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

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Introduction

The importance of isotopically labeled internal standards (I.S.) in a quantitative biological LC/MS/MS cannot be overstated. Analogue I.S. often fail to adequately parallel-extract, chromatographic separation and MS/MS transition. Gentamicin, an aminoglycoside antibiotic, is synthesized by Micromonospora fermentation and labeled as a mixture of components C₂a, C₁a, C₂, and C₃. Analytical approaches often involve derivatizations of the five basic nitrogen making chromatophores for HPLC-UV or HPLC-FL. For an LC/MS/MS analysis identifying a suitable I.S. is critical. Analogue aminoglycosides I.S. have been deemed unsatisfactory. No isotopically labeled I.S. is commonly 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, C₂a, C₁a, C₂, and C₃. Rabbit plasma with the previously synthesized I.S. acid is precipitated using 4% perchloric acid. The supernatant is recovered, neutralized with potassium carbonate buffer and reacted with propyl chloroformate (PCF) in acetic acid to produce non-labeled 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% NH₄. TFA promoted the derivatization with PCF gave way to much stronger yields of product ions in argon collision induced fragmentation. The chosen mobile phase modifiers (TFA and NH₄+ adducts of propyl chloroformate derivatives

Representative Chromatograms

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 benefit to treatment with PCF. The chosen mobile phase modifiers (TFA and NH₄+ adducts while attenuating other adducts. Most significantly though for overall sensitivity, the derivatization with PCF promoted the derivatization step itself must be robust and reliable and not susceptible to analyte conversion from the labeled derivative. Secondly, the 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 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 analyze conversion from the labeled derivative into the non-labeled derivative. On the design of the method meets these requirements, all of the benefits of parallel extraction, chromatographic separation and LC/MS/MS transition are realized just like would be with a synthetically labeled drug analogue. Though derivatizations are more labor intensive than would otherwise be required using the labeled components or analogues, the quality of the data depicted by this successful validation and the very sensitive LLOQ would not have otherwise been obtainable.

Evaluation of the Lower Limit of Quantification

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 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 analyze conversion from the labeled derivative into the non-labeled derivative. On the design of the method meets these requirements, all of the benefits of parallel extraction, chromatographic separation and LC/MS/MS transition are realized just like would be with a synthetically labeled drug analogue. Though derivatizations are more labor intensive than would otherwise be required using the labeled components or analogues, the quality of the data depicted by this successful validation and the very sensitive LLOQ would not have otherwise been obtainable.