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Original Article

High-Performance Liquid Chromatography Determination of Methotrexate in Plasma

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Abstract

This article describes a simple and fast high-performance liquid chromatography method for the determination of methotrexate (MTX) in plasma. Samples were collocted from children receiving high-dose MTX at shafa Hospital (Ahvaz University of Medical sciences, Ahvaz, iran) at various times after the end of eachinfusion. Plasma was deproteinized with trichloroacetic acid and the supernatant was injected into a 250×4.6 mm octadecylsilane column. Mobile phase was made of TRIS-phosphate buffer (pH 5.7): methanol: acetonitrile (82:11:7) with a flow rate of 1.8 ml/min. Ultraviolet detection was done at 313 nm and at ambient temperature. Para-aminoacetophenone was used as internal standard. Methotrexate and internal standard retention times were 4.4 and 6.5 minutes, respectively. Results showed that reproducibility (precision) of method within a day was 2.6 to 6 percent and between days was 5.5 to 9.5 percent. The recovery of the method was between 61.5 and 72.7 percent. The quantitation limit of the method for methotrexate was 0.1 μM. This method is suitable for quantitation of methotrexate after infusion of high doses of this drug and has good accuracy, precision and quantiatation limit.

Key Words: *Methotrexate; HPLC; Plasma Concentration.*

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Introduction

Methotrexate (MTX) is a folate antagonist with cytotoxic activity that is used in the treatment of diseases such as acute lymphocytic leukemia, acute non-lymphocytic leukemia, non-Hodgkin's lymphoma, choriocarcinoma, osteosarcoma, some other tumors and also acute psoriasis, rheumatoid arthritis and steroid dependent asthma (1, 2).

Methotrexate concentration in plasma and other biological fluids is determined to study its pharmacokinetics and also to predict and prevent its toxicity when administered in high-dose intravenous infusion of the drug by modification of the calcium folinate administration schedule according to the plasma concentration of methotrexate (1-3).

Different methods have been described for the determination of methotrexate in plasma and other biological fluids including enzyme inhibition methods, radiometry and radio immunoassay, enzyme multiple immunoassay technique (EMIT), fluorescence polarization immunoassay (FPIA) (3, 4), and high-performance liquid chromatography (HPLC) (5-13). The aim of this study is to describe a simple, fast, accurate and precise method for the determination of methotrexate in plasma for pharmacokinetic studies and routine therapeutic drug monitoring in high-dose intravenous infusion of this drug.

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Experimental

Materials

Chemicals: Methotrexate (+)-amethopterine, (>98% purity for HPLC) and p-aminoacetophenone were purchased from

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Sigma (USA). Methanol and acetonitrile (Merck, Germany), trichloroacetic acid (BDH, UK), TRIS (tris-hydroxymethyl–aminomethane (Merck, Germany), sodium dihydrogen phosphate, $2H_2O$ (from Merck, Germany) were all of analytical grade.

Methods

Apparatus: A Cecil CE1100 liquid chromatography system equipped with a CE 1000 pump, a CE1200 variable wavelength uv-visible detector and a Rheodyne 20 microliter loop injector system was used (Cecil Instruments, England). The chromatography column was a 250x 4.6 (i.d)-millimeter Spherisorb ODS2 with 5-micron particles (Hichrom, England).

Chromatographic conditions: The mobile phase consisted of phosphate –TRIS buffer (0.1 M dihydrogen phosphate and 0.01 M TRIS; pH 5.7): methanol: acetonitrile, with the ratio of 82:11:7, respectively. The flow rate was 1.8 ml/minute and the eluent was monitored spectrophotometrically at room temperature.

Solutions of external and internal standards: Due to the possible instability of methotrexate in aqueous phosphate buffer solutions (14), a stock standard solution of methotrexate (200.2 µM) was prepared using TRIS-HCl buffer (pH 8): acetonitrile, 9:1, respectively to provide adequate stability and solubility. Stock solutions of internal standard (p-aminoacetophenone) were also prepared in the same solvent at the concentrations of 15 and 50 µg/lit. Further dilution of methotrexate stock solution was done with drug free plasma to prepare different concentrations of methotrexate (0.1 to 5 µM and 5 to 100.1 µM).

Sample collection and preparation: received methotrexate at doses of 0.5, 1 or 2 g/m^2 as 4, 6 or 24-h infusions, as part of protocols for the treatment of various oncologic or hematological diseases at shafa Shafa Hospital (Ahvaz University of Medical Sciences,Ahvaz, Iran). Blood samples were collected at various times after the end of each infusion. To each 200 µL of patient's or standard sample, 20 µL of stock solutions of internal standard 50µg/lit and 15 mg/litwere added to samples to samples with methotrexate concent of above and lees than 5µm, respect After complete mixing of samples with internal standard, 40 µL of trichloroacetic acid (2 M in ethanol) was added and vortex mixed for 2 minutes, then centrifuged at 3000 rpm for 15 minutes.10 or 20 µL aliquots of the supernatant was directly injected into the chromatography column. Each sample was analyzed in duplicate. All samples or standard solutions were stored at –20ºC until analyzed.

f) Recovery and precision: the recovery was studied by preparation of the various amounts of MTX in blank plasma (spiked blank). MTX was determind according to the described method. The recovery was calculated by comparison of the found amounts with the added ones.

Results

Under the conditions used for the chromatography, the retention times for methotrexate and the internal standard were 4.44±0.08 and 6.47±0.14 minutes, respectively. Figure 1 shows the chromatograms of human blank plasma used for preparation of different

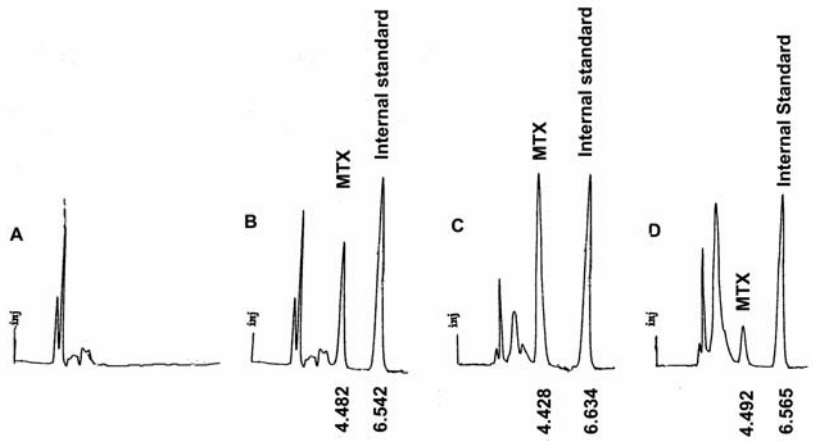

Figure 1. Chromatograms: A-human blank plasma, B-MTX standard (25 µM), C-Patient plasma sample at the end of a 4-h infusion of 2 g/sqm of MTX, D-patient plasma sample 7 hours after the end of a 4-hours infusion of 2 g/sqm of MTX. (detector sensitivity: 0.005 a.u.f.s; injection volume: 10 µl)

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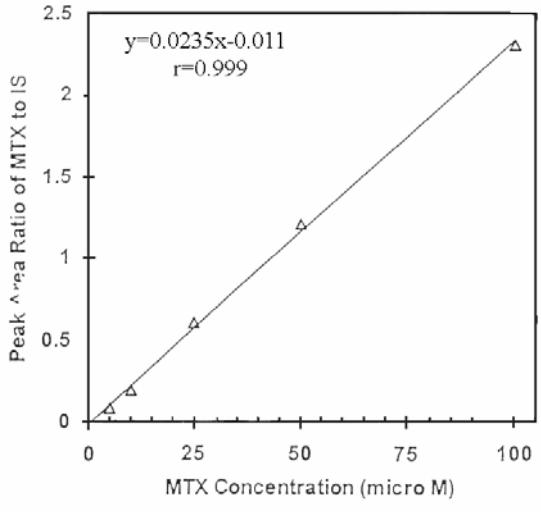

Figure 2. Linear standard curve determination of methotrexate in plasma (concentration range 0.1-5 µM/L)

concentrations of methotrexate and also from patients (1A), standard solution (1B) and samples (1C and 1D) withdrawn from patients who received 2 g/m^2 of methotrexate as a 4-h infusion.

The chromatographic condition employed was quite specific for methotrexate and p-aminoacetophenone. Other drugs that might be administered concomitantly with methotrexate such as leucovorine, adriamycin, bleomycin, cyclophosphamide, mercaptopurine, vincristine, metocloperamide, prednisolone, diazepam, aspirin and trimethoprim-sulfamethoxazole could not interfere with MTX Peaks because either they have no significant absorption at 313 nm or their retention times are quite different. This fact was proved in this study by adding each of these drugs to plasma samples containing methotrexate and analyzed by chromatography.

According to the previous reports, the major and most important metabolite of methotrexate is 7-hydroxymethotrexate but its pharmacological effect is not significant. (4-7, 9-12). We did not have any pure standard of this metabolite and unable to obtain it from elsewhere, therefore it was not possible to identify its retention time positively and to quantitate its concentration in plasma.

In order to determine plasma concentration of methotrexate, internal standardization method was used. After preparation of various concentrations of methotrexate and analyzing chromatography each standard solution, two standard curves were prepared by plotting the ratio of peak area of methotrexate to internal standard (p-aminoacetophenone) versus concentration of methotrexate. A good linearity was seen for both the standard curves (Figure 1, 2).

Table 1. Asessment of recovery of the method

Substance	Concentraton	Recovery (%)
MTX	0.1 (µM)	61.5±9.0
MTX	5 (µM)	72.7±7.2
MTX	10 (µM)	67.1±10.8
MTX	100 (µM)	65.6±7.1
Internal Standard	15 (µg/ml)	90.9±12.0
Internal Standard	50 (µg/ml)	91.9±17.0

To assess the accuracy of the method, recovery of methotrexate from plasma samples with known concentrations was compared with the solutions of methotrexate at the same concentrations as shown in table 1.

For the assessment of method precision, reproducibility of the results obtained for different concentrations of methotrexate was determined at 5 different days and 5 times in one day. The results of reproducibility study are shown in Table 2 as coefficient of variations.

The limit of detection for this method with signal to noise ratio of 3 was 0.01-µM.The limit of quantitation of methotrexate in plasma with the above sample pretreatment method was 0.1 µM.

Discussion

Various methods of high-performance liquid chromatography for the determination of methotrexate in biological fluids have been described so far which differ in chromatography type (reverse phase or ion-pair chromatography) or detection system (uv or fluorescence). (4-12). Reverse-phase high-performance liquid chromatography with uv detection has been most recommended. (5-8, 10, 11, 13). But, an important point is that most of them are tedious and expensive because they use more material and have many stages of experiment. Besides, they are not suitable for a routine and quick therapeutic drug monitoring

Table 2. Assessment of reproducibility of the method (five replicate analyses).

MTX Concentration (µM)	Intraday Coefficient of Variation (%)	Interday Coefficient of Variation (%)
0.1	6.0	9.5
5	4.5	5.5
10	2.6	7.2
100.1	2.1	7.3

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(TDM) test which is necessary for a child cancer patient in a high dose infusion therapy at a research university hospital, or reference laboratories. In this article a simple and fast method for the determination of methotrexate in plasma is described that has equal precision and accuracy to other similar methods (6-8, 10, 11, 13). A full chromatography takes 10 minutes. To include sample preparation time it may need 30 minutes for the whole of each analysis, which is comparatively a short time. The short duration of assay time is of quite importance in routine monitoring of the drug in plasma to predict and prevent future toxicity in high-dose methotrexate intravenous infusion. On the other hand this method has a satisfactory quantitation limit that makes it ideal for pharmacokinetic studies and therapeutic drug monitoring of methotrexate after the administration of high doses of this drug. To improve the quantitation limit further we could use solid phase extraction technique along with fluorescence detection after post column derivitization of the methotrexate to fluorescent compounds so that the method become suitable for determination of methotrexate after the administration of lower doses of the drug but such methods are more tedious, time consuming and expensive.

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