A Novel Approach to Internal Standardization in LC/MS/MS Analysis; Sensitive LC/MS/MS Analysis of Gentamicin

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ASMS • Salt Lake City, UT • May 2010

Introduction

The importance of isotopically labeled internal standards (I.S.) in quantitative bioanalytical LC/MS/MS cannot be overstated. Analogue I.S. often fail to adequately parallel extraction, chromatographic separation and MS/MS ionization. Gentamicin, an aminoglycoside antibiotic, is synthesized by Micromonospora fermentation and isolated as a mixture of components C_{1a}, C_2, C_{2a}, and C_1. Analytical approaches often involve derivatizations of the five basic nitrogens making chromophores for HPLC-UV or HPLC-FL. For an LC/MS/MS analysis identifying a suitable I.S. is critical. Analogue aminoglycoside I.S. have been deemed unsatisfactory. No isotopically labeled I.S. is commercially available and synthetic routes to such a standard would not yield a mixture that reflects the fermentation derived relative concentrations. A novel approach must be considered.

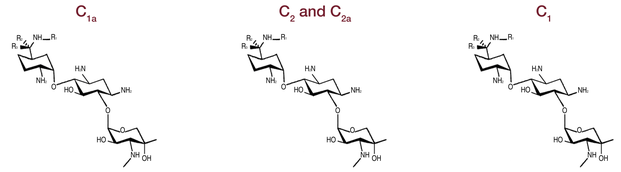
Methods

Gentamicin is derivatized with propyl-d7 chloroformate making an I.S. containing analogues for each component, C_1, C_2, C_{2a}, and C_{1a}. Rabbit plasma with the previously synthesized I.S. added is precipitated using 4% perchloric acid. The supernatant is recovered, neutralized with potassium carbonate buffer and reacted with propyl chloroformate (PCF) in acetone to produce non-deuterated analogues from each of the gentamicin components in the plasma sample. The derivatives are extracted with ethyl acetate in hexane, dried and reconstituted for LC/MS/MS analysis on a Waters Quattro Ultima in ESP+ mode. Chromatographic separation is performed on a Restek Allure PFP Propyl, 5µm 100x2.1 mm column using a gradient elution of water vs. ACN both containing 0.05% TFA and 0.025% NH_4TFA.

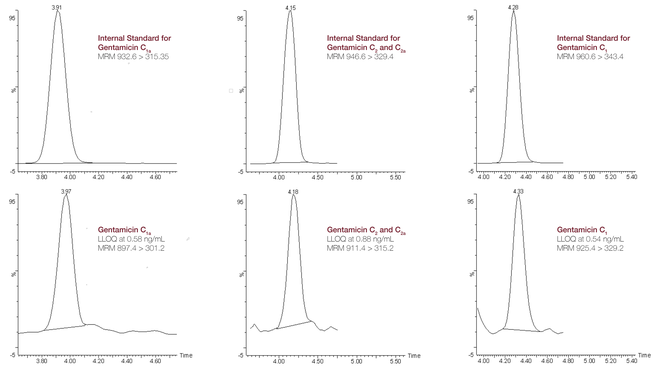
Preliminary Data

Though the initial intent of the derivatization was to provide a mechanism to employ an ideal I.S., the benefits to treatment with PCF proved to be many. Each of the 5 tags on the gentamicin molecule adds a net 86 amu. The increased hydrophobicity allowed for solvent extraction clean-up and chromatographic retention not possible for the naive molecule. Electrospray positive ionization of the PCF derivatives is very prone to sodium adduct formation. The chosen mobile phase modifiers (TFA and NH_4TFA) promoted formation of NH_4+ adducts while attenuating other adducts. Most significantly though for overall sensitivity, the derivatization with PCF gave way to much stronger yields of product ions in argon collision induced fragmentation. The precursor-product transitions chosen for detection of the NH_4+ adducts were m/z 897.40 > 301.25, m/z 911.4 > 315.25 and m/z 925.4 > 329.3 for gentamicin C_{1a}, C_2 and C_{2a}, and C_1, respectively. Analogous transitions were used for the internal standard components offset by 35 amu on the parent mass (due to 5 PCF tags at 7 deuteriums per each): m/z 932.60 > 315.35, m/z 946.6 > 329.40 and m/z 960.6 > 343.40 for gentamicin C_{1a}, C_2 and C_{2a}, and C_1, respectively. Isomeric forms C_2 and C_{2a} were not chromatographically resolved and were treated as a single compound. Other than this coelution a gradient elution provided single sharp peaks for the other components with a total run time of 7 minutes. Calibration standards were prepared in rabbit plasma ranging from 2.0-5,000 ng/mL for total gentamicin concentration. QC's samples in rabbit plasma were prepared at 6.00, 100 and 4,000 ng/mL. The method was successfully validated per USFDA guidance requirements.

Structure of Gentamicin Components

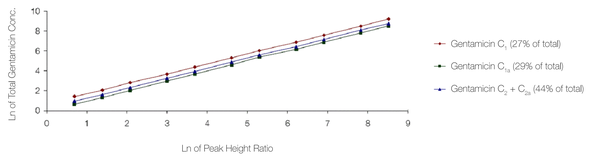


Representative Chromatograms



Gentamicin components are detected as NH_4+ adducts of propyl chloroformate derivatives
Internal Standard components are detected as NH_4+ adducts of propyl-d7 chloroformate derivatives

Example Calibration Curves



Interday Quality Control Sample Data and Statistics

Mean results of 24 replicates of each QC over 4 days

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	58.0	1,160
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

Evaluation of the Lower Limit of Quantification

Mean of six replicates

	Gentamicin C_1	Gentamicin C_{1a}	Gentamicin C_2 + C_{2a}
Theory (ng/mL)	0.540	0.580	0.880
Mean (ng/mL)	0.501	0.575	0.870
%CV	9.65	13.4	10.9
%DEV	-7.22	-0.862	-1.14

Summary & Conclusions

A novel approach to internal standardization in LC/MS/MS bioanalysis has been demonstrated. A deuterium labeled derivatization reagent was used to make an ideal internal standard for a complicated multi-component drug substance, Gentamicin, for which no satisfactory internal standard was available. Derivatization within the assay is required to make the drug substance analogous to the I.S. There are two critical characteristics for successful use of this unorthodox approach when the I.S. does not undergo derivatization within the assay. The steps of sample pre-treatment ahead of derivatization must be equally suitable for both derivatized and non-derivatized forms of the analyte. Secondly, the derivatization step itself must be robust and reliable and not susceptible to analete conversion from the labeled derivative into the non-labeled derivative. Once the design of the method meets these requirements, all of the benefits of parallel extraction, chromatographic separation and LC/MS/MS ionization are realized just like would be with a synthetically labeled drug analogue. Though derivatizations are more labor intensive than would otherwise be required using true labeled compounds or analogues, the quality of the data depicted by this successful validation and the very sensitive LLOQ would not have otherwise been obtainable.

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