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응용 자료

UPLC-MS/MS Analysis of Methotrexate in Plasma and Serum for Clinical Research

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Abstract

The antifolate methotrexate is a widely used drug for the treatment of various malignancies. Traditionally, enzyme and fluorescence based immunoassay tests are used to analyze methotrexate concentrations, but they are known for their cross-reactivity to the inactive metabolite DAMPA. Immunoassay techniques can overestimate methotrexate concentrations post-glucarpidase administration due to elevated levels of DAMPA, emphasizing the importance of using a selective detection method when performing clinical trials of high dose methotrexate. Here we describe a clinical research method using deproteination of plasma or serum samples with methotrexate-$^{2}H_{3}$ internal standard in methanol. Isocratic separation was achieved within five minutes using an ACQUITY UPLC HSS C_{18} SB Column on an ACQUITY UPLC I-Class System followed by detection on a Xevo TQD Mass Spectrometer.

Benefits

- Analytical selectivity afforded by highly selective mass detection
- Wide (400 fold) dynamic range
- Simple, inexpensive sample preparation using low sample volumes
- Speed of analysis

Introduction

The antifolate methotrexate is a widely used drug for the treatment of various malignancies. Traditionally, enzyme and fluorescence based immunoassay tests are used to analyze methotrexate concentrations, but they are known for their cross-reactivity to the inactive metabolite 2,4-Diamino-N^{10}-methylpteroic acid (DAMPA). Off-label use of high dose methotrexate (>500 mg/m^2) for patients with CNS lymphoma[1] is usually accompanied with the administration of glucarpidase (Voraxaze) or leucovorin rescue therapy to reduce methotrexate concentrations to below 1 mmol/L. Immunoassay techniques can overestimate methotrexate concentrations post-glucarpidase administration due to elevated levels of DAMPA, emphasizing the importance of using a selective detection method when performing clinical trials of high dose methotrexate.

Here we describe a clinical research method using deproteination of plasma or serum samples with

methotrexate-$^{2}H_3$ internal standard in methanol. Isocratic separation was achieved within five minutes using an ACQUITY UPLC HSS C_{18} SB Column (2.1 x 30mm, 1.8 µm) on an ACQUITY UPLC I-Class System followed by detection on a Xevo TQD Mass Spectrometer (Figure 1).

Figure 1. The Waters ACQUITY UPLC I-Class System with the Xevo TQD.

Experimental

Sample preparation

Methotrexate certified reference solution and its stable labeled internal standard ($^{2}H_3$) were purchased from Cerilliant (Round Rock, TX, USA). Calibrators were prepared in a surrogate matrix of pooled plasma purchased from Sera Laboratories International Ltd (West Sussex, UK). The calibration range for methotrexate was 0.025–10 µmol/L (0.01–4.5 µg/mL). QC materials were prepared using this same pooled plasma at 0.1, 2.5, 10, and 75 µmol/L (0.045, 1.1, 4.5, and 34 µg/mL). The two highest concentrations of QC samples were diluted tenfold in plasma, prior to analysis, to demonstrate acceptability of a dilution protocol.

Sample extraction

To 50 µL of sample, 250 µL of 0.1 µmol/L internal standard (ISTD) in methanol was added as a precipitation solution, vortex mixed for 30 seconds, and centrifuged for 2 minutes at 16,100 g. 50 µL of supernatant was added to 950 µL water to prepare the final extract for analysis on a conditioned UPLC-MS/MS system.

LC conditions

System:	ACQUITY UPLC I-Class (FTN)
Needle:	30 µL
Column:	ACQUITY UPLC HSS C_{18} SB (P/N 186004117)
Mobile phase A:	Water + 2 mM ammonium acetate + 0.1% formic acid
Mobile phase B:	Methanol + 2 mM ammonium acetate + 0.1% formic acid
Needle wash solvent:	80% aqueous methanol + 0.1% formic acid
Purge solvent:	Mobile phase A
Seal wash:	20% aqueous methanol
Column temp.:	45 °C
Injection volume:	20 µL
Flow rate:	0.40 mL/min
Isocratic gradient:	77:23 mobile phase A:mobile phase B
Run time:	5.0 min (5.7 min injection to injection)

MS conditions

System:	Xevo TQD
Resolution:	MS1 (0.7 FWHM) MS2 (0.7 FWHM)
Acquisition mode:	Multiple reaction monitoring (MRM) (see Table 1 for details)
Polarity:	ESI positive ionization
Capillary:	0.8 kV
Source temp.:	150 °C
Desolvation temp.:	500 °C
Dwell time:	0.2 s
Inter-scan delay:	0.02 s
Inter-channel delay:	0.01 s

Data management

MassLynx Software v4.1 with TargetLynx Application Manager

Method conditions

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage	Collision energy
Methotrexate (quantifier)	455.2	175.1	36	42
Methotrexate (qualifier)	455.2	134.1	36	34
Methotrexate-$^{2}H_{3}$ (internal standard)	458.2	175.1	36	42

Table 1. Guideline MRM parameters for methotrexate and its internal standard, methotrexate- $^{2}H_{3}$ *used in this study.*

Results and Discussion

Under the chromatographic conditions mentioned previously, methotrexate is separated chromatographically from its principal metabolites 7-hydroxymethotrexate and DAMPA. Figure 2 shows a typical lowest concentration calibrator and metabolites.

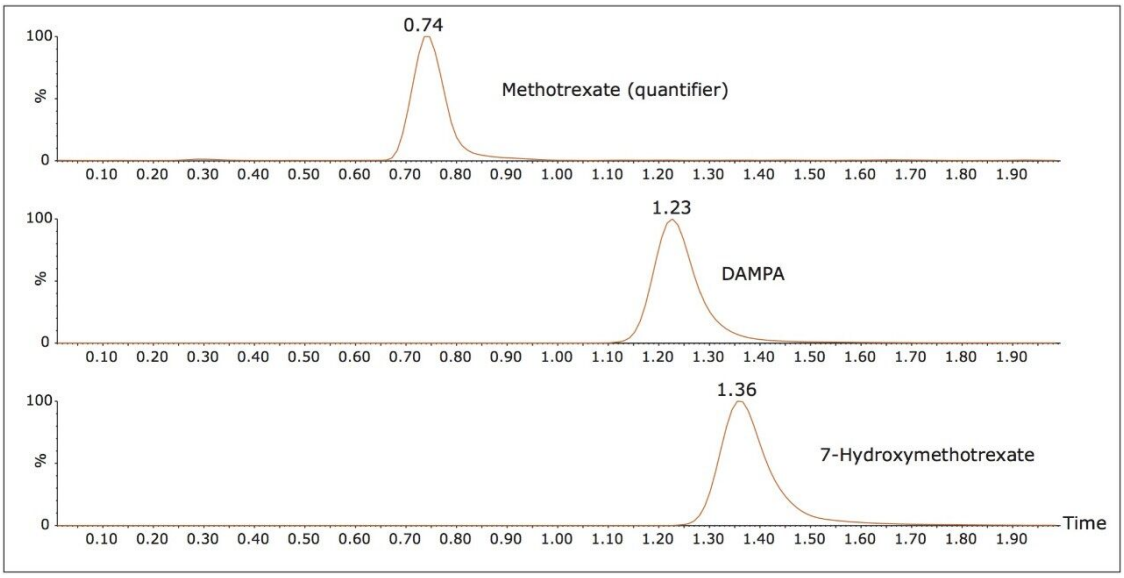

Figure 2. Example chromatograms of methotrexate (0.025 µmol/L), 7-hydroxymethotrexate (5 µmol/L), and DAMPA (5 µmol/L) in plasma.

No system carryover was observed following analysis of plasma samples with methotrexate levels of up to 100 µmol/L.

Analytical sensitivity investigations indicate that quantification (<20% RSD) at 0.0025 µmol/L for methotrexate is achievable.

Total precision was determined by extracting and quantifying five replicates of four concentrations of QC material over five separate days (n=25). Repeatability was assessed by analyzing five replicates at each QC level. Table 2 presents the results of these experiments, where total precision and repeatability at the four concentrations assessed was ≤5.5% RSD for methotrexate. Samples of 10 µmol/L and 75 µmol/L concentration were diluted tenfold prior to analysis to enable quantification.

Nominal concentration (µmol/L)	Total precision (% RSD)	Repeatability (% RSD)
0.1	5.1	4.0
2.5	4.6	1.8
10	5.4	2.9
75	5.5	2.7

Table 2. Total precision and repeatability for analysis of methotrexate.

The method was shown to be linear over the range of 0.0175–13.0 µmol/L when different ratios of high and low concentration pools of methotrexate were combined and analyzed.

A tenfold dilution was performed on spiked plasma samples (20 and 75 µmol/L), with a mean accuracy of 102.1% (n=3) relative to the expected concentration.

Matrix effects were evaluated at low (0.1 µmol/L) and high (7.5 µmol/L) methotrexate concentrations in plasma and serum samples (n=7). The matrix factor range was 0.94 to 1.40 for serum samples compared with 3.12 to 3.77 for the plasma samples. The use of methotrexate internal standard adjusted response compensated for this enhancement with matrix factor ranges of 1.01 to 1.11 and 1.01 to 1.12 for serum and plasma samples, respectively.

Equivalence between plasma and serum matrix samples was determined by fortifying six individual matched plasma and serum samples with 0.1 µmol/L, 2.5 µmol/L, 10 µmol/L, and 75 µmol/L of methotrexate. Mean % differences between plasma and serum in the range -1.2% to 6.5% were observed.

Potential interference from endogenous compounds (albumin, bilirubin, cholesterol, triglycerides, and uric acid) and the exogenous material intralipid (20% emulsion) spiked at high concentrations was assessed by determining the recovery of methotrexate (n=3) from low and high pooled plasma samples (0.1 µmol/L and 1.0 µmol/L). Recovery ranged from 90.4–102.9%, and when assessing metabolites 7-hydroxymethotrexate and DAMPA at 5 µmol/L and 50 µmol/L the recovery range was 98.8–103.3%. A substance was deemed to interfere if a recovery range of 90–110% was exceeded.

An initial assessment of method accuracy was made by analyzing 12 External Quality Assurance (WEQAS, Cardiff, UK and LGC, Teddington, UK) serum samples spanning a concentration range from 0.057–3.80 µ mol/L. Samples were analyzed over five separate days. Mean % deviations from absolute reference values

ranged from -7.4% to +0.4%, with an overall mean of -5.7%.

Methotrexate measurements were made on a plasma series drawn from an individual 27 hours pre- to 147 hours post-glucarpidase administration. Methotrexate measurements by immunoassay differed to UPLC-MS/MS values by 36–99% <45 hours post-glucarpidase; the magnitude of the difference was proportional to the DAMPA peak area. Between 34–57 hours post-glucarpidase, the UPLC-MS/MS method detected an increase in methotrexate concentration which was not observed by immunoassay. Figure 3 presents a comparison of data.

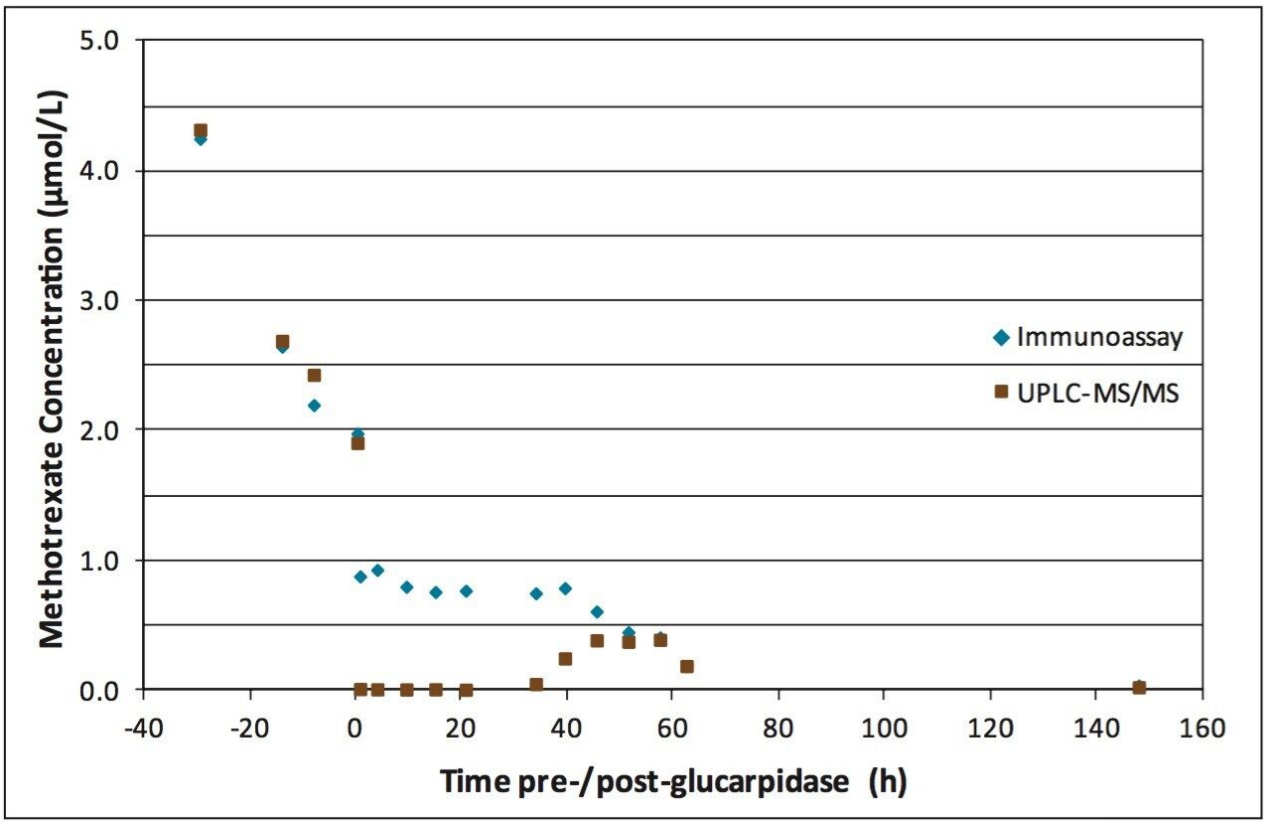

Figure 3. Comparison of methotrexate concentration determined by UPLC MS/MS and immunoassay from an individual undergoing glucarpidase treatment.

Conclusion

An analytically sensitive and selective clinical research method has been developed for the analysis of

plasma and serum methotrexate.

Using only 50 µL of sample with fast and inexpensive sample preparation, this analytical method provides sufficient analytical sensitivity to analyze low levels of methotrexate (0.025 µmol/L). Methotrexate is separated from its principal metabolites both chromatographically (UPLC) and selectively (mass detection using multiple mass monitoring), enabling accurate and precise quantification of methotrexate across the investigated concentration range.

Applying the UPLC-MS/MS method to research samples, post-glucarpidase administration shows a methotrexate 'rebound' phenomenon, not detected by immunoassay. This highlights the importance of using this selective detection method when performing clinical trials of high dose methotrexate in combination with glucarpidase rescue therapy to further understanding the pharmacokinetics of methotrexate.

References

1. Ahmed and Hasan, *J Cancer Sci* Ther. 2013;5(3):106–12.

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