MicroConstants
REGULATED BIOANALYSIS & DMPK

A Novel Approach to Internal Standardization in LC/MS/MS Analysis

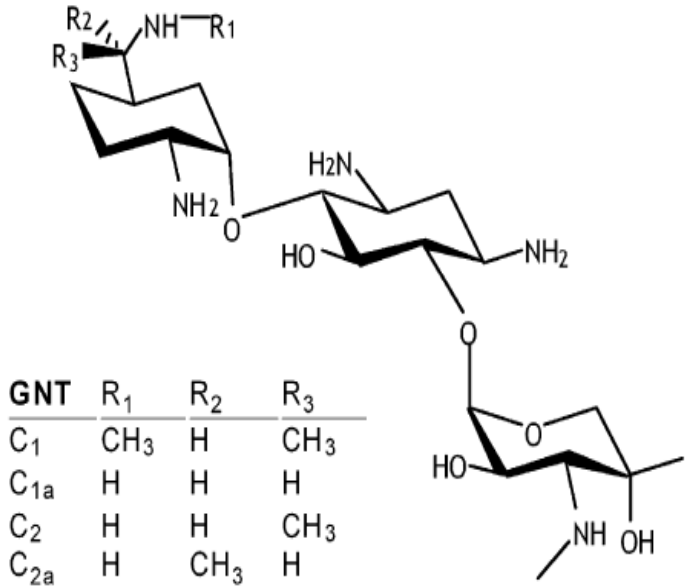
SENSITIVE LC/MS/MS ANALYSIS OF GENTAMICIN

CACO-PBSS Mini-Symposium:
Bioanalytical and Analytical Applications and Problem Investigation Case Studies
August 10, 2012

Background & Introduction

Gentamicin is an aminoglycoside antimicrobial agent produced by fermentation of Micromonospora purpurea or M. echinospora. It has a wide spectrum of antimicrobial activity. Gentamicin is not a single molecule but a complex of three major and several minor components. Gentamicins C_1, C_{1a} and C_2 are the three major components of the drug complex. The C_2 component consists of two stereoisomers (C_2 and C_{2a}).

Structures of Gentamicin Components



GNT	R_1	R_2	R_3
C_1	CH_3	H	CH_3
C_{1a}	H	H	H
C_2	H	H	CH_3
C_{2a}	H	CH_3	H
C_{2b}	CH_3	H	H

Molecular Weights and Approximate Composition

Gentamicin C_{1a}	MW 449.5	10-35%
Gentamicin C_2 & C_{2a}	MW 463.6	25-55%
Gentamicin C_1	MW 477.6	25-50%

The Analytical Challenge

- Gentamicin is associated with severe side effects
- Use is subject to therapeutic drug monitoring, especially in patients with renal impairment
- Product is being developed for subcutaneous injection at surgical incision sites to prevent post surgery infections
  - High systemic exposure not intended and hopefully avoided
  - Circulating plasma levels likely to be very low relative to IM, IV or oral routes of dosing
- LLOQ of 1.00 ng/mL requested
- Very low sample volumes are available
- Gentamicin is co-administered with Vancomycin requiring sample volume for a separate assay

Approaches in the Literature

Pharmaceutical Product Analyses:

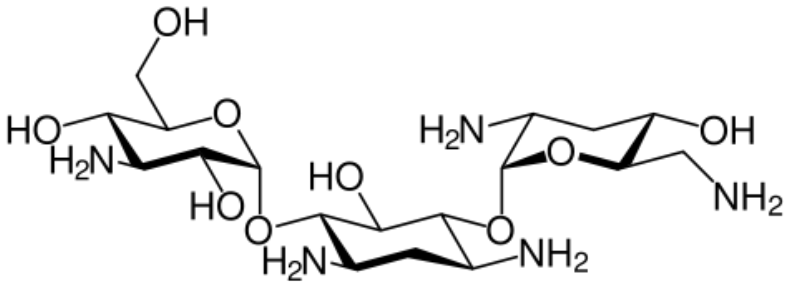
- USP monograph. Pre-column labeling with o-phthalaldehyde (OPA). Methodology makes a somewhat flawed assumption for equal response of components as they have different number of reactive primary amines.
- Similar OPA derivative with micellar electrokinetic chromatography and UV @ 340 nm
- HPLC 2012 poster by Thermo with ion-paired RP-HPLC (HFBA, TFA) charged aerosol detection
- Reversed phase ion-pairing (octane sulfonic acid) pulsed electrochemical detection
- Reversed phase ion-pairing TFA and methanol and electrospray LC/MS/MS

Bioanalytical Analyses of Plasma or Tissues:

- SPE with on-column derivatization with 1-fluoro-2,4-dinitrobenzene RP-HPLC ultraviolet absorbance at 365 nm
- Derivatization with phenyl isocyanate and RP-HPLC UV
- Fluorenylmethyloxycarbonyl chloride (FMOC) derivatization, UV 265 nm or fluorescence
- Tissues: HPLC with post column derivatization with OPA and fluorescence detection limits 400 ng/gm
- Hydrophilic interaction chromatography (HILIC) with MS/MS detection

Our Approach

- Common themes: Ion Pairing or Derivatization
- No precedent in literature to achieve required 1.00 ng/mL LLOQ using small plasma volumes
- Initial approach = LC/MS/MS
- No isotopically labeled I.S. would ever be available
  - Even if a single isoform could be synthesized would it track the other isoforms?
  - Spent considerable time and effort with an analogue I.S Tobramycin (MW 467.51)

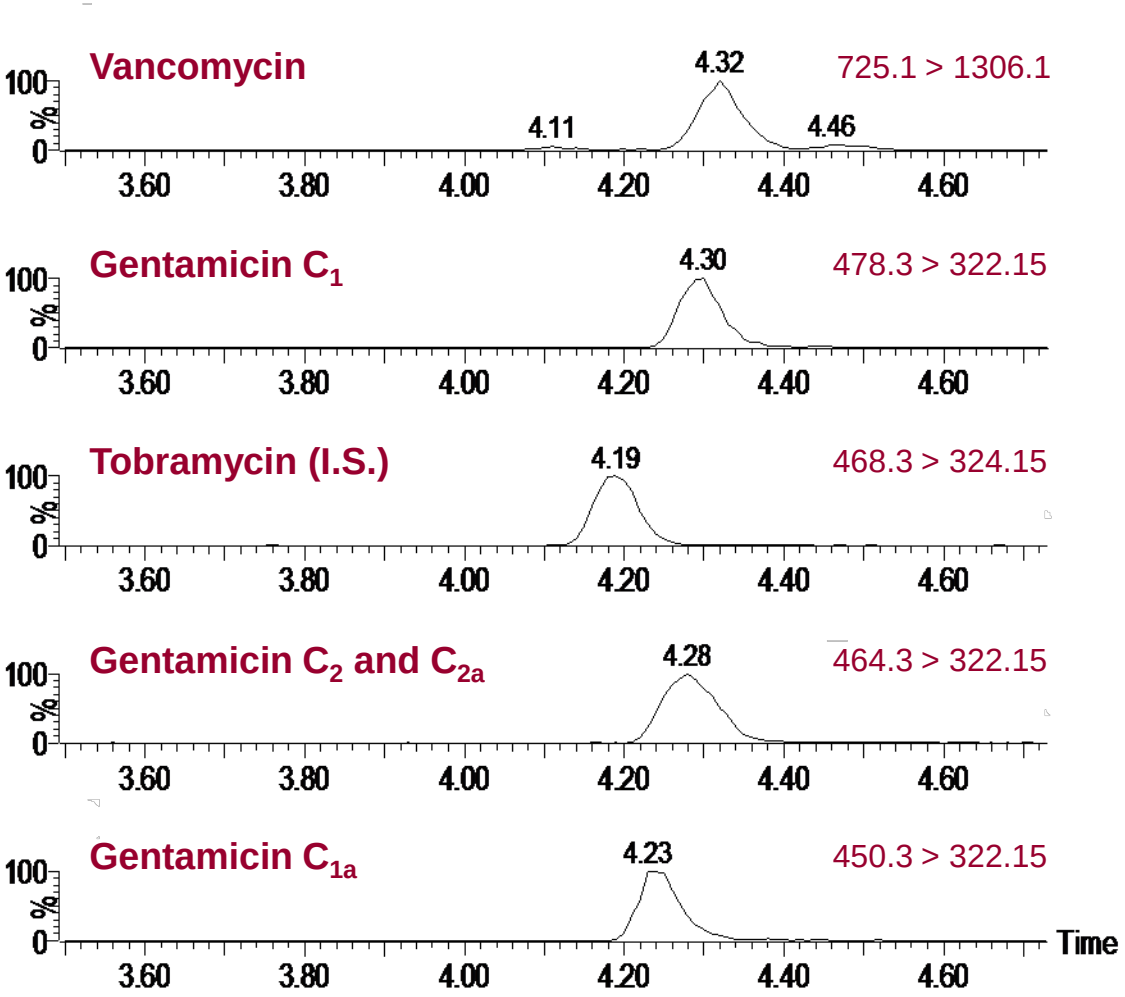


Tobramycin

- Ion – Paired SPE with HFBA pretreatment on micro-elution C18 followed by LC/MS/MS
- Decided to quantify C_2 and C_{2a} together (same MW and not readily resolved chromatographically)

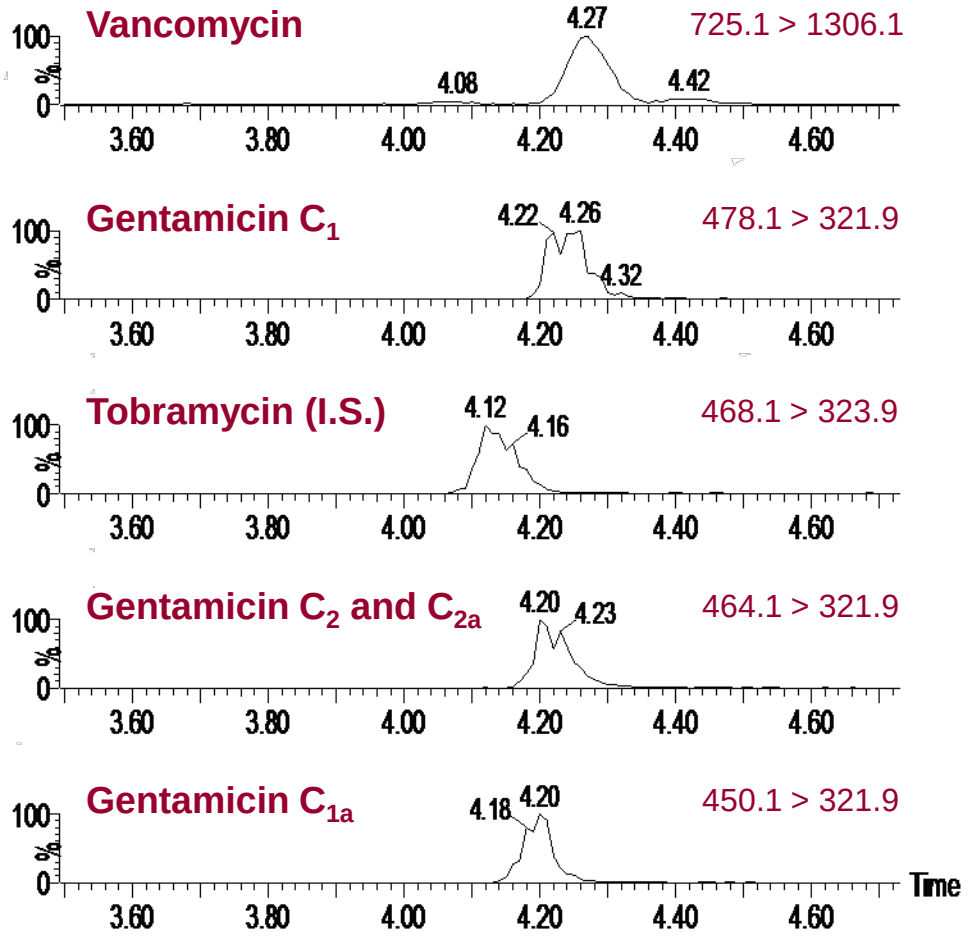
Initial Results

- Made considerable progress
- Sensitivity looked promising
- HFBA modifier in a HILIC-type separation
- Attempted to include Vancomycin



Results Over Time

- Vancomycin was successfully validated using an analogue I.S. (Ristomycin). LLOQ was 0.500 ng/mL using 20 µL of plasma.
- Gentamicin exhibited poor ionization stability in the source
- Good progress, but intuition suggested the technique would not validate per FDA Guidance
- Other options were pursued



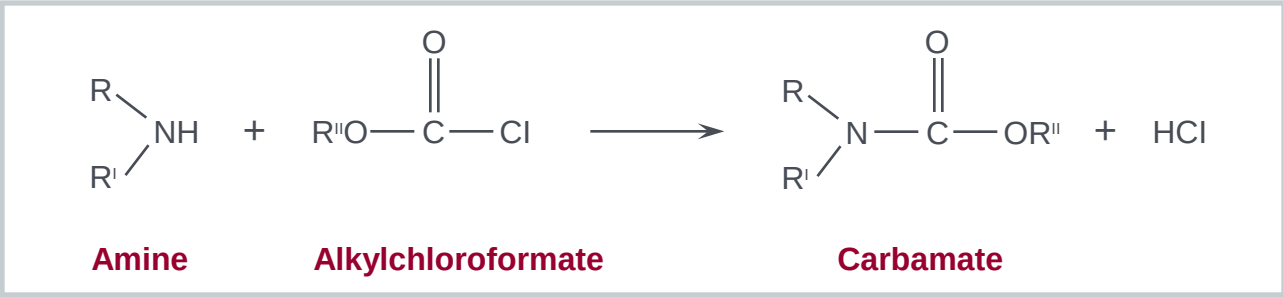
How About Derivatization with LC/MS/MS?

For this application, what are the attributes of a good derivative?

1. Needs to tag all 5 amines efficiently and robustly
2. Need a stable derivative, one not affected by moisture during formation or stability post reaction
3. Pre-column derivatization for simplicity... let's not add unnecessary mechanical complexity
4. Derivative should impart added hydrophobicity allowing more selective clean-up and chromatographic options
5. Bigger, but not too big! With 5 amines to tag, things could get out of hand quickly.
6. Improves LC/MS/MS sensitivity and ionization consistency – potentially new product ions and greater yield could vastly improve overall response
7. I.S. tracking – could the correct derivative make an analogue I.S. track better?
8. Manageable excess – a derivatization reagent for which excess reagent would either not be a problem or be readily eliminated

We Gave it a Try…

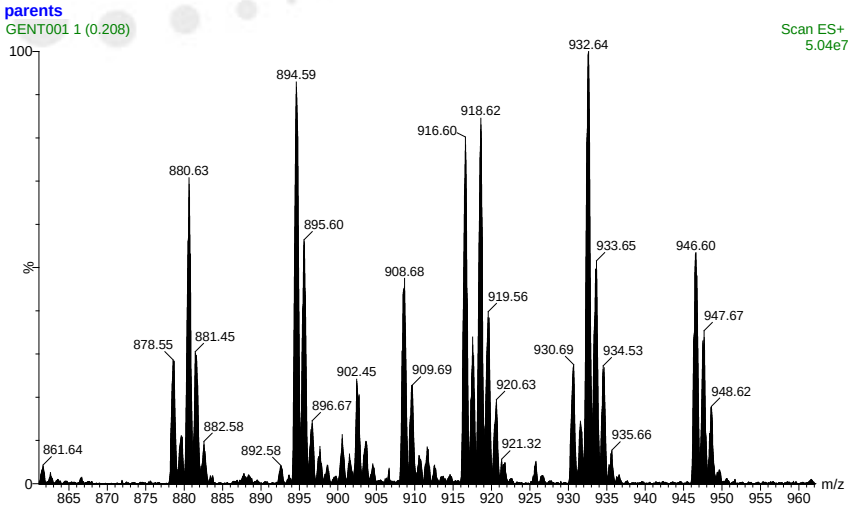
- Given the attributes of a good derivative (previous slide), could all the criteria be met using alkyl chloroformates?
- Reaction on primary and secondary amines:



- Explored both ethyl chloroformate and propyl chloroformate
  - Reaction is base-catalyzed and works in aqueous phase
  - Acetone is a good co-solvent
- Have previous experience with the derivatization of Amikacin (related amino glycoside) using ethyl chloroformate
- Only obvious initial disadvantage was ionization - chloroformate derivatives of Gentamicin can find sodium where it doesn't exist!

Initial Outcome

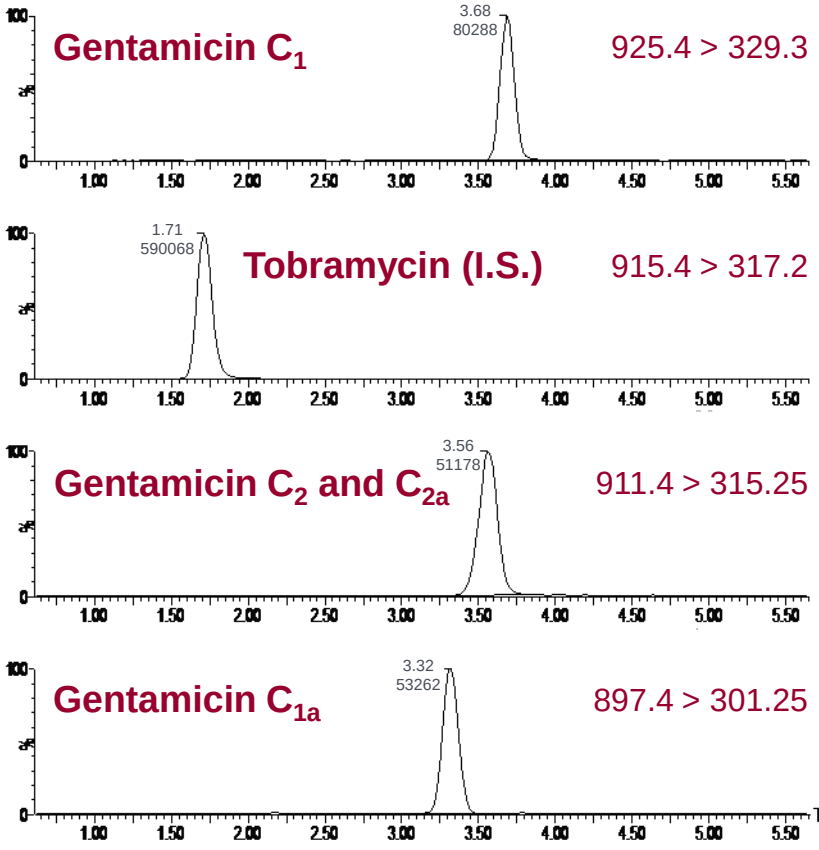
Direct MS Infusion of PCF Reaction Product



What masses can we expect for a 5 X PCF reaction?

Gentamicin Form:	C_{1a}	C_2 & C_{2a}	C_1
Original Parent	450	464	478
Derivative (no adduct)	880	894	908
Sodium Adduct	902	916	930
Potassium Adduct	936	933	947
Ammonium Adduct	**897**	**911**	**925**

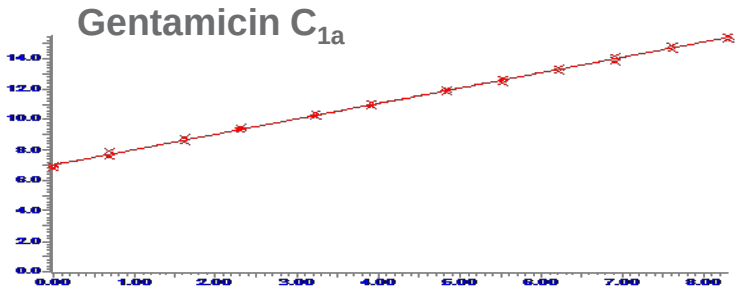
PCF derivatives of isomeric forms C_2 and C_{2a} were not chromatographically resolved and were treated as a single compound:

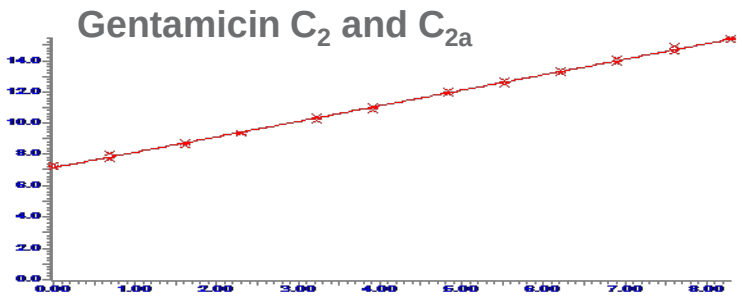


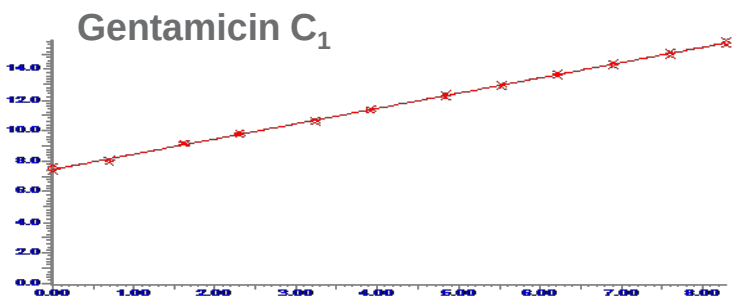
Extracted standard representing 50 ng/mL total Gentamicin

Proof of Concept… Does it Work?

- Tested extraction of 50 µL of plasma and calibration range from 1.00 to 4,000 ng/mL
- Calculations by external standard showed promise!



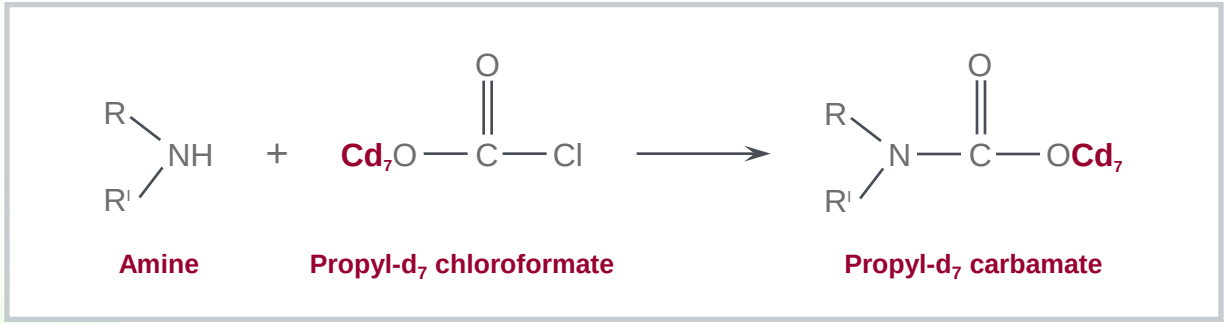




GOOD NEWS
Great assay

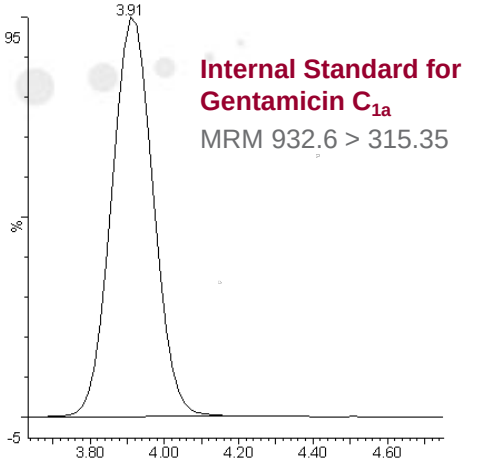
BAD NEWS
No internal standard

The Solution

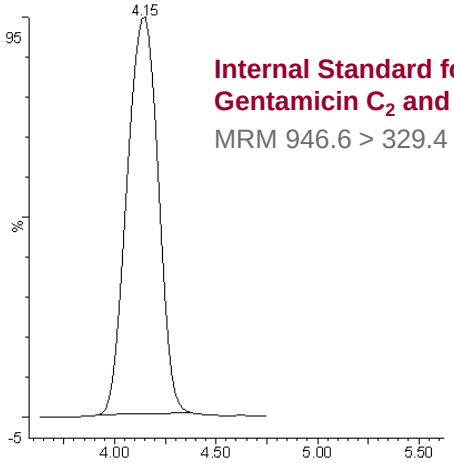


- Stable labeled I.S. is synthesized by pre-derivatization with d_7-PCF
- I.S. is added at the beginning of extraction but is not derivatized *within* the assay
- Gentamicin isoforms in samples are derivatized within the assay by non-labeled PCF

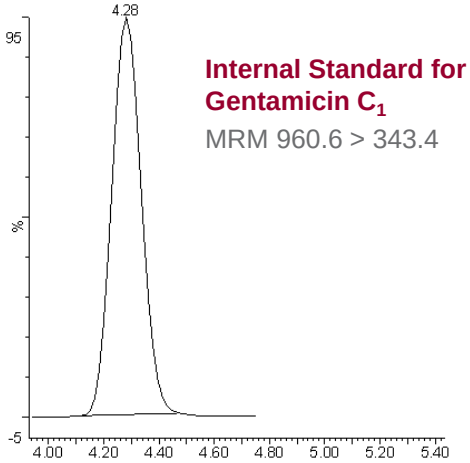
Final Outcome



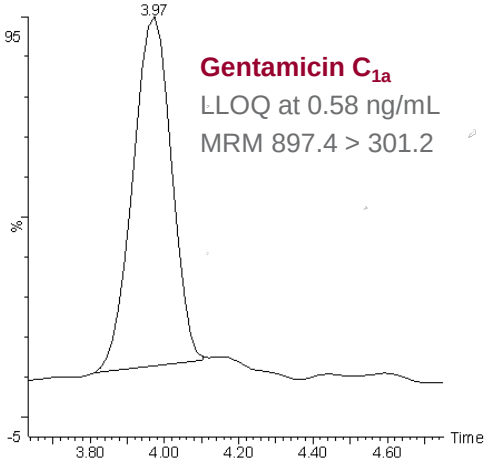
Internal Standard for Gentamicin C_{1a}
MRM 932.6 > 315.35



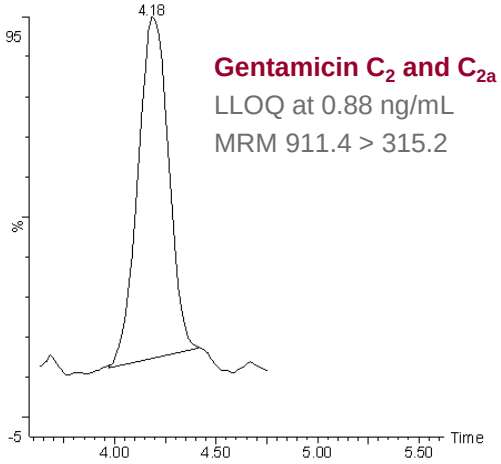
Internal Standard for Gentamicin C_2 and C_{2a}
MRM 946.6 > 329.4



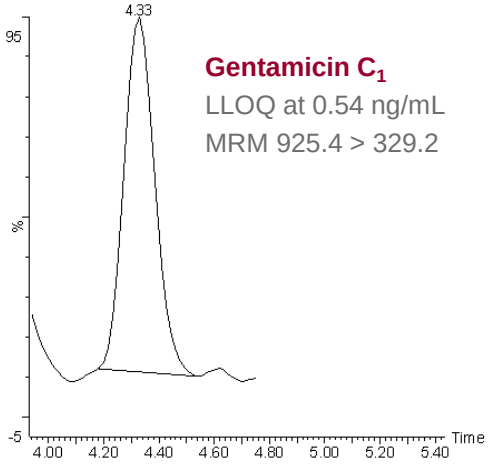
Internal Standard for Gentamicin C_1
MRM 960.6 > 343.4



Gentamicin C_{1a}
LLOQ at 0.58 ng/mL
MRM 897.4 > 301.2



Gentamicin C_2 and C_{2a}
LLOQ at 0.88 ng/mL
MRM 911.4 > 315.2

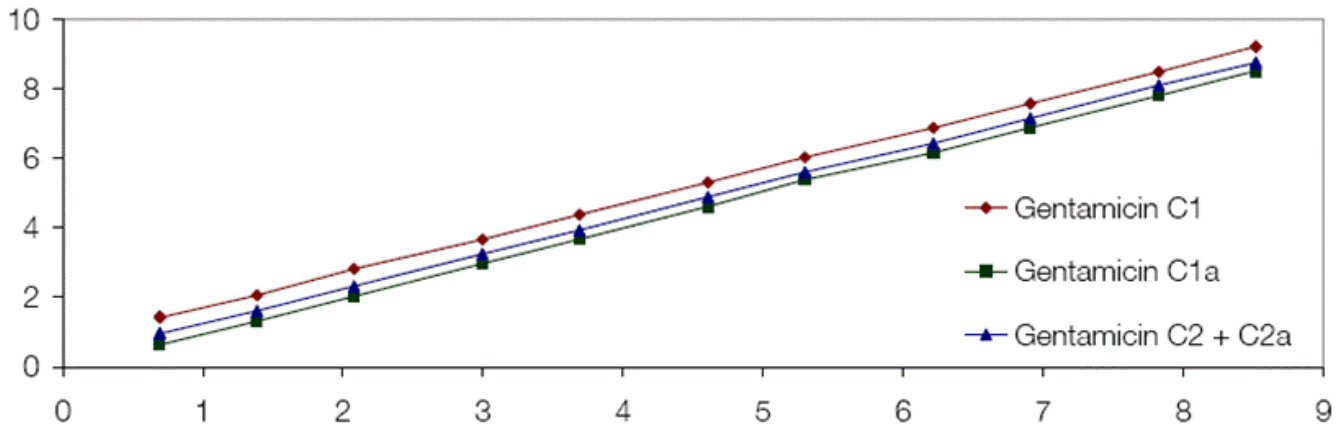


Gentamicin C_1
LLOQ at 0.54 ng/mL
MRM 925.4 > 329.2

Note: Each isoform has its own I.S in the same relative abundance as the drug substance because they originate from the same reference material.

Validation Summary

Example Calibration Curves



Interday Quality Control Sample Data and Statistics

Gentamicin C_1 Concentration (ng/mL)			
	1.62	**54.0**	**1,080**
Mean	1.60	54.1	1,100
%CV	5.41	2.89	4.02
%DEV	-1.23	0.185	1.85

Gentamicin C_{1a} Concentration (ng/mL)			
	1.74	**58.4**	**1,180**
Mean	1.76	58.4	1,180
%CV	6.89	2.93	3.78
%DEV	1.15	0.690	1.72

Gentamicin C_2 + C_{2a} Concentration (ng/mL)			
	2.64	**88.0**	**1,760**
Mean	2.65	88.9	1,780
%CV	5.25	4.11	3.09
%DEV	0.379	1.02	1.14

Final validated range was 2.00 – 5,000 ng/mL total Gentamicin

Summary & Conclusions

- Novel application of deuterium labeled derivatization reagent to make an ideal I.S.
- Multiple benefits to chloroformate derivatization
- Critical aspects for success:
  - Parallel extraction for analyte and pre-derivatized I.S.
  - Robust derivatization – analyte has to “catch up” with I.S.
  - Analyte conversion – labeled derivative cannot exchange for unlabeled derivative
- Further applications:
  - General approach for other aminoglycoside antibiotics
  - Fluoro-β-alanine (FBAL)
  - Doxorubicin and metabolite Doxorubicinol

Acknowledgements

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