



# Difficulties in Developing a Sensitive Assay for the Quantification of Rifampin in Multiple Biological Matrices by LC-MS/MS

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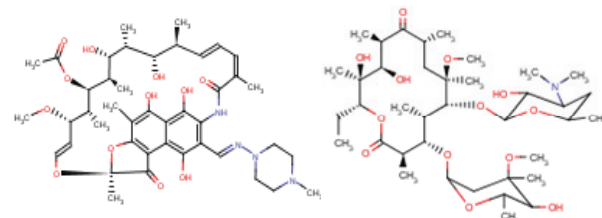
## Introduction and Purpose

BASi previously developed an LC-MS/MS method for the quantification of rifampin in human plasma with a lower limit of quantification (LLOQ) of 200 ng/mL.

The intent of this research was to develop a validated analytical method for the detection of rifampin in rabbit plasma and cerebrospinal fluid (CSF) with a lower limit of quantification 200 fold lower than the human method or 1 ng/mL.

Several difficulties arose while attempting to decrease the lower limit of quantification while keeping the sample size constant.

## Structures of Rifampin and Internal Standard



Rifampin

Internal Standard

## Binding in Aqueous Solutions

Early experiments showed that rifampin experienced binding in plasma. Further experiments discovered that this phenomenon was not limited to biological matrices. The following data shows the results of several successive transfers of aqueous solutions of rifampin between polypropylene tubes (n=6).

	Rifampin in MQ after 0 Transfers	Rifampin in MQ after 6 Transfers
	31156	8077
	29264	6991
	19183	2956
	7424	2016
	10135	3165
	11277	3349
AVE	18135	4460
%Decrease		75

Previously experience in this laboratory has indicated that binding can be reduced by the introduction of an organic solvent. The following data shows the results of several successive transfers of 50% methanol solutions of rifampin between polypropylene tubes (n=6).

	Rifampin in 50% MeOH after 0 Transfers	Rifampin in 50% MeOH after 6 Transfers
	78134	72750
	79609	72522
	81906	73366
	81622	77477
	80580	78855
	81814	80754
AVE	80664	77752
%Decrease		4

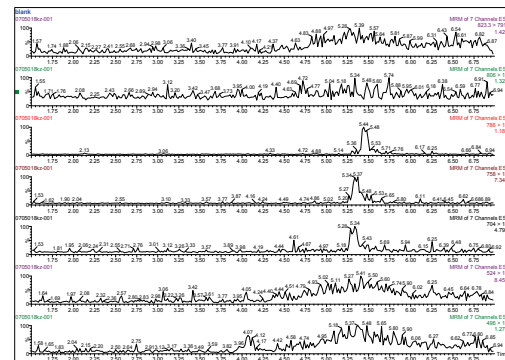
Binding was also observed in urine.

## Phospholipid Interferences

Early experiments examining samples at or near the LLOQ showed a large variability (%CV) between samples. To determine if the variability was due to co-eluting phospholipids, a 1 µg/mL solution of rifampin was infused via a T-union into the mass spectrometer while extracted plasma blanks were injected using the autosampler. Several common phospholipid transitions were monitored along with a transition for rifampin.

Compound	Transition Monitored
Rifampin	823 > 791
	806 > 184
	786 > 184
	758 > 184
Phospholipid	704 > 184
	524 > 184
	496 > 184

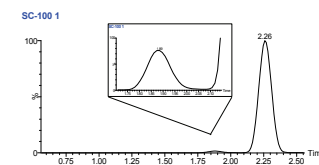
The data below suggests that phospholipids are not the cause of the variability between samples.



Rifampin and phospholipid transitions monitored during the injection of an extracted plasma blank while infusing rifampin.

## Multiple Chromatographic Peaks

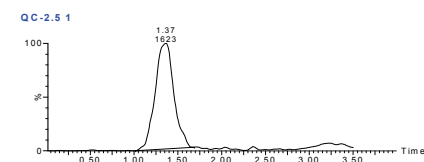
The internal standard produced multiple peaks when examined by LC-MS/MS. The peaks were present under a variety of chromatographic conditions. Reference material from two different lots from two different suppliers (including USP) produced two peaks. Monitoring multiple transitions produced two peaks for both reference materials. Each peak was evaluated for quantitation and no significant differences were detected. The larger peak was chosen for use in this method. Both peaks were evaluated for chromatographic purposes and there was no significant difference between the two. The larger peak was chosen for use in quantitation.



Chromatogram of the internal standard showing two peaks. The later peak was used for quantitation.

## Increasing Sensitivity

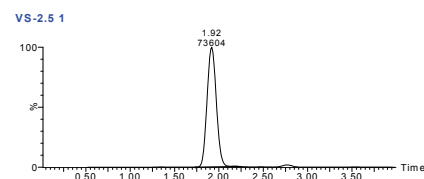
Initial experiments attempted to extend the LLOQ from 200 ng/mL to 1.0 ng/mL in plasma. The original analytical method for the extraction of rifampin from plasma utilized liquid-liquid extraction using MTBE. Experiments determined that liquid-liquid extraction did not provide sufficient sensitivity near the desired LLOQ of 1.0 ng/mL. The extraction method was changed to protein precipitation with 200 µL of methanol using a 100 µL sample size. The supernatant would then be evaporated and the dried residue would be reconstituted in 100 µL of mobile phase. 10 µL of the reconstituted samples were injected onto the column.



Typical chromatogram of 2.5 ng/mL rifampin extracted from rabbit plasma.

Unfortunately, there was a large variation in the samples (>15% CV). It was determined that the source of this variation was the evaporation and reconstitution step. Direct injection of the supernatant did not provide adequate sensitivity to achieve the target LLOQ. In addition, the supernatant was very dirty as evidenced by the buildup of residue on the sampling cone of the mass spectrometer.

Sensitivity was increased by utilizing column switching to concentrate and cleanup the sample prior to the analytical column. Samples prepared by protein precipitation were injected onto a cleanup column at a flow rate of 2.0 mL/min. After 45 seconds, samples were backflushed off of the cleanup column onto the analytical column for analysis. The injection volume was increased to 50 µL. Using the column switching method, the variability between samples decreased from >15% to <10%.



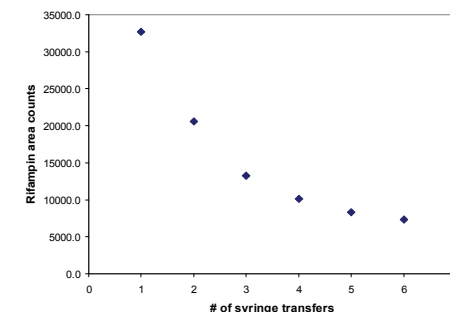
Typical chromatogram of 2.5 ng/mL rifampin extracted from rabbit plasma. Sample was analyzed using the column switching technique.

## Rifampin in CSF

Binding of rifampin in artificial CSF solutions was also evaluated. Rifampin prepared in artificial CSF showed area counts decreasing by 58% with just one transfer between polypropylene tubes. The addition of methanol to the artificial CSF reduced signal decreases to less than 5%.

With previous experiments indicating that rifampin would bind to plastic, experiments were undertaken to determine the extent of binding of rifampin to a syringe. A solution of artificial CSF was fortified with rifampin and placed in a 96-well plate. Fractions of the solution were transferred to new wells using a fresh 3 mL polypropylene syringe with a 1.5 inch, 22G metal needle. Methanol and internal standard were then added and the solutions were analyzed by LC-MS.

The following figure shows the results of this experiment with a 37% decrease in signal following a single transfer of rifampin in CSF with a syringe.

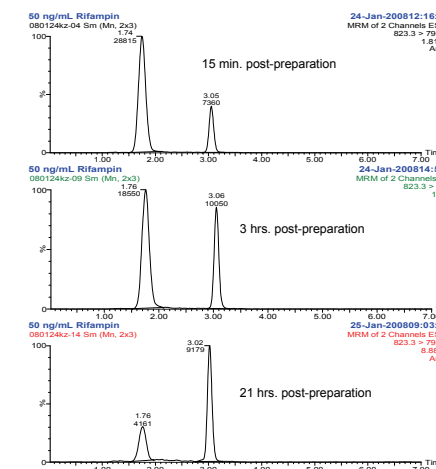


## Rifampin in Urine

Rabbit urine was also assessed as a potential matrix for use with this method. Rifampin prepared in urine showed binding similar to what was observed in aqueous solutions. This could be prevented by the addition of methanol to the urine, as was performed with CSF.

Rifampin samples prepared in urine produced a second peak in the rifampin channel approximately 1.2 minutes after the elution of the primary rifampin peak. The MS<sup>2</sup> spectra of the secondary peak was identical to rifampin. The area counts of the peak were dependant on the spiked concentration of rifampin, confirming that this peak was due to rifampin and not an endogenous compound.

Additionally, the primary rifampin peak was not stable in prepared urine samples. As depicted in the following figure, the primary rifampin peak was unstable in prepared urine samples. The secondary peak showed limited stability. Acidification, basification, or addition of organic solvent to the precipitation solution did not stabilize the primary peak.



Instability of Rifampin in rabbit urine. Rifampin was spiked into rabbit urine at 50 ng/mL. The sample was analyzed 15 minutes (top), 3 hours (middle), and 21 hours (bottom) after preparation.

## Method Performance

The method has been validated in rabbit plasma. The following is an example of the accuracy and precision of the method with rabbit plasma and additional matrices

### Example Method Performance Quality Calibrator Samples in Plasma

	QC-5	QC-25	QC-75
Mean Concentration Found (ng/mL)	5.15	26.4	75.2
S.D.	0.26	1.2	3.2
%CV	5	4.6	4.3
%Theoretical	103	105.5	100.3
n	6	6	6

### Example Method Performance Quality Calibrator Samples in Artificial CSF

	QC-5	QC-25	QC-75
Mean Concentration Found (ng/mL)	4.78	24.13	72.24
S.D.	0.22	1.17	3.23
%CV	4.6	4.9	3.2
%Theoretical	95.6	96.5	96.3
n	6	6	6

The method has also been used to analyze rabbit tissue samples. The following is an example of the accuracy and precision of the method with homogenized rabbit skin (10mL/g dilution).

	VS-5	VS-25	VS-75
Mean Concentration Found (ng/g)	5.1	27.6	82.4
S.D.	0.4	0.6	2.6
%CV	7.4	2.1	3.2
%Theoretical	101.2	110.5	109.9
n	4	4	4

## Conclusions

Several challenges were encountered when attempting to decrease the LLOQ for a method to quantitate rifampin in plasma and CSF. Binding to storage vessels was alleviated by the addition of methanol to solutions. Samples were prepared by protein precipitation. Sensitivity was increased and variability decreased by loading the samples onto a cleanup column before backflushing onto an analytical column.

The new method was validated in rabbit plasma with an LLOQ of 2.5 ng/mL. The method was used to analyze plasma, artificial CSF, and tissue samples successfully. Tissue samples were analyzed using an LLOQ of 2.5 ng/g with a 10 mL/g dilution factor

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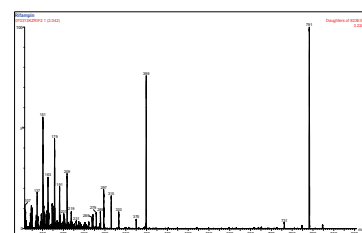
## Method

The method for the analysis of rifampin in rabbit plasma involves adding 200 µL of precipitating solution containing internal standard (IS) to 100 µL of plasma or tissue homogenate. The samples are vortexed and centrifuged before and the supernatant is transferred to 96 well format. The samples are analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) with MS/MS detection. This involves injecting 50 µL of the sample onto a 4.6 x 25 mm, 5 µm, Zorbax C18 cleanup column maintained at ambient conditions. The mobile phase conditions for sample loading are 30/70 methanol/10 mM ammonium formate at a flow rate of 2 mL/min. Samples are backflushed off the cleanup column at 0.75 seconds onto a 2.1 x 50 mm, 5 µm, Symmetry Shield C18 analytical column. Samples are eluted with a 55-95% methanol gradient with 10 mM ammonium formate as the aqueous mobile phase. The retention times of rifampin and the internal standard are 2.1 and 2.5 minutes, respectively. The total run time is approximately 10 minutes. Detection by MS/MS incorporates an electrospray interface in positive ion mode. The range of the validated assay is 2.5-100 ng/mL.

## MS/MS

Mass spectrometric analysis was performed on a MicroMass Quattro Ultima triple quadrupole mass spectrometer using an electrospray interface. The MS was operated in positive ion mode. Detection was by multiple reaction monitoring (MRM) observing the following transitions:

Rifampin	823.3 > 791 amu
Internal Standard	748 > 158 amu



MS<sup>2</sup> Spectra of Rifampin