A Highly Sensitive and Specific LC/MS/MS Method for Quantification of Methotrexate and Its 7-Hydroxy Metabolite in Human Plasma

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Overview

A sensitive and specific liquid chromatographic-tandem mass spectrometric (LC/MS/MS) method capable of quantifying methotrexate and its 7-hydroxy metabolite in human plasma is described.

In this method, methotrexate was extracted from plasma using a protein precipitation method. The separation was performed on a reverse phase C18 column with special mobile phases, such that the sensitivity was dramatically increased. It also greatly improved the ion chromatography separation and peak shape.

The method has been successfully applied to pharmacokinetic studies in human plasma.

Introduction

Methotrexate is one of the oldest chemotherapy drugs, in use for many years. It was found effective in treating certain diseases associated with abnormally rapid cell growth, such as cancer of the breast and psoriasis. Recently, methotrexate has shown effectiveness in treating rheumatoid arthritis. Several analytical methods have been developed for pharmacokinetic studies or clinical trials. In these methods, the lowest quantitative concentration of methotrexate in plasma is 5 ng/mL or higher. Solid phase extraction was used in sample preparation, which is costly and time consuming. Protein precipitation extraction used in this method simplified the sample preparation procedure. Our method employed special mobile phases, so that the ion [M+H]⁻, became a dominate peak (Figures 2 and 3).

Experimental

Sample Preparation

The methotrexate, 7-hydroxy metabolite, and the internal standard were extracted from the human plasma by protein precipitation method. The supernatant was directly injected for LC/MS analysis.

Liquid chromatography:

- LC System: Pump Shimadzu LC-10AD
- Autosampler Shimadzu SIL-HT
- System Controller Shimadzu SCL-10A
- Analytical Column: Lux C18 column, 2.0 x 30 mm, 5 µm
- Gradient: 0.5 mL/min
- Injection Volume: 10 µL

Mass Spectrometry

MS System: AB Sciex API-3000 or API-4000
Condition: LC(/+)ESI-MS/MS (MRM)

The method has been validated, and successfully applied in clinical sample analysis.

Results and Discussion

In previous work, solid phase extraction was used in the sample preparation, which is costly and time consuming. Protein precipitation extraction used in this method simplified the sample preparation procedure. Our method employed special mobile phases, so that the ion [M+H]⁻ became a dominate peak (Figures 2A and 3).

- Excellent linearity was obtained with correlation coefficient greater than 0.996. The inter-day precision (CV%) and accuracy (RE%) for all QC samples, including LLOQ were <14% and <10%, respectively (Table I).

Concluding statement

A rapid and specific LC/MS/MS method was developed and validated for quantifying methotrexate and 7-OH methotrexate in a single experiment, with a lower limit of quantitation (LLOQ) of 1 ng/mL in human plasma. The method has been used successfully in clinical sample analysis.

Table I. Human plasma validation results

<table>
<thead>
<tr>
<th>Calibration Range (ng/mL)</th>
<th>Correlation coefficient (r)</th>
<th>Accuracy &amp; Precision</th>
<th>Method Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLOQ 1.00</td>
<td>0.9960 to 0.9996</td>
<td>Inter-Batch (n=15)</td>
<td>Bioanalytical&lt;br&gt;QC Conc. ± RE% ± CV%</td>
</tr>
<tr>
<td>Low</td>
<td>3.00</td>
<td>3.60 ± 2.13</td>
<td>4.45 ± 5.50</td>
</tr>
<tr>
<td>High</td>
<td>600</td>
<td>2.60</td>
<td>5.56</td>
</tr>
</tbody>
</table>

Figure 1. The structure of methotrexate (A), and 7-hydroxy metabolite (B). Figure 2. (A) Methotrexate LC(/+)ESI-MS spectrum. (B) Methotrexate LC(/+)ESI-MS/MS product ion spectrum. Figure 3. (A) 7-OH methotrexate LC(/+)ESI-MS spectrum. (B) 7-OH methotrexate LC(/+)ESI-MS/MS product ion spectrum. Figure 4. (A) Representative chromatograms of methotrexate in plasma, and (B) Representative chromatograms of 7-OH methotrexate in plasma.