Method Development and GLP Validation for the HPLC/MS/MS Bioanalysis of Vancomycin Extracted from Rat Plasma

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Overview

- **Purpose** - Develop and validate a selective HPLC/MS/MS method to determine the concentration of Vancomycin in rat plasma
- **Methods** – Solid Phase Extraction (SPE) in 96 well-plate format, analyzed on a HPLC - triple-quadrupole mass spectrometer (API -3000, -4000 and -5000)
- **Results** – A robust HPLC/MS/MS method was developed and validated following FDA guidelines for the analysis of vancomycin extracted from rat plasma from 10.0 to 10,000 ng/mL.

Introduction

The glycopeptide vancomycin is a broad spectrum antibiotic for gram positive bacteria, administered intravenously for systemic infections. To accurately assess the efficacy of dosing regimes and to study the pharmacokinetics, vancomycin should be quantified from plasma.

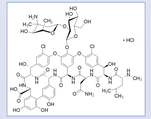
Bioanalysis of vancomycin often utilizes an acid precipitation, which dilutes the sample, limits the specificity, and offers poor recovery (< 20 %). Sample preparation via solid phase extraction (SPE) offers a clean and specific sample preparation technique. Several SPE media and various internal standards were investigated to obtain optimal recovery of both the analyte and the internal standard.

Here we report an accurate and precise LC/MS/MS assay with > 80% recovery for the determination of vancomycin from rat plasma.

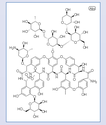
Method Development

Internal Standard Selection

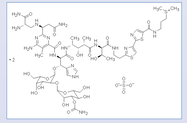
An isotopically labeled internal standard was not available for the analyte vancomycin at the time of development; therefore several compounds which are considered similar were evaluated as suitable internal standards: bleomycin and its analog phleomycin, and ristomycin. Phleomycin showed very poor MS signal and was quickly eliminated from further development.



Vancomycin



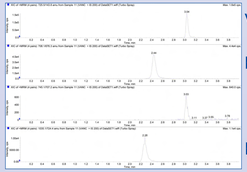
Ristomycin



Bleomycin

Bleomycin offered suitable MS response and good chromatography so was selected as the internal standard. However, as a SPE extracted internal standard it offered poor accuracy possibly due to the marked structural differences. Ristomycin is structurally similar to vancomycin, so was chosen for the internal standard, even though it offered an extraction efficiency of ~30%.

Chromatography of compounds evaluated as an internal standard



SPE phases

Once an internal standard was selected, three different SPE media were evaluated, using the recovery of the analyte and internal standard for selection.

C18 was selected because it offered the highest recovery upon initial assessment. Further method development was able to increase the recovery to 80% for vancomycin, yet the recovery of ristomycin was only about 10%. A post-spiking experiment showed that the loss of ristomycin was due to matrix effects and not due to the SPE media.The response even with the matrix effect was reproducible thus the accuracy and precision was still acceptable.

Table 1. % Recovery with SPE Media

	Media			
Compund	**C2**	**C8**	**C18**	**LMS**
Vancomycin	14.3	27.1	50.3	34.8
Ristomycin	28.0	39.9	10.6	62.9
Precision (Peak Area Ratio)	25.0	15.4	12.4	4.6

Method

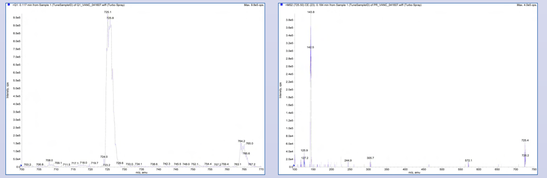
Summary: Prepare an aliquot of the sample with C18 SPE, and evaporate excess solvent to concentrate the extract. Re-constitute with aqueous mobile phase and inject onto HPLC/MS/MS instrument.

HPLC

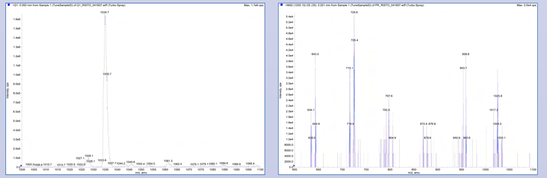
Reverse Phase HPLC
0.5 mL/min flow rate
Mobile Phases:
1% Formic Acid Aqueous Solution
Acetonitrile with 1% Formic Acid
LC Column: HSC18, 5 µm, 2.1 x 50 mm (Supelco)
30µL injection

Mass Spectrometry

ABI API-3000, -4000 or -5000 mass spectrometer with positive-mode ESI
Vancomycin (+2 charge): 725.50→143.80
3.00 min retention time
Ristomycin (+1 charge): 1035.10→724.40
1.75 min retention time



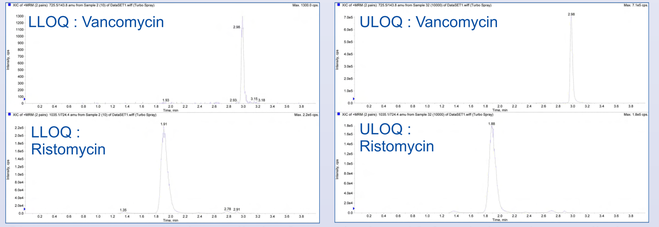
Q1 and Product ion mass spectra of Vancomycin



Q1 and Product ion mass spectra of Ristomycin

Validation

Following the more resent Guidelines, the determination of vancomycin from rat plasma was validated over six days.



Chromatogram at the lower limit of quantitation, 10.0 ng/mL and upper limit of quantitation, 10000 ng/mL.

Table 2. Validation Summary

QC Level (ng/mL)	Intraassay Accuracy ± Precision (%)	Interassay Accuracy ± Precision (%)	Freeze Thaw Stability (% Difference from Nominal)	Benchtop Stability (Hours)	Extract Stability (Hours)
10.0	94.6 ± 1.5	---	---	---	---
30.0	101 ± 2.7	104 ± 12.1	-0.3	> 2.5	> 35
1000	111 ± 2.9	96.5 ± 12.8	4.4	> 2.5	> 35
8000	102 ± 12.1	97.0 ± 12.5	1.6	> 2.5	> 35
10000	96.2 ± 12.0	---	---	---	---

Table 3. Recovery for the validated method

	Nominal concentration (ng/mL)		
Recovery	**30.0**	**1000**	**8000**
Vancomycin	**90.8**	**94.9**	**83.7**
Ristomycin		**29.8**	

Conclusion

With thorough method development, the validation of the method was straightforward. To date, several thousand plasma samples have been analyzed with the validated method. To further improve the method, the purchase or synthesis of a stable labeled internal standard is being investigated.