

Overview

A sensitive and specific 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. To support clinical trials, a short run time and lower detection limit is needed. We report a rapid, specific, and highly sensitive LC/MS/MS method capable of quantifying methotrexate and its 7-hydroxy metabolite at levels as low as 1 ng/mL. This method has been validated, and successfully applied in clinical sample analysis.

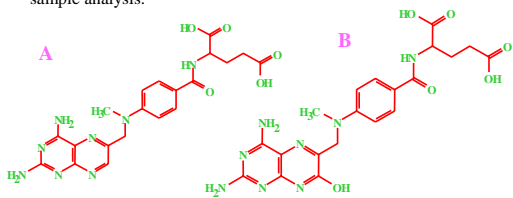


Figure 1. The structure of methotrexate (A), and 7-hydroxy metabolite (B).

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: Luna C18 column, 2.0 x 30 mm, 5 μm
 Gradient
 Flow rate: 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 mass spectrometer was set up for the following transition:
 Methotrexate: 455.3 → 308.1
 7-Hydroxy Metabolite: 471.2 → 191.0
 IS: 442.2 → 295.2

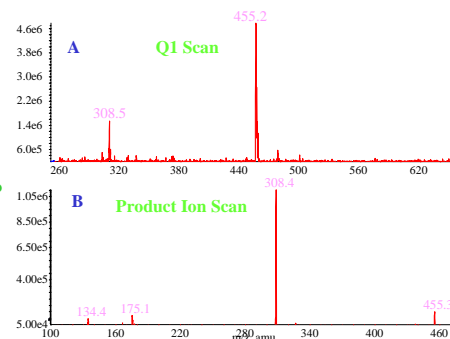


Figure 2. (A) Methotrexate LC(+)/ESI-MS spectrum. (B) Methotrexate LC(+)/ESI-MS/MS product ion spectrum.

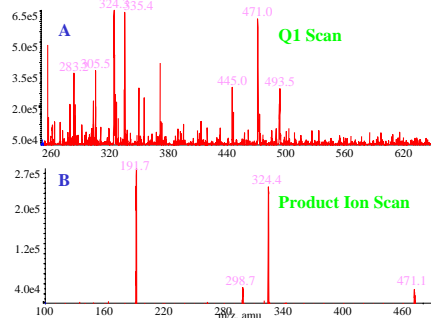


Figure 3. (A) 7-OH methotrexate LC(+)/ESI-MS spectrum. (B) 7-OH methotrexate LC(+)/ESI-MS/MS product ion spectrum.



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 dominant peak (Figures 2A, and 3A). Intense and consistent ion signals were achieved.

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).

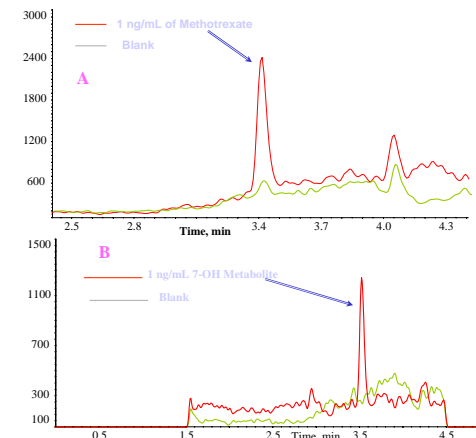


Figure 4. (A) Representative chromatograms of methotrexate in plasma, and (B) Representative chromatograms of 7-OH methotrexate in plasma.

Table I. Human plasma validation results

Calibration Range		1.00 to 500 ng/mL				
Correlation coefficient(r)		0.9960 to 0.9996				
		Methotrexate		7-OH Methotrexate		
Accuracy & Precision		Accuracy	Precision	Accuracy	Precision	
		RE%	CV%	RE%	CV%	
Inter-Batch (n=15)	LLOQ	1.00	-3.00	13.92	6.00	7.26
	Low	3.00	-6.33	4.45	-5.00	4.18
	Medium	250	3.60	5.56	4.40	6.70
	High	450	3.56	5.32	0.44	6.57
Method Recovery				%		
Methotrexate				103.60	to	106.77
7-OH Methotrexate				78.90	to	91.85
Stability				OCL(%)		OCH(%)
Freeze and Thaw		Methotrexate		103.33		110.44
		7-OH Methotrexate		98.67		108.44
Bench-Top (~4 hours)		Methotrexate		96.00		104.67
		7-OH Methotrexate		94.33		102.67

Conclusion

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.