Journal of Pharmaceutical Analysis 6 (2016) 103–111

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Journal of Pharmaceutical Analysis

journal homepage: www.elsevier.com/locate/jpa
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Original Article

# Optimized high performance liquid chromatography–ultraviolet detection method using core-shell particles for the therapeutic monitoring of methotrexate ☆

Milagros Montemurro, María M. De Zan *, Juan C. Robles

*Laboratorio de Control de Calidad de Medicamentos, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina*

ARTICLE INFO

*Article history:*
Received 15 May 2015
Received in revised form
10 December 2015
Accepted 15 December 2015
Available online 17 December 2015

*Keywords:*
HPLC
Core–shell particles
Methotrexate
Drug monitoring

ABSTRACT

Methotrexate (MTX) is an antineoplastic drug, and due to its high toxicity, the therapeutic drug monitoring is strictly conducted in the clinical practice. The chemometric optimization and validation of a high performance liquid chromatography (HPLC) method using core–shell particles is presented for the determination of MTX in plasma during therapeutic monitoring. Experimental design and response surface methodology (RSM) were applied for the optimization of the chromatographic system and the analyte extraction step. A Poroshell 120 EC-C18 (3.0 mm × 75 mm, 2.7 μm) column was used to obtain a fast and efficient separation in a complete run time of 4 min. The optimum conditions for the chromatographic system resulted in a mobile phase consisting of acetic acid/sodium acetate buffer solution (85.0 mM, pH=4.00) and 11.2% of acetonitrile at a flow rate of 0.4 mL/min. Selectivity, linearity, accuracy and precision were demonstrated in a range of 0.10–6.0 μM of MTX. The application of the optimized method required only 150 μL of patient plasma and a low consumption of solvent to provide rapid results.



## 1. Introduction

Methotrexate (MTX, 4-amino-N10-methylpteroylglutamic acid) is a cytotoxic drug used since the 1940s in the therapy of acute lymphoblastic leukemia (ALL), non-Hodking lymphoma and cerebral tumors [1].

The structure analogy between MTX and folic acid (Fig. 1) is the basis of its mechanism of action. Its antiproliferative action is developed by the competitive inhibition of dihydrofolate-reductase (DHFR), an enzyme involved in folic acid metabolism. At high doses, MTX follows different metabolic pathways of detoxification, producing two main metabolites, i.e., 7-hydroxy-MTX (7-OH-MTX) and 4-amino-4-deoxy-N10-methylpteroic acid (DAMPA) [2].

Since MTX inhibits a key cellular function, it is an important cytotoxic compound, especially on actively replicating cells, but also on other tissues [1–3]. Thus, MTX is one of the very few antineoplastic drugs for which therapeutic drug monitoring (TDM) is currently conducted in clinical practice, especially in high-dose protocols.

In order to allow the use of protocols with very high doses of MTX, a strategy called "leucovorin rescue therapy" has been devised. Administration of leucovorin at a scheduled time after the infusion of high-dose MTX is beneficial to healthy cells, and protects them from the cytotoxic action of MTX [1].

Several methods have been developed for the determination of MTX and its metabolites in human fluids. Firstly, bioanalytical methods using antibodies, such as radioimmunoassay [4,5], fluorescent polarization immunoassays (FPIA from Abbott) [6] and the enzyme multiplied immunoassay technique (EMIT from Behring Diagnostics) [7], have been used. These techniques have many advantages in simplicity, speed and cost. However, specificity can be compromised because antibodies can present cross-reactivity, leading to an overestimation of the actual MTX concentration. Recently, an electrochemical approach has been presented for the determination of MTX, which uses a poly (L-lysine) modified electrode in the presence of sodium dodecyl benzene sulfonate. This sensor provided satisfactory results for a wide linear concentration range, low detection limit, high selectivity and good stability. Its practical applicability has been proven by quantifying MTX in medicinal tablets, but it was not applied to detect the analyte in biological samples [8].

Among the separation methods, high-performance liquid chromatography (HPLC) using different detection modes, such as electrochemistry [9,10], fluorescence through pre- or post-column oxidation [11], UV [12,13] and mass spectrometry [14,15], is widely

☆Peer review under responsibility of Xi'an Jiaotong University.

* Corresponding author.
*E-mail address:* mmdezan@fbcb.unl.edu.ar (M.M. De Zan).

http://dx.doi.org/10.1016/j.jpha.2015.12.001

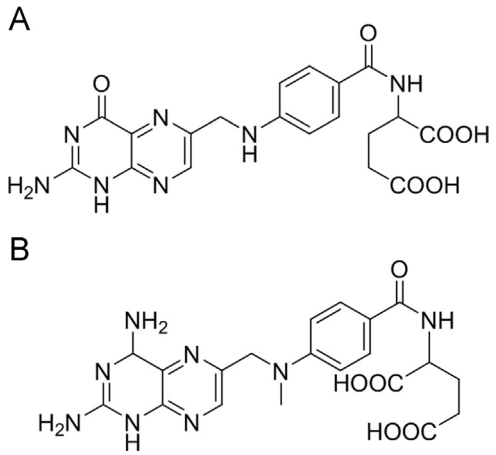


**Fig. 1.** Chemical structures of (A) folic acid and (B) methotrexate.

used for the determination of MTX in plasma for clinical purposes.

In the past few years, sub-3 μm core–shell particles columns for HPLC have been developed. The premise which drove their development is the reduced diffusion length for analytes inside the core–shell particles compared to their fully porous counterparts. As the thickness of the porous shell decreases, the faster mass transfer can lead to improved column efficiency and shorter elution time, reducing both total analysis time and organic solvent consumption [16,17].

Core–shell particles columns for biological samples have been reported for the determination of 25-hydroxymetabolites of vitamin D2 and D3 in serum [18], N-(ω)-hydroxy-nor-L-arginine, L-arginine and N-(ω)-ethyl-L-arginine in rat plasma [19] and boldine in rat plasma, urine and bile [20].

As for sample preparation, protein precipitation as the first step is highly recommended, as it is simple, fast and inexpensive. An extraction step can contribute to further clean-up, in which an organic solvent is employed to back-extract the solvent used for protein precipitation, leaving a smaller water volume that can be directly injected into the chromatographic system.

The development of a new analytical method may involve optimization approaches, for which experimental design, especially response surface analysis and Derringer's desirability function, are valuable tools [21,22].

Response surface methodology (RSM) is a statistical and mathematical technique used to model the experimental data and obtain the polynomial equation that best fits the response behavior [23]. When more than two responses are to be optimized simultaneously, the Derringer's desirability function is a useful strategy for finding the operative conditions that satisfy the optimization criteria for all the responses taken into account [24].

Once the method is developed and optimized, a full validation should be performed. The main characteristics of a bioanalytical method, which are essential for ensuring the acceptability of the performance and the reliability of analytical results, are selectivity, lower limit of quantification, response function and calibration range, accuracy, precision, matrix effects, stability of the analyte (s) in the biological matrix, and stability of the analyte(s) in the stock and working solutions [25].

In this study, a fast and efficient high performance liquid chromatography–ultraviolet (HPLC–UV) method was developed for the determination of MTX in human plasma applicable to the TDM. Both sample preparation and chromatographic separation were optimized and the method was validated according to the European Medicines Agency (EMA) guideline on bioanalytical method validation [25].

## 2. Materials and methods

### 2.1. Apparatus and software

The HPLC analyses were accomplished using an Agilent 1100 Series system, equipped with a quaternary pump, a membrane degasser, a thermostated column compartment, an autosampler and a diode array detector (DAD) (Agilent Technologies, Waldbronn, Germany). For data acquisition and processing, the Chemstation version B 0103 was used.

Experimental design, surface response modeling and desirability function calculations were performed using the Stat–Ease Design–Expert 8.0.0.

### 2.2. Chemicals, reagents and samples

Methotrexate sodium ($Na_2$MTX) freeze-dried preparation for injection was supplied by Microsules (Buenos Aires, Argentina). Acetonitrile (HPLC grade) was purchased from Aberkon Química (José León Suarez, Argentina). Glacial acetic acid (analytical grade) and chloroform (analytical grade) were purchased from Laboratorios Cicarelli (San Lorenzo, Argentina), while methylene chloride (analytical grade) and sodium acetate (analytical grade) were supplied by Anedra (Buenos Aires, Argentina). Purified HPLC grade water was obtained from a Milli-Q® system (Millipore, Milford, MA, USA).

Real unknown samples containing MTX were obtained from the remaining volume of plasma used in the laboratory for the TDM in patients hospitalized at "J.M. Iturraspe Hospital" in city of Santa Fe (Argentina). These samples were conserved at 4 °C for less than 6 h until analysis. Blank human plasmas were obtained from non-treated unidentified volunteers.

During the method development, pooled plasma samples prepared by mixing several samples from different hours post infusion were used to obtain an average concentration of MTX and 7-OH-MTX.

### 2.3. Calibration solutions and quality control (QC) samples

MTX stock standard solution at 1250 μM was prepared by weighing and dissolving an appropriate amount of $Na_2$MTX in 50.0 mL of purified water. Calibration solutions were prepared by adding appropriate volumes of stock standard solution to blank human plasma. The final MTX concentrations in the calibration solutions were 0.10, 0.50, 1.00, 1.50, 2.00, 4.00, and 6.00 μM.

QC samples were prepared by diluting the stock solution with blank plasma at 0.10, 0.30, 3.0 and 4.5 μM, to obtain low limit of quantification (QC-LLOQ, 0.10 μM), and low (QC-L, 0.30 μM), medium (QC-M, 3.0 μM) and high (QC-H, 4.5 μM) concentration levels, respectively.

### 2.4. Sample preparation

The procedure used for the extraction of the analyte from plasma samples consisted of two steps, i.e., protein precipitation using acetonitrile (ACN) with a ratio of 2:1 (ACN:plasma), and then the back-extraction of the solvent used for protein precipitation. For this purpose, chloroform and methylene chloride were evaluated. The performance of this procedure was optimized by experimental design and RSM.

### 2.5. Chromatographic separation

The separation was achieved using a Poroshell 120 EC-C18 (3.0 mm × 75 mm, 2.7 μm) column (Agilent Technologies) and monitored at 305 nm. The mobile phase consisted of acetic acid/