Journal of Chromatography B, 1002 (2015) 107–112

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A reversed-phase high performance liquid chromatography method for quantification of methotrexate in cancer patients serum

Yuan-dong Li*, Yan Li, Ning-sheng Liang, Fan Yang, Zhi-peng Kuang

Research Department, Affiliated Tumor Hospital of Guangxi Medical University, Nanning, 530021 Guangxi, People's Republic of China

ARTICLE INFO

Article history:
Received 15 April 2015
Received in revised form 11 August 2015
Accepted 12 August 2015
Available online 20 August 2015

Keywords:
Method validation
HPLC
Methotrexate
Silver nitrate
Serum

ABSTRACT

A simple, rapid and sensitive reversed-phase high performance liquid chromatography (HPLC) method has been developed for the determination of methotrexate in human serum. After deproteinization of the serum with 40% silver nitrate solution, methotrexate and internal standard (IS) were separated on a reversed-phase column with a mobile phase consisting of 10 mM sodium phosphate buffer (pH6.40)-methanol (78:22%, v/v) and ultraviolet detection at 310 nm. The linearity is evaluated by a calibration curve in the concentration range of 0.05–10.0 μg/mL and presented a correlation coefficient of 0.9995. The absolute recoveries were 97.52 ± 3.9% and 96.87 ± 3.7% for methotrexate and ferulic acid (internal standard), respectively. The intra- and inter-day precision were less 6.19 and 5.89%, respectively ($n = 6$). The limit of quantitation was 0.02 μg/mL and the limit of detection was 0.006 μg/mL. The complete analysis was achieved less than 10 min with no interference from endogenous components or 22 examined drugs. This method was validated by using serum samples from high-dose methotrexate treated patients with osteosarcoma, breast cancer, acute leukemia and lymphoma. The method was demonstrated to be a simple, rapid and reliable approach in quantification of methotrexate in serum samples from patients with high-dose methotrexate therapy.

1. Introduction

Methotrexate (MTX; Fig. 1) is an antifolate cytotoxic agent used to treat certain types of hematological cancers, solid tumors, and rheumatoid arthritis. High-dose methotrexate (HDMTX) has for decades been in clinical use as a cytotoxic drug for solid tumors and leukemias [1,2]. HDMTX has many serious toxic effects, such as myelosuppression, hepatic, renal and pulmonary disorders [3,4]. For safe and effective use of HDMTX, certain precautions should be followed. Routine monitoring of MTX concentrations in serum with early detection of abnormal clearance has permitted countermeasures, such as adjustments of leucovorin doses and intensified hydration regimes, to prevent excessive host toxicity [5,6]. Therefore, the routine monitoring of MTX concentrations in serum is important in guiding leucovorin rescue and is considered to be imperative for both patient safety and evaluation of therapeutic concentrations of MTX [5,7].

Several chromatographic methods for determination of MTX in serum have been reported [8–17]. Among these methods, high sensitivity tandem mass spectrometry had been employed for MTX monitoring [8]. However, tandem mass spectrometry facilities are not always available as standard equipment in hospital laboratories, thereby limiting its application in clinical routines. Consequently, several HPLC methods have been developed to determine the levels of MTX in human serum following clean-up procedures, such as solid-phase extraction or protein precipitation [9–17]. Among these methods, solid-phase extraction method is the most used method, but it requires tedious solid phase-extraction procedures. Protein precipitation method usually uses trichloroacetic acid and shows lower recovery rate than solid-phase extraction methods [17]. There is still a need for a sensitive, rapid and inexpensive method broadly applicable to clinical routines for therapeutic drug monitoring of MTX.

The aim of this study was to develop a simple, rapid and highly specific and sensitive HPLC method for quantitation MTX in serum samples after deproteinization and clean-up steps. The method was validated in a cohort of routine drug monitoring in different cancer patients under HDMTX treatment. This method involved the isolation of MTX from 200 μL serum by deproteinization by Silver nitrate and subsequent HPLC-ultraviolet quantification of MTX. This study included the evaluation of several frequently co-

* Corresponding author at: Research Department, Affiliated Tumor Hospital of Guangxi Medical University, 71 Hedi Road, Nanning, 530021 Guangxi, People's Republic of China. Fax: +86 771 5310593.
E-mail address: lyd641209@163.com (Y.-d. Li).

http://dx.doi.org/10.1016/j.jchromb.2015.08.017
1570-0232/

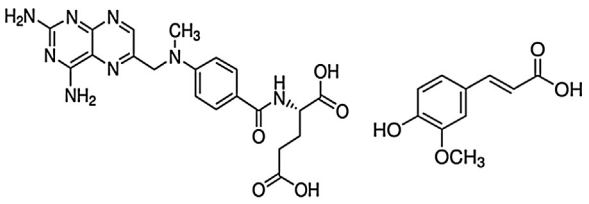

Fig. 1. Molecular structures of methotrexate and ferulic acid.

administered drugs to minimize errors resulting from the possible interference of these drugs with MTX, and thus, to enhance valuable analytical properties such as specificity.

2. Experimental

2.1. Reagents and chemicals

Methotrexate, ferulic acid (internal standard, IS; Fig. 1), hydrocortisone, gentamycin, caffeine, ibuprofen, acyclovir, etoposide, allopurinol, 5-flurouracil, aspirin, paracetamol, dexamethasone, prednisolone, caspofungin, amoxicillin, aztreonam, clarithromycin, folic acid, gentamycin, levofolinate, methylprednisolone, naproxen, omeprazole, salicylate, cytarabine, aminopterin, acyclovir, inosine, ferulaic acid, silver nitrate, cisplatin, potassium dihydrogen phosphate and potassium iodide were analytical-reagent (AR) grade (Sinopharm Chemical Regent Corporation, Beijing, China). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q Ultra-Pure Water System (Millipore, Bedford, MA).

2.2. Chromatographic conditions

The HPLC system (Shimadzu, Japan) consisted of a CBM-20 pump, a SIL-20 autosampler, a CTO-20A column oven and SPD-20A UV/VIS detector set at 310 nm. Samples were analyzed on a Zorbax-ODS C18 column (4.6 × 250 mm, 5 μm, Shimadzu), protected by a guard column (4.6 × 15 mm i.d.) of the same material. The mobile phase (10 mM sodium phosphate buffer (pH6.40): methanol, 78:22% (v/v) was vacuum degassed and filtered through a 0.2 μm Millipore membrane filter before use. The flow-rate was 1.0 mL/min and chromatography was performed at 35 °C temperature. The injection volume was 20 μL for all injections.

2.3. Preparation of standard solutions, calibration standards and quality control samples

Stock solution of MTX was prepared by dissolving 25.0 mg of MTX in 50 mL of in mobile phase. The IS solution was prepared by dissolving 20.0 mg of ferulic acid in 100 mL of the same solvent. Working solutions of each analyte at different concentration levels were freshly prepared by appropriate dilution with the previous solvent mixture in amber-glass vials. Aliquots of these working solutions together with 4 μL IS were added to blank human serum prior to prepare calibration standards resulting in 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10 μg/mL, respectively. Low, medium and high quality control (QC) samples were similarly prepared containing MTX concentrations in serum of 0.1, 1.0 and 5.0 μg/mL, respectively.

2.4. Sample preparation

Serum samples (200 μL) were placed in polypropylene centrifuge tubes followed by 5 μL of ferulic acid solution (200 μg/mL) as internal standard (IS). 20 μL of 40% (w/v) silver nitrate solution was added to precipitate serum proteins. The tubes were vigorously mixed for 1 min then heated at 45 °C for 5 min to optimize the precipitation. Subsequently, 25 μL of 50 % (w/v) potassium iodide solution was added to the mixture, then the mixture was vortexed again for 1 min and centrifuged at 15,000 × *g* for 10 min. The supernatant solution (20 μL) was injected into the chromatographic system for analysis.

2.5. Method validation

The method was validated for specificity, linearity, precision, accuracy, and recovery according to the Drug Evaluation and Research guideline recommended by the US Food and Drug Administration Center for bioanalytical method validation [18].

2.5.1. Specificity

Specificity was determined by testing 6 drug-free serum samples from seven different healthy volunteers for interference with the analyte and the IS. Furthermore, the possibility of chromatographic interference with the most frequently co-administered drugs was examined by analyzing several commercially available medications. The following substances were dissolved in the appropriate solvent for each medication filtered and analyzed: hydrocortisone, amoxicillin, aztreonam, caspofungin, clarithromycin, doxycycline, gentamycin, methylprednisolone, prednisolone, naproxen, omeprazole, etoposide, 5-flurouracil, caffeine, dexamethasone, folic acid, paracetamol, salicylate, levofolinate, acyclovir and allopurinol.

2.5.2. Recovery, limit of quantitation and limit of detection

The extraction recoveries of MTX in human serum at three QC levels were determined by comparison of the peak area of the QC samples ($n = 3$) from extracted samples with standard solutions and without extraction procedure at equivalent concentrations. The method recovery was determined for QC samples spiked at the 3 concentrations by comparing the calculated concentrations with the corresponding spiked concentrations.

The limit of quantitation (LOQ) of the method was defined as the point where the signal to noise ratio was equal to 10. The limit of detection (LOD) was defined as the point where the signal to noise ratio equaled 3.

2.5.3. Linearity

The calibration curve was generated with the concentrations ranging from 0.05 to 10.0 μg/mL. The calibration curve was constructed via linear regression, and the linear range of the analyte was calculated using the summed peak area ratio of MTX to that of the internal standard (ferulic acid).

2.5.4. Precision and accuracy

Intra-day precision and accuracy was performed with six replicates of the low (0.1 μg/mL), medium (1.0 μg/mL) and high (5.0 μg/mL) QC samples, whereas inter-day evaluation was assessed by the analysis of one replicate at each QC sample concentration on six different day. Intra- and inter-day precisions were determined by assessing the measurement of QC samples at low, medium and high concentration. Precisions were expressed by the coefficient of deviation (CV%) was defined as the ratio of the standard deviation to the mean, while accuracy (%) was expressed as the percentage of observed value to true value.

2.6. Biological samples

Blood samples were obtained from patients diagnosed with osteosarcoma, breast cancer, acute leukemia and lymphoma (Department of Clinical Oncology, Affiliated Tumor Hospital of