QPS, LLC
Delaware Technology Park
3 Innovation Way, Suite 240
Newark, DE 19711

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WEB www.qps-usa.com
TEL (302) 369-5601
FAX (302) 369-5602

CONFERENCE

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TITLE

Simulataneous Quantiation of Methotrexate and its Metabolite 7-Hydroxymethotrexate in Human Plasma by LC/MS/MS Combined with Solid Phase Extraction

AUTHORS

Xiaoping Ao; Jamie Zhao; Lina Tang; Hsun-Wen Chou; Yuan-shek Chen; Kumar Ramu

ABSTRACT

Novel Aspect: A challenging LC/MS/MS method for amphoteric analytes having sensitivity and severe carryover issues.

Introduction

Methotrexate has been used to treat various malignancies for many years. Recently, methotrexate is used to treat nonmalignant conditions such as rheumatoid arthritis and psoriasis. In order to better understand the pharmacological properties, it is critical to quantify methotrexate and its major metabolite, 7-hydroxymethotrexate accurately. Several bioanalytical methods have been developed to quantify these two analytes for pharmacokinetic studies or clinical studies. These methods have one or more of the following limitations: high lower limit of quantitation, narrow assay range, severe carryover, or necessity to use large sample volume up to 1 mL. Our purpose is to develop and validate a highly sensitive and specific bioanalytical method using LC/MS/MS combined with solid phase extraction to support clinical trials.

Methods

Methotrexate, 7-hydroxymethotrexate and their deuterated internal standards were extracted from 300µL human plasma by solid-phase extraction on 96-well Oasis HLB SPE plate. The plate was washed with ammonium acetate in water. The analytes and internal standards were then eluted using 0.1% formic acid in methanol. Following solvent evaporation and reconstitution, the samples were injected for LC/MS/MS analysis. Liquid chromatography was carried out on a Prodigy Phenyl-3 (50x2 mm, 5µ, Phenomenex) column with isocratic elution using Shimadzu LC-10A pumps and CTC PAL autosampler. Methotrexate and 7-hydroxymethotrexate were quantified on a Sciex API 4000 mass spectrometer under positive mode using multiple reaction monitoring (MRM): methotrexate: 455.3→308.2; methotrexate-d3: 458.3→311.2; 7-hydroxymethotrexate: 471.2→190.9; [methyl-2H3,U-13C]-7-hydroxymethotrexate: 475.2→190.9.

Preliminary results

Both methotrexate and 7-hydroxymethotrexate are weak diacids with multiple amine groups. The amphoteric molecules showed some quite interesting characteristics. 7-hydroxymethotrexate ionization is particularly sensitive to source temperature while methotrexate is not. At high temperature, methotrexate is more than 10-fold more sensitive than its metabolite. 350 ºC was found to be the optimal temperature for 7-hydroxymethotrexate. Many LC columns (such as Sepax GP-C18, Gemini C18, Phenyl-hexyl, etc.) can provide nice chromatography for the two analytes under acidic mobile phase using gradient elution. However, severe carryover was observed on both column and injector especially for 7-hydroxymethoxate, which significantly limited the assay range. The carryover was finally resolved by using isocratic elution on a Prodigy Phenyl-3 column, which provided satisfactory peak shape and retention. The method was validated for an assay range of 1 to 500 ng/mL for both analytes. The assay performance through three core run validation was characterized as: intra-day assay accuracy including LLOQ from -12.8 to 1.1% and precision from 1.6-9.3%; inter-day accuracy from -9.0 to -1.3 and precision

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from 3.4 to 7.9% for these two analytes. The recovery, matrix effect, stability including freeze-thaw, bench-top, and long-term storage were also validated.