# A Sensitive Method for Quantitation of Rifabutin and Its Desacetyl Metabolite in Human Biological Fluids by High-Performance Liquid Chromatography (HPLC)

**Richard C. Lewis,[1] Naomi Z. Hatfield,[1] and Prem K. Narang[1,2]**

*Received March 27, 1991; accepted June 12, 1991.*

Sensitive HPLC-UV methodology has been developed and validated for quantitating rifabutin, an antimycobacterial, and its 25-desacetyl metabolite, LM-565, in human plasma and urine. The HPLC separation for both plasma and urine samples was performed on an ODS, 5-μm, reverse-phase column (25 cm × 4.6-cm ID) using a mobile phase of acetonitrile/0.05 *M* potassium phosphate, pH 4.2, with triethylamine, (38:61.5:0.5, v/v), at a flow rate of 1.0 ml/min. The separation eluate was monitored by absorbance at 275 nm. Plasma samples (1 ml) were spiked with an internal standard (medazepam), buffered at pH 7.4 and extracted with 80:20 (v/v) hexane:ethyl acetate, and then back extracted with acidified water (0.05 *M* $H_3PO_4$). Linearity was established between 5.0–800 and 2.5–400 ng/ml for rifabutin and LM-565, respectively. Intraday imprecision for rifabutin and LM-565 plasma quality controls prepared at 7.3 and 3.2 ng/ml, respectively, was <15% relative standard deviation (RSD). Absolute recovery for parent drug and metabolite, from plasma, was >90% throughout the respective dynamic ranges and >70% for medazepam. Urine samples (1 ml) were acidified with 50 μl of 3.6 *M* $H_2SO_4$ and diluted with 0.1 *M* ammonium acetate. Linearity was established between 100 and 5000 ng/ml for both rifabutin and LM-565. Intraday imprecision for a urine control at 200 ng/ml was ≤12% RSD for either component. The method is currently being used to support Phase I kinetics program for rifabutin in prophylaxis of MAC infection of AIDS patients. Application of this method to a bioavailability assessment is presented.

KEY WORDS: high-performance liquid chromatography (HPLC); method validation; rifabutin and metabolite; sensitive; biological fluids.

## INTRODUCTION

Rifabutin, 4-deoxo-3,4-[2-spiro-(*N*-isobutyl-4-piperidyl)-2,5-dehydro-1H-imidazo]rifamycin S (Fig. 1), is a semisynthetic derivative of rifampicin S with broad-spectrum antibacterial activity against Gram-positive and negative microorganisms and mycobacteria (1). The drug is currently being evaluated in the United States and Europe for its effectiveness against mycobacterial infections afflicting patients with acquired immune deficiency syndrome (AIDS). The major metabolite of rifabutin is LM-565, the 25-desacetyl derivative (Fig. 1), which also exhibits antibacterial activity but is much less lipophilic than the parent compound (2). Quantification of both components in plasma and urine is deemed necessary to assess pharmacokinetics of the parent drug adequately.

Both normal and reverse-phase HPLC methods have been developed for the related antibiotic, rifampin, and its 25-desacetyl metabolite (3–6). These methods employed liquid/liquid extraction with UV absorbance detection. Another method employed direct injection of serum samples into an HPLC system that exploited the selectivity offered by column switching and electrochemical detection (7). The lower limit of quantitation (LLOQ) for these methods ranged from 100 to 500 ng/ml. For rifabutin, a more sensitive (25 ng/ml LLOQ) HPLC method employing UV detection has also been reported (8), but this methodology did not utilize an internal standard.

We describe here the development and validation of HPLC methodology for rifabutin and LM-565 in plasma and urine with improved sensitivity and an enhanced precision due to inclusion of an internal standard, medazepam (Fig. 1).

## EXPERIMENTAL

### Materials

Rifabutin and LM-565 reference standards (free base) were obtained from Farmitalia Carlo Erba (Milan, Italy). The internal standard, medazepam (IS), was kindly provided by Dr. D. Abernathy of Roger Williams General Hospital, Providence, RI, but can also be purchased from Sigma (St. Louis, MO).

HPLC-grade acetonitrile, ethyl acetate, hexane, and water were purchased from either EM Science (Cincinnati, OH) or American Burdick and Jackson (Muskegon, MI), Triethylamine (TEA; Gold Label) was obtained from Aldrich (Milwaukee, WI) and 1-heptane sulfonate sodium (HPLC grade) was procured from Kodak (Rochester, NY). All other chemicals were reagent grade and used without further purification. Blank human plasma used to prepare spiked standards and controls was purchased from the American Red Cross (Columbus, OH).

### Analytical Instrumentation and System Conditions

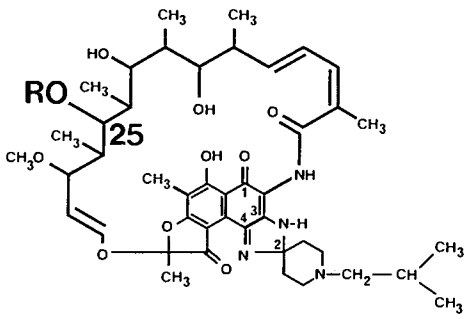
The HPLC system consisted of a Spectroflow 400 pump, a Spectroflow 783 absorbance detector (Applied Biosystems, Ramsey, NJ), and an ISS-100 autosampler (Perkin-Elmer, Norwalk, CT). The detector analog signal (1 V) was digitized by a Nelson 941 Interface, then acquired and processed by ACCESS*CHROM data acquisition software (both supplied by PE Nelson, Cupertino, CA) residing on a MicroVAX 2000 computer (Digital, Maynard, MA).

The chromatographic separation was performed on a 5-μm Ultrasphere ODS reverse-phase column (25 cm × 4.6-mm ID; Beckman, Fullerton, CA) with a RP-18 NewGuard precolumn (15 × 3.2 mm; Brownlee, Santa Clara, CA). Both columns were maintained at 40°C by a column heater (TC-50, Fiatron Systems, Oconomowoc, WI). The mobile phase

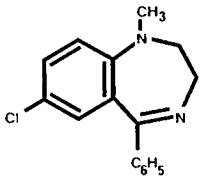
[1] Bioanalytical Research Section, Department of Pharmacokinetics/Dynamics, Medical Research Center, Adria Laboratories, Division of Erbamont, Dublin, Ohio 43017.

[2] To whom correspondence should be addressed at Department of Pharmacokinetics/Dynamics, Adria Laboratories, 7001 Post Road, Dublin, Ohio 43017.

0724-8741/91/1100-1434$06.50/0 



| | Rifabutin | LM565 |
|---|---|---|
| R= | $CH_3CO$- | H- |
| M.W.= | 847.12 | 805.08 |



Medazepam, Internal Standard

Fig. 1. Molecular structures for rifabutin, LM-565, and medazepam.

for both plasma and urine bioanalytical separations was acetonitrile/0.05 *M* monobasic potassium phosphate, pH 4.2/TEA (38:61.5:0.5, v/v). Using a flow rate of 1.0 ml/min, a typical back pressure of 80 bar was generated. Column effluent was monitored by absorbance at 275 nm. A vortex evaporator (Buchler, Fort Lee, NJ) was used for drying samples.

### Standards and Controls

Rifabutin and LM-565 methanolic stock solutions, and subsequent serial dilutions were prepared using amber volumetric flasks to minimize photodegradation. The methanolic spiking solutions (working standards) were aliquoted into leakproof polyethylene vials and stored at 4°C until use.

*Plasma Standards and Controls.* Aliquots of working standards (20.0 μl) were spiked fresh daily into blank plasma (1 ml) to provide plasma standards in the ranges of 2.5–400 and 5–800 ng/ml for LM-565 and rifabutin, respectively. Pooled controls were prepared in human blank plasma in the ranges of 3 and 300 and 7 and 700 ng/ml for LM-565 and rifabutin, respectively. Aliquots of these control samples were pipetted into polypropylene vials and stored at −20°C until assayed. A methanolic solution of medazepam (5 μg/ml) was employed as an internal standard.

*Urine Standards and Control.* Urine standards were prepared fresh daily by spiking 20.0-μl aliquots of working standards to 1 ml of blank urine. A dynamic range of 100–5000 ng/ml was chosen for both rifabutin and LM-565. A pooled control containing 200 ng/ml of each component was also prepared, and aliquots were stored in polypropylene vials at −20°C until use. A methanolic solution of medazepam (50 μg/ml) was employed as an internal standard.

### Sample Workup Procedure

*Plasma and Urine Samples.* Twenty microliters of internal standard solution, and where applicable, 20 μl each of the appropriate rifabutin and LM-565 methanolic working standards were added to a 15-ml polypropylene tube. After adding 1 ml of blank plasma or urine for standards, or 1 ml of unknown or quality control sample to respective tubes, processing proceeded as follows.

*Plasma Samples.* One milliliter of aqueous buffer containing 0.25 *M* potassium phosphate, monobasic, and 0.05 *M* 1-heptane sulfonate sodium (pH adjusted to 7.4) was added to each sample then extracted twice with 7 ml of hexane: ethyl acetate, 80:20 (v/v). The tube was vortex-mixed (10 sec) and centrifuged (10 min at 1200 rpm) for each extraction. The organic layers were combined in a clean 15-ml polypropylene tube and evaporated to dryness at 40°C under vacuum (20 in. Hg). The dried extract was reconstituted with 250 μl of extraction solvent, and back extracted with 200 μl of 0.05 *M* aqueous phosphoric acid. After vortex-mixing (10 sec) and centrifuging (1–2 min at 1200 rpm), the aqueous layer was frozen at −20°C in a dry ice/2-propanol bath. The organic layer was discarded and the frozen aqueous layer was rinsed with hexane (1 ml), thawed, and dried (10 min at ambient temperature) in a vortex evaporator to eliminate residual ethyl acetate. The remaining aqueous layer (ca. 200 μl) was buffered with 100 μl of 0.25 *M* methanolic ammonium acetate, then transferred to a polypropylene autosampler microvial, and 100 μl was injected.

*Urine Samples.* Urine standards and samples (1 ml) were acidified with 50 μl of 3.6 *M* aqueous sulfuric acid. A 200-μl aliquot of the acidified urine was mixed with 800 μL of 0.1 *M* ammonium acetate in water:methanol, 60:40 (v/v). After transferring the sample to a polypropylene autosampler vial, a 100-μl aliquot was injected.

### Calculations

Peak-height ratios were calculated for both rifabutin and LM-565 relative to medazepam. Standard curves were constructed for both components using an unweighted least-squares linear regression of peak-height ratio versus component concentration in the plasma or urine standards. To accommodate the large dynamic range in plasma, a split curve was used. This approach improved accuracy for low level samples relative to a single curve approach. A single curve was employed for the urine. The peak-height ratios for the unknown and quality-control samples were converted to concentration estimates by employing the appropriate linear regression parameters.

### Linearity

Linearity of rifabutin and LM-565 was evaluated in both plasma and urine on each of at least 4 and 2 days, respectively. Correlation coefficient and analysis of residuals by scattergram were used to assess linearity and bias in the linear regression model fit.

### Accuracy and Precision

Accuracy and precision were assessed by assaying qual-

ity-control samples in a replicate of six on each of at least 2 days.

### Detection Limits

The instrumental limit of detection (ILOD) and the lower limit of quantitation (LLOQ) were evaluated for both rifabutin and LM-565. ILOD was defined as the lowest amount of the component in clean solutions, which could produce a discernible peak [signal-to-noise (S/N) ratio = 3]. LLOQ was defined as the lowest amount of the component in the sample matrix which could be quantified with acceptable precision and accuracy.

### Absolute Recovery

*Plasma Assay.* The absolute recovery of rifabutin and LM-565 was determined by spiking human plasma at three concentrations in replicate of five. The spiked samples were extracted as described previously, with the IS added just prior to injection. To calculate absolute recovery, the peak-height ratios for rifabutin and LM-565 from the extracted samples were compared to those from unextracted solution standards. In similar fashion, the absolute recovery of medazepam was determined at 100 ng/ml in human plasma. Rifabutin was added as an IS just prior to injection to compensate for volume variations.

*Urine Assay.* The absolute recovery of rifabutin, LM-565, and medazepam was determined by comparing component peak heights from spiked urine to those from an unextracted solution standard.

### Specificity

To verify the specificity of the method, several drugs which could be administered concomitantly with rifabutin, including amikacin sulfate, clofazimine, isoniazid, streptomycin sulfate, zidovudine, and its glucuronide metabolite, were injected using the same chromatographic conditions.

### Stability

The stability of rifabutin, LM-565, and medazepam in methanolic solution at 4°C, in extracted plasma samples at room temperature, and in plasma at −20°C was also evaluated.

## RESULTS AND DISCUSSION

### Chromatography

Typical retention times for LM-565, medazepam, and rifabutin were 9, 19, and 22 min, respectively. The rigorous extraction procedure for plasma provided chromatographic windows consistently free from endogenous plasma interferences. Representative chromatograms for a human plasma blank and low- and midrange standards are shown in Fig. 2. For urine, solvent front interferences required quantification of LM-565 from a sloping baseline. Representative human urine blank and low standard chromatograms are shown in Fig. 3. Typical run time for both plasma and urine samples was 27 min each; late-eluting peaks were only seldom encountered throughout this validation. The retention time for medazepam was found to be highly dependent upon the mobile-phase pH. Therefore, the aqueous buffer/TEA solution was adjusted to pH 4.2 with $H_3PO_4$ prior to the addition of acetonitrile. Peak resolution could be altered by decreasing the pH by 0.1, which reduced medazepam's retention without affecting that of rifabutin.

Column temperature was adjusted to 40°C, which improved the selectivity for LM-565 relative to ambient conditions. At this elevated temperature, LM-565's capacity factor increased, while the solvent front interferences compressed toward the void volume. Chromatographic ruggedness was demonstrated throughout the validation exercise as component retention times remained constant, and only a minor increase in back pressure was observed over several hundred injections.

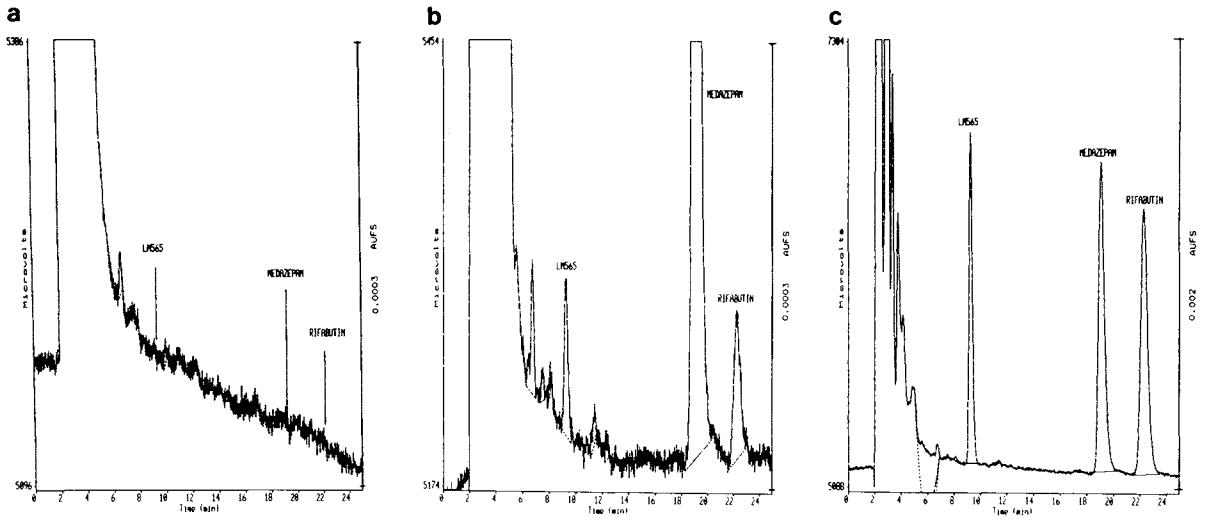



Fig. 2. (a) Typical extracted human plasma blank. (b) Low human plasma standard: LM-565 (2.61 ng/ml), medazepam (103 ng/ml), and rifabutin (4.83 ng/ml). (c) Midrange human plasma standard: LM-565 (52.2 ng/ml), medazepam (103 ng/ml), and rifabutin (96.7 ng/ml).

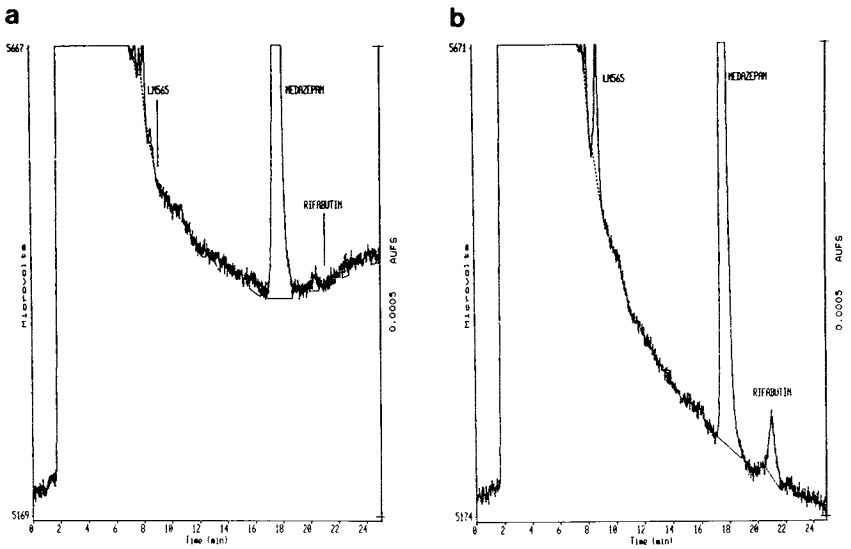



Fig. 3. (a) Typical human urine blank. (b) Low human urine standard: LM-565 (97.9 ng/ml), medazepam (1048 ng/ml), and rifabutin (98.7 ng/ml).

### Linearity

*Plasma Assay.* For rifabutin, the low and high standard curve ranges were 5–60 and 60–800 ng/ml. For LM-565, the low and high standard curve ranges were 2.5–30 and 30–400 ng/ml. Using a single curve for the entire dynamic range produced unacceptable bias (>25%) for the back-calculated values of the low standard. Acceptable accuracy was obtained when a split curve approach was used. Correlation coefficients for rifabutin ranged from 0.995 to 0.999, while those for LM-565 ranged from 0.989 to 0.999. The evaluation of back-calculated standard values showed random scatter of residuals with no obvious directional runs. Mean slopes (±%RSD) of low and high curves were $8.70 \times 10^{-3}$ (±3.5%) and $8.19 \times 10^{-3}$ (±4.9%), respectively, for rifabutin, and $20.7 \times 10^{-3}$ (±8.1%) and $18.9 \times 10^{-3}$ (±6.5%), respectively, for LM-565.

*Urine Assay.* The entire dynamic range for both rifabutin and LM-565 in urine (100–5000 ng/ml) resulted in back-calculated standard values with acceptable accuracy (<±15% bias). Correlation coefficients were greater than 0.999 for rifabutin and its metabolite. Mean slopes (±%RSD) were $6.12 \times 10^{-4}$ (±5.1%) and $1.45 \times 10^{-3}$ (±2.1%) for rifabutin and LM-565, respectively, with negligible mean $y$ intercept.

### Accuracy and Precision

The accuracy and precision of bioanalytical methods employed during early Phase I/II drug development program are fast becoming recognized as critical components of such investigations.

*Plasma Assay.* As shown in Tables Ia and b, the intra- and interday imprecisions, expressed as percentage RSD for both rifabutin and LM-565 were <20% over the dynamic ranges between 7–700 and 3–300 ng/ml, respectively. Method accuracy expressed as percentage bias was <±15% for the low control (7.2 and 3.2 ng/ml for rifabutin and LM-565, respectively) and generally <±10% at the other control levels. The average intraday imprecision for rifabutin ranged from 13% at the low end to 6.4% RSD at the upper end.

*Urine Assay.* The intra- and interday imprecision was generally <10% RSD for both rifabutin and LM-565 at 200 ng/ml (Table Ic). For these control samples, the experimentally found component levels were within ±9% of the label claim.

### Detection Limits

Instrumental limit of detection (ILOD) for rifabutin and LM-565 was 0.8 and 0.4 ng on column, respectively, while the lower limits of quantitation (LLOQ) for the plasma assay were 5 and 2.5 ng/ml, respectively. At these levels, S/N for either component was >5, with accuracy and imprecision of <±15 as estimates of percentage bias and percentage RSD.

For the urine assay, the LLOQ for both components was established at 100 ng/ml. Performance of the low-level urine standards equaled or exceeded the above criteria used to define LLOQ for the plasma assay.

### Absolute Recovery

*Plasma Assay.* The absolute recoveries for rifabutin and LM-565 in the ranges of 10–800 and 5–400 ng/ml, respectively, were >90% with <5.0% RSD. The addition of an ion pairing agent, 1-heptane sulfonate sodium, to the aqueous buffer–plasma mixture substantially increased the extraction efficiency of the parent drug and its metabolite (LM-565) into the organic phase (hexane:ethyl acetate, 80:20). For plasma standards below 50 ng/ml, a decrease in absolute recovery was observed when glass was used for storage of working standards and for sample processing. This problem was circumvented by employing methanol as a diluent for working standards and polypropylene and polyethylene labware for storage of working standards, sample processing, and storage of extracted samples in the autosampler.

The absolute recovery for medazepam was 72% with

Table I. Method Accuracy and Precision for Rifabutin (a) and LM-565 (b) in Plasma and for Rifabutin and LM-565 in Urine (c)[a]

| Label conc. of control sample (ng/ml) | Day | n | Accuracy | | Precision | | |
|---|---|---|---|---|---|---|---|
| | | | Mean found (ng/ml) | % bias | SD | % RSD (intraday) | Pooled % RSD (interday) |
| | | | (a) Rifabutin in plasma | | | | |
| 7.26 | 5 | 6 | 8.12 | +11.8 | 0.858 | 10.5 | |
| | 6 | 5 | 6.27 | −13.6 | 0.878 | 14.0 | 17.4 |
| 29.0 | 1 | 6 | 27.6 | −4.8 | 1.82 | 6.61 | |
| | 2 | 5 | 30.8 | +6.2 | 3.29 | 10.7 | |
| | 3 | 6 | 27.8 | −4.1 | 2.88 | 10.4 | |
| | 4 | 6 | 25.6 | −11.7 | 2.03 | 7.93 | 10.8 |
| 144 | 1 | 6 | 142 | −1.4 | 13.1 | 9.23 | |
| | 2 | 6 | 151 | +4.9 | 7.84 | 5.19 | |
| | 3 | 6 | 147 | +2.0 | 21.9 | 14.9 | |
| | 4 | 6 | 138 | −4.2 | 8.78 | 6.36 | 9.82 |
| 726 | 1 | 6 | 810 | +11.5 | 41.8 | 5.16 | |
| | 2 | 6 | 730 | +0.5 | 45.2 | 6.20 | |
| | 3 | 6 | 775 | +6.7 | 80.2 | 10.3 | |
| | 4 | 6 | 716 | −1.3 | 28.2 | 3.94 | 8.20 |
| | | | (b) LM-565 in plasma | | | | |
| 3.19 | 5 | 6 | 3.69 | +15.6 | 0.495 | 13.4 | |
| | 6 | 6 | 2.76 | −13.4 | 0.350 | 12.7 | 19.7 |
| 12.7 | 1 | 6 | 11.9 | −6.3 | 0.840 | 7.06 | |
| | 2 | 5 | 13.4 | +5.5 | 1.27 | 9.48 | |
| | 3 | 6 | 12.8 | +0.8 | 1.12 | 8.78 | |
| | 4 | 6 | 10.6 | −16.5 | 0.879 | 8.29 | 12.0 |
| 63.4 | 1 | 6 | 61.4 | −3.1 | 7.31 | 11.9 | |
| | 2 | 6 | 70.0 | +10.4 | 4.30 | 6.14 | |
| | 3 | 6 | 64.7 | +2.1 | 9.18 | 14.2 | |
| | 4 | 6 | 61.6 | −2.8 | 3.97 | 6.45 | 10.9 |
| 319 | 1 | 6 | 396 | +24.1 | 32.5 | 8.23 | |
| | 2 | 6 | 322 | +0.9 | 21.3 | 6.63 | |
| | 3 | 6 | 345 | +8.2 | 39.6 | 11.4 | |
| | 4 | 6 | 316 | −0.9 | 16.6 | 5.25 | 12.1 |
| | | | (c) Rifabutin and LM-565 in urine | | | | |
| Rifabutin, 197 | 1 | 6 | 188 | −4.5 | 16.3 | 8.67 | |
| | 1 | 6 | 212 | +7.6 | 15.6 | 7.35 | |
| | 2 | 5 | 190 | −3.5 | 8.58 | 4.51 | |
| | 2 | 6 | 206 | +4.5 | 24.7 | 12.0 | 9.78 |
| LM-565, 195 | 1 | 6 | 212 | +8.7 | 18.9 | 8.91 | |
| | 1 | 6 | 208 | +6.6 | 18.8 | 9.03 | |
| | 2 | 6 | 203 | +4.1 | 8.14 | 4.01 | |
| | 2 | 6 | 206 | +5.6 | 7.89 | 3.83 | 6.72 |

[a] Two standard curves were run on each day along with two groups of controls.

<10% RSD. This estimate appeared more variable than those of the parent drug and metabolite. We attribute this to trace amounts of ethyl acetate in the acidified water layer after the back extraction step. As stated earlier under Sample Workup Procedure, the acidified aqueous layer was dried for 10 min to eliminate the majority of the residual ethyl acetate from the back extraction step. Without this drying step, the prepared sample was often turbid and the absolute recovery of medazepam depressed (i.e., <50%).

*Urine Assay.* For all components, absolute recoveries were greater than 90%. Simply diluting urine (1:5) with water or mobile phase resulted in variable and low recovery of all three components. Low recovery was also observed when water was spiked with rifabutin and medazepam, then diluted with mobile phase. This suggested a solubility related problem. Quantitative recoveries were achieved when urine samples (1 ml) were acidified with 50 μl of 3.6 *M* $H_2SO_4$ and then diluted with buffer (pH 4.5). Simply buffering spiked urine or water to pH 4.5 without first acidifying resulted in low recovery, especially for rifabutin (<60%). This observation suggests that factors other than pH-dependent ionization could be responsible for rifabutin aqueous solubility. Micelle formation has been reported (9) for rifampin under acidic conditions (pH 0–4). This phenomenon could partially explain rifabutin's unusual recovery behavior from urine and water.

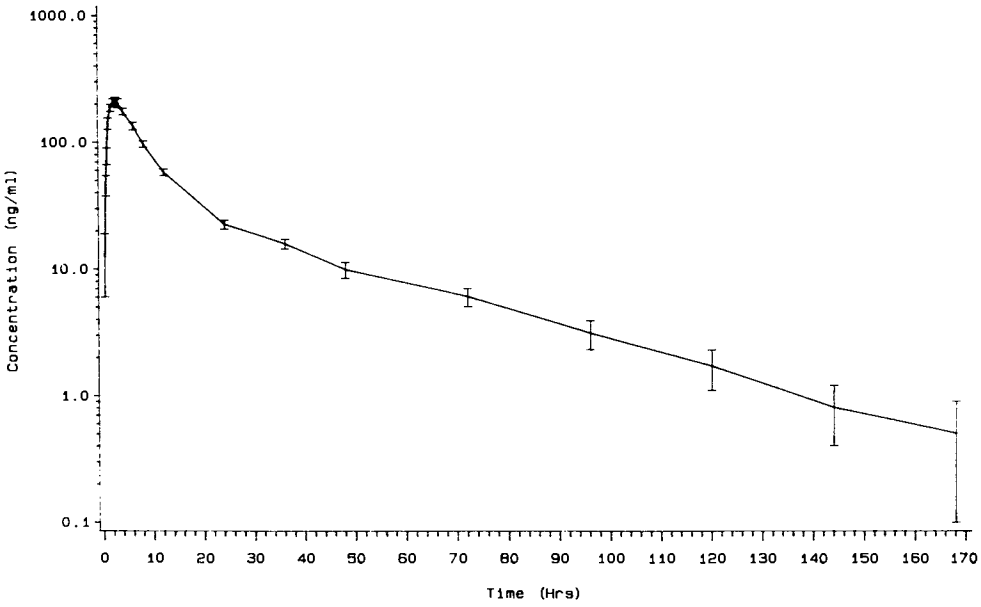



Fig. 4. Semilogarithmic plot of mean plasma rifabutin concentration as a function of time following a single oral dose of 150 mg of rifabutin as a solution in fasted healthy volunteers.

### Specificity

Amikacin sulfate, isoniazid, streptomycin sulfate, zidovudine, and its glucuronide metabolite were either undetected (solution concentrations in the therapeutic range) or eluted at the void volume when injected on column. Clofazimine which survives the extraction procedure eluted approximately 90 min after injection. Therefore, the use of these concomitant medications in AIDS patients should not interfere with the quantification of rifabutin or LM-565.

### Stability

No degradation was observed in methanolic solutions for any of the components in stock or working standard solutions for at least 1 month when stored at 4°C. A positive bias was observed for stored methanolic standards relative to those freshly prepared. This bias was attributed to gradual evaporation of the working standards during a month's storage at 4°C. Leakproof polyethylene vials eliminated this problem. Rifabutin showed no observable degradation in extracted plasma samples over 48 hr when stored in polypropylene autosampler vials at room temperature. Rifabutin plasma controls prepared at 10 and 250 ng/ml exhibited no quantifiable degradation in plasma over 3 months at −20°C. These same controls, subjected to five freeze/thaw cycles over a 1-day period, demonstrated no observable degradation.

### Method Application

This new assay procedure has recently been applied to a Phase I study conducted in healthy human volunteers, assessing the bioavailability of the capsule dosage form relative to a solution and the effect of food. Figure 4 shows a semilogarithmic plot of mean plasma rifabutin concentration as a function of time in 12 subjects. The mean maximum concentration ($C_{max}$), time to achieve maximal concentration ($T_{max}$), and terminal elimination half-life ($t_{1/2},\lambda_z$) estimates for rifabutin, following a solution dosage form under the fasted state, were 237 ng/ml, 2.5 hr, and 41 hr, respectively. The coefficients of variation (%CV) for these parameters ranged from 16 to 53%. The relatively small magnitude of %CV not only reflects a controlled population of healthy volunteers but also the performance of this assay within the criteria established by the rigorous validation detailed in this report. These data show that the assay method can be effectively applied to the kinetic evaluation of this drug.[3]

## CONCLUSIONS

A significant improvement in plasma assay sensitivity has been achieved by implementing a back extraction into acidified water and by employing polypropylene and polyethylene labware in place of glassware. The defined LLOQs for this method are 5 and 2.5 ng/ml for rifabutin and LM-565, respectively, representing a 20-fold increase over previous work with rifampin (3–7). The rigorous extraction workup from plasma was necessary to assure consistently clean chromatographic windows. Addition of an internal standard (medazepam) and quantitative extraction efficiencies for rifabutin and LM-565 suggest adequate method control. In addition, a simple and specific assay for quantifying rifabutin and LM-565 in urine has been developed and validated within the range of 100–5000 ng/ml. These two methods are intended to provide bioanalytical support for current human clinical pharmacology and pharmacokinetic studies with rifabutin.

## ACKNOWLEDGMENTS

Harris Laboratories performed the plasma assays used

[3] The data beyond 96 hr represent information generated during method validation at the site. Use of an increased sample volume resulted in a two- to threefold increase in the LLOQ.

to construct the semilog plot in this report. The efforts of Mr. Alan Dzerk and Ms. Jean Calahan of Harris Laboratories in extending aspects of this work are particularly acknowledged.

## REFERENCES

1. C. Della Bruna, G. Schioppacassi, D. Ungheri, D. Jabes, E. Morvillo, and A. Sanfilippo. LM 427, a new spiropiperidylrifamycin: *In vitro* and *in vivo* studies. *J. Antibiot.* **36**:1502–1506 (1983).
2. D. Ungheri *et al.* Activity of LM 427 on Legionella spp.: *In vitro* study and intracellular killing. *14th International Congress of Chemotherapy*, Kyoto, June 23–28, 1985, abstract, p. 417, P-47-93.
3. J. B. Lecaillon, N. Febvre, J. P. Metayer, and C. Souppart. Quantitative assay of rifampicin and three of its metabolites in human plasma, urine, and saliva by high-performance liquid chromatography. *J. Chromatogr.* **145**:319–324 (1978).
4. B. Ratti, R. Parenti Rosina, A. Toselli, and L. F. Zerilli. Quantitative assay of rifampicin and its main metabolite 25-desacetylrifampicin in human plasma by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **225**:526–531 (1981).
5. M. Guillaumont, M. Leclercq, Y. Frobert, B. Guise, and R. Harf. Determination of rifampicin, desacetylrifampicin, isoniazid, and acetylisoniazid by high-performance liquid chromatography: Application to human serum extracts, polymorphonucleocytes and alveolar macrophages. *J. Chromatogr.* **232**:369–376 (1982).
6. M. Ishii and H. Ogata. Determination of rifampicin and its main metabolites in human plasma by high-performance liquid chromatography. *J. Chromatogr.* **426**:412–416 (1988).
7. S. Oldfield, J. D. Berg, H. J. Stiles, and B. M. Buckley. Measurement of rifampicin and 25-desacetylrifampicin in biological fluids using high-performance liquid chromatography with direct sample injection. *J. Chromatogr.* **377**:423–429 (1986).
8. E. Moro, V. Bellotti, P. Marrari, E. Pianezzola, S. Stocco, and G. Valzelli. Assay of LM 427 (rifabutin) and its metabolites by HPLC in human plasma and urine. *Tenth International Symposium on Column Liquid Chromatography*, San Francisco, May 18–23, 1986, abstract 2740.
9. G. G. Gallo and P. Radaelli. In K. Florey (ed.), *Rifampicin: Analytical Profiles of Drug Substances*, Vol. 5, 1976, pp. 490–491.