls at each concentration (20, 80, and 400 ng/ml of rifabutin in plasma) were analyzed with each calibration curve. The calibration curves were obtained by weighted ($1/C$) least-squares linear regression analysis of the peak height ratios of rifabutin/internal standard versus the concentration of rifabutin. The equations of the calibration curves were then used to calculate the concentration of rifabutin in the samples and controls from their peak height ratios.

## 3. Results and discussion

### 3.1. Separation

The molecular structures of rifabutin, its metabolite LM-565 and sulindac (internal standard) are shown in Fig. 1. The three compounds were well separated from each other as shown in Fig. 2c. Representative chromatograms from plasma extracts are also shown in Fig. 2. The mean retention times of rifabutin, and the internal standard were 10.8, and 6.9 min, respectively. The retention time for LM-565 was 5.8 min.

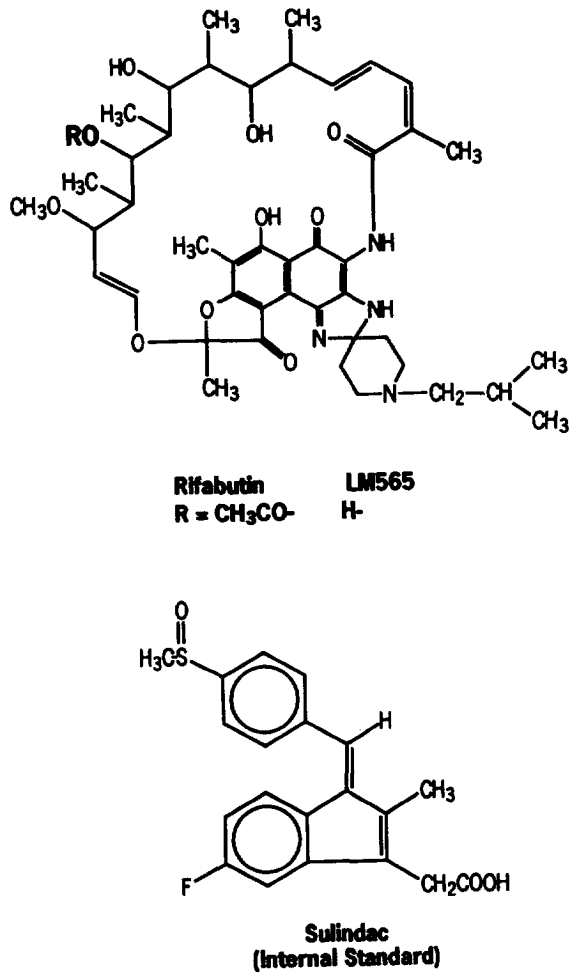


Fig. 1. Molecular structures of rifabutin, its metabolite, LM-565 and sulindac, internal standard.

### 3.2. Specificity

Blank plasma from seven pools was tested for endogenous interferences. When commercial plasma was used, the internal standard region was clean for all of the lots tested; however, the rifabutin region was not clean. The screening of the plasma donated by CHI was clean in both rifabutin and internal standard regions.

An assay specificity check with AIDS drug candidates atevirdine and its metabolite (U-89,255), as well as of delavirdine and its metabolite (U-96,183) was also conducted. Controls at each of the three concentration were spiked to give 10 and 100 $\mu M$ of these four compounds, and analyzed in duplicate. All four compounds were well separated from rifabutin and the internal standard. The retention times of atevirdine, U-89,255, delavirdine and U-96,183 are approximately 12.2, 5.4, 7.7 and 3.3 min, respectively. The presence of these four compounds does not

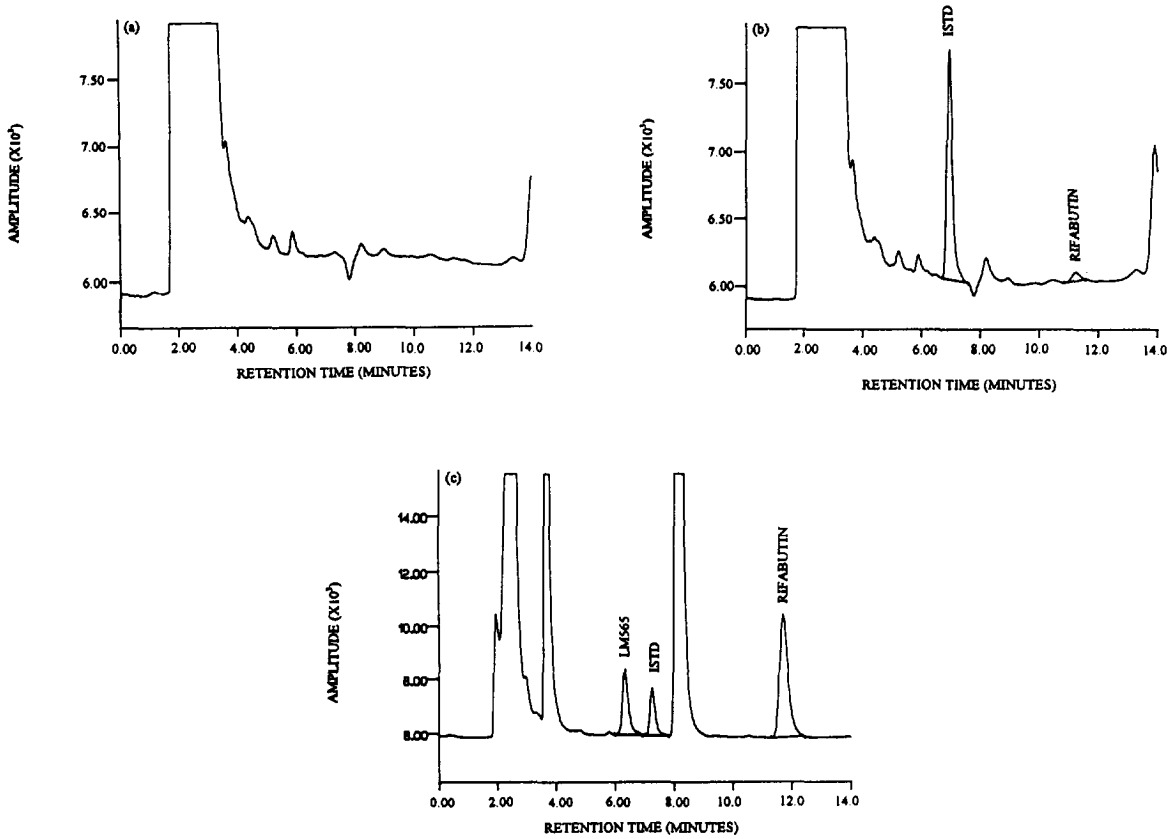


Fig. 2. Chromatograms of (a) blank human plasma, (b) 5 ng/ml calibration standard in human plasma, and (c) plasma from a patients taken 8 h after dosing with rifabutin.

significantly affect the accuracy of the control results.

### 3.3. Linearity, precision, and accuracy

Calibration curve data and parameters for rifabutin are given in Table 1 and Table 2. Calibration curves for rifabutin in plasma were linear over the concentration range of 5–600 ng/ml, with correlation coefficients ($r$) greater than 0.9977 for all curves.

Table 1
Calibration curve parameters for rifabutin in human plasma

| Day | Curve | Slope | Intercept | $r$ |
|---|---|---|---|---|
| 1 | 1 | $6.55 \cdot 10^{-3}$ | $2.59 \cdot 10^{-3}$ | 0.9994 |
| | 2 | $6.70 \cdot 10^{-3}$ | $-1.79 \cdot 10^{-2}$ | 0.9994 |
| 2 | 3 | $7.45 \cdot 10^{-3}$ | $-2.04 \cdot 10^{-2}$ | 0.9995 |
| | 4 | $6.82 \cdot 10^{-3}$ | $-1.67 \cdot 10^{-2}$ | 0.9999 |
| 3 | 5 | $7.08 \cdot 10^{-3}$ | $-1.02 \cdot 10^{-2}$ | 0.9977 |
| | 6 | $7.45 \cdot 10^{-3}$ | $-1.30 \cdot 10^{-2}$ | 0.9998 |

Results of validation studies over three-day periods.

Table 2
Calibration curve data for rifabutin in plasma

| Calibration standard concentration (ng/ml) | Calculated concentration (mean±S.D., $n=6$) (ng/ml) | R.S.D. (%) | Deviation (%) |
|---|---|---|---|
| 5 | 5.13±0.34 | 6.5 | 2.6 |
| 15 | 15.0±0.62 | 4.1 | 0.0 |
| 30 | 29.3±1.0 | 3.4 | −2.2 |
| 75 | 74.2±2.2 | 3.0 | −1.0 |
| 150 | 150±7.4 | 4.9 | −0.0 |
| 300 | 303±15.3 | 5.0 | 0.9 |
| 600 | 599±17.8 | 3.0 | −0.2 |

Results of validation study over a three-day period.

Table 3
Precision and accuracy of the assay for rifabutin in plasma

| Control concentration (ng/ml) | Calculated concentration (Overall mean±S.D., n=18) (ng/ml) | R.S.D. (%) | Deviation (%) |
|---|---|---|---|
| 20 | 21.1±1.23 | 5.8 | 5.4 |
| 80 | 83.9±5.7 | 6.8 | 4.9 |
| 400 | 415±25.9 | 6.2 | 3.8 |

Results of validation studies over a three-day period with six determinations per day.

Data from the spiked control samples are shown in Table 3. The within-day precision of the method as measured by the relative standard deviation (R.S.D.) of the daily mean ($n=6$) was less than 8.7% at the three control concentrations in human plasma. The overall precision was 5.8%, 6.8%, and 6.2% R.S.D. ($n=18$) for the 20-, 80-, and 400-ng/ml rifabutin controls, respectively.

The accuracy of the method was determined by comparing the means of the measured concentrations with the nominal (theoretical) concentrations of rifabutin in the plasma controls. All of the daily mean ($n=6$) and overall mean ($n=18$) values for the controls were within 9.3% of their expected values.

### 3.4. Limit of quantitation

The limit of quantitation (LOQ) was set at 5 ng/ml of rifabutin in plasma. Six replicates of the lowest standard (5 ng/ml) were analyzed to evaluate the LOQ. At the LOQ, the R.S.D. ($n=6$) of the peak height ratios was 7.1%, the R.S.D. ($n=6$) of the measured concentrations was 5.8%, and the deviation of the mean ($n=6$) of the measured concentrations from their nominal value was 5.6%.

### 3.5. Absolute recoveries

Absolute recoveries were determined by comparing the peak heights of extracted calibration standards with the peak heights of recovery standards prepared by mixing 25 μl of internal standard working solution and 50 μl rifabutin working solutions in 250 μl of methanol. The mean ($n=14$) recoveries for rifabutin, and the internal standard were 63.7% and 65.8%, respectively.

### 3.6. Parallelism

A control pool containing 1200 ng/ml rifabutin was prepared and analyzed at the partial volumes of 100 and 200 μl. The mean ($n=6$) values for both partial volumes were within 4.2% of their expected values. The precision was better than 3.3% R.S.D. ($n=6$) at both partial volumes.

In addition, the high control pool which contained 400 ng/ml rifabutin was prepared and analyzed at the partial volumes of 100 and 200 μl. The mean ($n=6$) values for both partial volumes were within 9% of their expected values. The precision was better than 3.8% R.S.D. ($n=6$) at both partial volumes.

### 3.7. Stability

Stock and working solutions of rifabutin in methanol (protected from light) were stable for at least two months when stored at 5°C. The stability of rifabutin was determined by measuring the concentration changes in the control samples over time. The plasma controls stored in polypropylene at −20°C were stable for more than two months. Stability was tested by subjecting the plasma controls to two and three freeze/thaw cycles, and storage for 24 h at room temperature. The thawing and refreezing of controls, and the storage of controls at room temperature had little effect on the precision or accuracy of the results. The mean ($n=3$) value was within 6.2% of their expected values.

Process stability was tested by extracting two sets of calibration standards with duplicate controls. One set was stored overnight at room temperature, and the other at 5°C before analyzing. The storage of extracted samples at room temperature or 5°C had little effect on the accuracy of the control results.

The mean value was within 10.5% and 13.3% of their expected values, at room temperature and 5°C, respectively.

## 4. Conclusions

This new assay procedure is precise and accurate for the quantitation of rifabutin in human plasma. The ruggedness of the assay has been demonstrated in assaying clinical specimens from patients dosed with rifabutin. Fig. 3 presents the mean pharmacokinetic profile of rifabutin from the study.

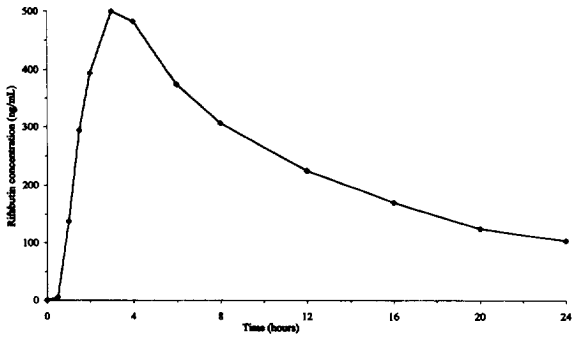


Fig. 3. Mean rifabutin plasma concentration from a clinical study.

## References

[1] L.B.Heifets, M.D. Iseman and P.J. Lindholm-Levy, in M. Casal (Editor), Mycobacteria of Clinical Interest, Elsevier, Amsterdam, 1986, p. 180.
[2] L.B. Heifets, M.D. Iseman and P.J. Lindholm-Levy, Drugs Exp. Clin. Res., 13 (1987) 529.
[3] M.H. Skinner, M. Hsieh, J. Torseth, D. Pauloin, G. Bhatia, S. Harkonen, T.C. Merigan and T. Blaschke, Antimicrob. Agents Chemother., 33 (1989) 1237.
[4] R.C. Lewis, N.Z. Hatfield and P.K. Narang, Pharm. Res., 8 (1991) 1434.
[5] J.B. Lecaillon, N. Febvre, J.P. Metayer and C. Souppart, J. Chromatogr., 145 (1978) 319.
[6] A.B.M. Jamaluddin, G. Sarwar, M.A. Rahim and M.K. Rahman, J. Chromatogr., 525 (1990) 495.
[7] K.J. Swart and M. Papgis, J. Chromatogr., 593 (1992) 21.
[8] M. Ishii and H. Ogata, J. Chromatogr., 426 (1988) 412.
[9] K. Chan, Methods Find. Exp. Clin. Pharmacol., 8 (1986) 721.
[10] M. Guillaumont, M. Leclercq, Y. Forbert, B. Guise and R. Harf, J. Chromatogr., 232 (1982) 369.
[11] A. Weber, K.E. Opheim, A.L. Smith and K. Wong, Rev. Infect. Dis., 5 (1983) S433.
[12] V. Vlasakova, J. Benes and K. Zivny, J. Chromatogr., 151 (1978) 199.