



# Challenges in Developing a Highly Sensitive LC-MS/MS Method for the Quantification of Rifampin

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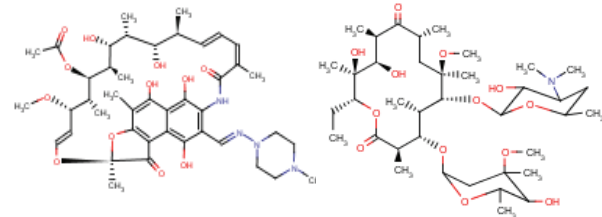
## Introduction and Purpose

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 quantitation 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

## Method

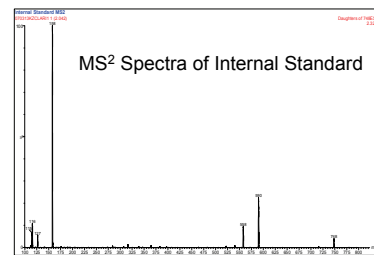
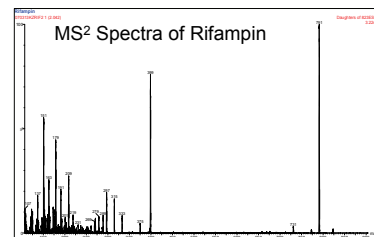
The method for the analysis of rifampin in rabbit plasma involves adding 200 µL of precipitating solution containing internal standard (IS) to 100 µL of plasma. The samples are vortexed and centrifuged and the supernatant is transferred to 96 well format. The clean up is attained by injecting 50 µL of the sample onto a 4.6 x 25 mm, 5 µm, Zorbax C18 pre-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 pre-column 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 samples are analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) with MS/MS detection. 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 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 (ESI). 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



## Labware Binding in Aqueous Solutions

Early experiments uncovered that rifampin was binding to labware when aqueous solutions. 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 6X Transfers
	37184	8077
	23624	6291
	19183	2959
	7424	2919
	10135	3185
	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).

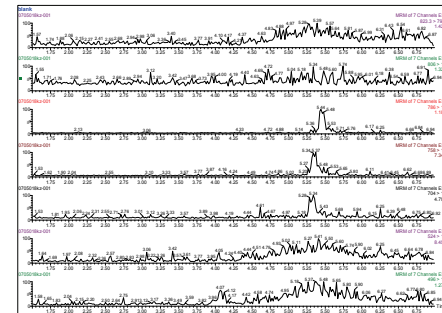
	Rifampin in 50% MeOH after 0 Transfers	Rifampin in 50% MeOH after 6 Transfers
	78134	72750
	79609	78262
	81926	78396
	81522	77477
	80580	78855
	81614	80754
AVE	80564	77752
%Decrease		4

## Phospholipid Interferences

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
Phospholipid	758 > 184
	704 > 184
	524 > 184
	496 > 184

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



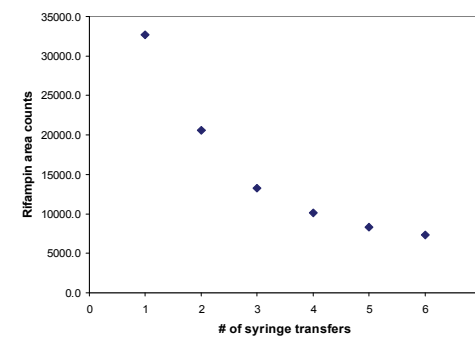
Rifampin and phospholipid transitions monitored during the injection of an extracted plasma blank while infusing rifampin.

## 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%.

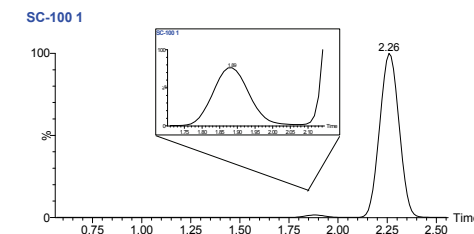
With previous experiments indicating that rifampin would bind to plastic, experiments were undertaken to determine the extent of binding of rifampin to a syringe. A solution of artificial CSF was fortified with rifampin and placed in a 96-well plate. Fractions of the solution were transferred to new 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.



## Multiple Chromatographic Peaks

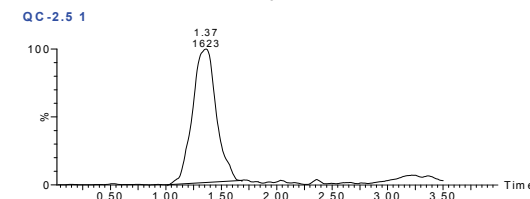
The internal standard produced multiple peaks when examined by LC-MS/MS. The peaks were present under a variety of chromatographic conditions. Reference material from two different lots from two different suppliers (including USP) produced two peaks. Monitoring multiple transitions produced two peaks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



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

## Increasing Sensitivity

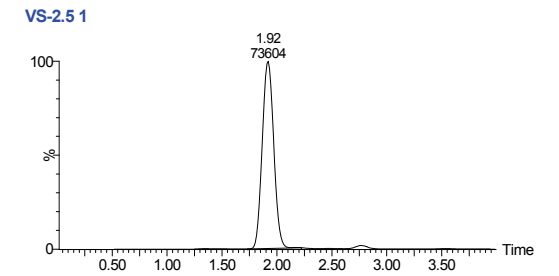
The analytical method for quantification of rifampin in human plasma prepared samples for analysis using liquid-liquid extraction. Experiments determined that liquid-liquid extraction did not allow sufficient recovery to meet the desired LLOQ of 1 ng/mL. The extraction method was changed to protein precipitation with 200 µL of methanol using a 100 µL sample size. The supernatant was evaporated and the dried residue 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. 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.

## Method Performance

### SC Samples

	SC-2.5 2.50 ng/mL	SC-5 5.00 ng/mL	SC-10 10.0 ng/mL	SC-15 15.0 ng/mL	SC-25 25.0 ng/mL	SC-50 50.0 ng/mL	SC-100 100 ng/mL
Mean	2.56	4.74	9.96	15.1	25.6	50.5	99.1
S.D.	0.184	0.298	0.613	0.959	1.4	2.85	5.84
%CV	7.2	6.3	6.2	6.4	5.5	5.6	5.9
%Bias	2.4	-5.2	-0.4	0.7	2.4	1	-0.9
n	8	8	8	8	8	8	8

### QC Samples

	VS-2.5 2.50 ng/mL	VS-25 25.0 ng/mL	VS-75 75.0 ng/mL
Mean Concentration Found (ng/mL)	2.68	26.5	77.2
Inter-run SD	0.162	2.22	4.62
Inter-run %CV	6	8.4	6
Inter-run %Bias	7.2	6	2.9
n	17	18	18

The above data was collected during pre-validation and validation runs for this method. The method has been validated in rabbit plasma.

## Conclusions

Several challenges were encountered when attempting to decrease the LLOQ for a method to quantitate rifampin in rabbit 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 and an LLOQ of 2.5 ng/mL achieved.

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