QUANTITATIVE LC ANALYSIS OF METHOTREXATE AND ITS APPLICATION IN PHARMACOKINETIC STUDY OF DRUG IN RAT SERUM SAMPLES

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Summary

Methotrexate (MTX) is an immunosuppressive medication used to treat a variety of inflammatory diseases such as rheumatoid arthritis, cancers and uveitis**.** MTX concentration in serum and other biological fluids is determined to study its pharmacokinetic and also to predict and prevent its toxicity**.** The objective of this study was to develop a simple and reliable liquid chromatographic method for quantitative analysis of MTX in rat serum samples with high linearity, and use of this method in pharmacokinetic studies of MTX**.** An Isocratic reversed-phase HPLC method with ultraviolet detection at 313 nm has been developed for analysis of MTX in serum samples. The mobile phase composition was Phosphate Tris Buffer: Methanol: Acetonitrile (82:11:7). P-aminoacetophenone (PAAP) and Trichloroacetic acid (TCA) were used as internal standard and de-proteinizing agent, respectively. The calibration curve for MTX in serum rat samples was linear over the concentration range of 0.25 to 5 μg mL^{-1} with a correlation coefficient of 0.9997. Intra-day and inter-day precision were 2.23-6.40% and 1.20-11.94, respectively. The accuracy, calculated to reflect the difference between nominal and observed concentrations, was in the range 91.1–109.4%, which is acceptable. The recovery of MTX in this calibrated analytical method was between 76 and 96%. The calibrated assay was successfully applied for pharmacokinetic study of MTX in rat after intraperitoneally administration of drug. The results indicated that LC calibrated method could be used for quantification and pharmacokinetic studies of MTX in rat serum samples.

Keywords: High-Performance Liquid Chromatography; Methotrexate; Pharmacokinetic Study; Serum Samples.

Introduction

Methotrexate (MTX) is an immunosuppressive medication that belongs to a family of medicines known as antimetabolites. Since the 1950's, MTX has been used for the treatment of certain cancers such as acute lymphoblastic leukemia, osteosarcoma, head and neck tumors and breast cancer (1, 2, 3).

The multidrug resistance protein 2 (MRP2), participates in active transport of compounds in the liver, small intestine and kidney. Studies demonstrated that, MRP2 has an important role in MTX efflux at cellular folate homeostasis (4). On the other hand, MTX treatment down-regulates expression levels of MRP2 (5). Thus, the quantification of MTX in serum samples and its kinetic study would be valuable to predict and prevent the probable toxicity of drug (6-8).

Numerous methods for MTX analysis have been reported in the literature. These include high-performance liquid chromatography (HPLC) analysis with ultraviolet detection and fluorescence detection, radioimmunoassay, dihidrofolate reductase inhibition assay, enzyme-multiplied immunoassay, fluorescence polarization immunoassay, enzymeimmunoassay and capillary zone electrophoresis with laser –induced fluorescence detection. Among the above mentioned approaches, HPLC is one of the greatest sensitive and selective analytical methods (9-11).

The objective of this study was to develop a simple and reliable liquid chromatographic method for quantitative analysis of MTX in rat serum samples with high linearity, and use of this method in pharmacokinetic studies of MTX. The method was validated by determination of the concentration of the drug in male rat serum samples.

Materials and Methods

MTX (> 98% pure, HPLC) and P-aminoacetophenone (PAAP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA); TRIS (tris-hydroxymethyl–aminomethane) and Sodium dihydrogen phosphate dihydrate (extra pure) were obtained from Merck (Darmstadt, F.R. Germany); Acetonitrile (CAN) and Methanol were of HPLC grade and purchased from Duksan (Ansan, South Korea).

Preparation of stock and standard solutions

Stock solutions of MTX (5 mg mL^{-1}) and PAAP (5 mg mL^{-1}) were prepared in methanol. Standard solutions (0.25, 0.5, 1, 2.5 and 5 mg mL^{-1} MTX containing 5 mg mL^{-1} PAAP) were prepared from stock solutions by dilution with mobile phase. The value of $\frac{AUC_{(MTX)}}{AUC_{(PAAP)}}$ was plotted over the MTX concentration range as standard curve.

Calibration

Standard solutions of MTX (2.5–50 μg mL^{-1}) and PAAP (50 μg mL^{-1}) in water, used for spiking calibration samples, were also prepared from stock solution. For calibration, 20μL of MTX (2.5–50 μg mL^{-1}) and 20 μL of PAAP (50 μg mL^{-1}) as internal standard were added to 160 μL serum samples to obtain calibration solutions (0.25 – 5 μg mL^{-1} MTX containing 5 μg mL^{-1} PAAP). Trichloroacetic acid (40 μL , 2 M in ethanol) was added to precipitate

proteins. Samples were then centrifuged (10000 g) and supernatant was stored at -20 °C with dark cover until analysis. Quantification of the MTX was performed by plotting nominal MTX concentrations against the corresponding $\frac{AUC_{(MTX)}}{AUC_{(PAAP)}}$ values for spiked samples.

Animals and treatment

Sprague-Dawley rats (180-250 g) were obtained from Razi Institute (Mashhad, Iran) and housed in groups of five under standard laboratory conditions of constant temperature (21±2 °C) and a 12/12 h light/dark cycle for at least 10 days prior to testing. Commercial food pellets and tap water was freely available. Animals were transferred to the testing laboratory at least one day before the start of the experiments. The use of the animals was carried out in accordance with the regulations of the Mashhad University of Medical Sciences (MUMS) Ethics Committee.

Serum Sample Preparations

Twenty microliters of PAAP was added to 180 µL serum as internal standard. Trichloroacetic acid (40 µL, 2 M in ethanol) was also added to precipitate proteins. After vortex for 2 minutes and then centrifugation at 10000 g for 5 minutes, 20 µL aliquots of the supernatant was directly injected for HPLC analysis.

Precision and accuracy

Assay precision was determined by spiking samples with known concentration of MTX. Drug concentrations were determined in batches of four on the same day and on different days. Intra and inter day assay precision was determined by calculating relative standard deviation (RSD %) of the obtained data(Table 1 and 2).

Relative accuracy was determined using the following equation:

$$\text{Accuracy (\%)} = \frac{\text{Observed concentration}}{\text{Nominal concentration}} \times 100$$

Chromatographic conditions

High-performance liquid chromatography of MTX was performed with a Young Lin (Anyang, South Korea) Acme 9000 system, consisting of an SP930D solvent delivery module, an SDV50A Solvent Mixing Vacuum Degasser, a CTS30 Column Oven, a UV730 Dual Wavelength UV/VIS detector and an ODSA C18 (4.6 × 250 mm, 5µm) column. Data were analysed by use of Autochro-3000 software supplied by Young Lin (Anyang, South Korea). The injection volume was 20 µL, the flow rate was 1 mL min^{-1}, and the column temperature was fixed at 30 °C. The UV detector was set to 313 nm. An isocratic method was used for chromatographic analysis of MTX. The composition of the mobile phase was: ACN 7%, methanol 11% and phosphate-TRIS buffer (0.1 M dihydrogen phosphate and 0.01 M TRIS; pH 5.7) 82%.

Application of Analytical Method for serum Samples

MTX (10 mg/kg) was injected, intraperitoneally (i.p), into the male rats. The rats were then anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (6 mg/kg) (i.p). Polyethylene (PE-50, Clay Adams) cannulas tipped with 2.5 cm of silastic tubing (Dow Corning, Midland, USA) were inserted into the left jugular vein and exteriorized by subcutaneous tunneling in the interscalpular area. At various time points following drug administration (1, 2, 3 and 4 h), 500 µL blood samples were taken into heparinized syringes from the cannulated vein (12). The same volume of strile saline 0.9% was injected through cannula. During the surgery, the body temperature was monitored and maintained at 37.5 ±

0.5°C by means of a heating lamp. Samples were centrifuged at 6000 rpm immediately and serum was collected and kept in -20ºC until analysis. Handling and experimental procedures on all animals were in accordance with the Mashhad University of Medical Sciences Ethics Committee Acts.

Data Analysis

The peak serum concentration (C_{max}) and the time to peak concentration (T_{max}) were obtained from visual observation. The pharmacokinetic calculations were based on standard methods. The areas under the serum concentration-time curves were calculated using conventional method (Table 3).

Results

Studies revealed that the quantification of MTX in serum samples and its kinetic study would be valuable to predict and prevent the probable toxicity of drug (6-8). Therefore, we developed an HPLC method for quantification of MTX in rat serum samples and its application in pharmacokinetic studies. Multiple blood sampling is sometimes necessary for pharmacokinetic studies in small animals. We calibrated the method for sample volume as low as 180 µL which could be obtained from 400 to 500 µL blood sample. In spite of many HPLC methods (11, 13, 14, 15), the dryness and re-dissolvation step (which decrease the precision and accuracy of the method) was not necessary in this work. The acid-deproteinized sample was directly injected for HPLC analysis. As described in other studies, sample-freezing and light protecting was necessary to increase the stability of MTX and PAAP for HPLC analysis (15). Under these conditions used for the chromatography, the retention times of MTX and the PAAP were around 10 and 19 minutes, respectively. Figure 1 shows the chromatograms of rat blank serum (a) and a calibration sample spiked with 5 µg mL^{-1} MTX (b). The chromatographic condition employed in this work was quite specific for MTX and PAAP. Under these conditions no interfering peaks were observed in the chromatogram.

Method validation

Linearity and specificity

A calibration curve for standard solutions ($y = 0.0868x + 0.0109$, $R^2 = 0.9927$) was plotted over the range 0.25 to 5 µg mL^{-1}. A calibration curve for assay of MTX in Rat Serum samples ($y = 0.0685x - 0.0073$, $R^2 = 0.999$) was plotted over the same range. This range of concentrations was within the acceptable limit for analysis of real serum samples. Linearity was tested using serum samples spiked with standard solutions containing known concentrations of the drug. The correlation coefficient obtained for the calibration curve demonstrated the linearity of the results in this analysis. The limit of detection (LOD), defined as the concentration that produces a signal-to-noise ratio of 3, was 8 ng mL^{-1} and the limit of quantification (LOQ), defined as the concentration that produces a signal to noise ratio of 20, was 54 ng mL^{-1} .Recovery of MTX in this calibrated analytical method was between 76 and 96%. To verify the specificity of the method, blank serum samples were analyzed to evaluate the absence of endogenous compounds at the retention time of MTX. The results indicated that no interference was observed at retention time of MTX.

Precision and Accuracy

Table 1 shows the precision and accuracy of the assay for serum samples. Intra-day and inter-day precision were determined as the relative standard deviation (RSD %) of the results obtained. Intra-day precision for MTX concentrations of 0.25 to 5 μg mL^{-1} ranged from 2.23 to 6.40%, and inter-day precision for these concentrations was between 1.20 and 11.94. In other studies, intra-day and inter-day precision ranged between 0.75 and 21.87% (18). Accuracy, calculated to reflect the difference between nominal and observed concentrations, was in the range 91.1–109.4%, which is acceptable.

Stability of the Analyte

MTX was unstable at room temperature and in daylight. Thus, solutions were prepared freshly every day, protected from light by wrapping in foil and stored at -20 ^{o}C until analysis. These conditions were necessary to minimize the degradation of the analyte and repeatability of the HPLC method.

Ruggedness and Robustness of the Method

The repeatability of data obtained by analysis of the same sample under a variety conditions such as instruments or experimenters, indicated that the method was rugged.

To study the robustness of the method, deliberate slight variations were made in method conditions, for example changes of flow rate, MTX content of the mobile phase, and column temperature. These slight variations had no significant effect on the area and shape of the peak. The results indicated that the resolution of the peak is more sensitive to changes in column temperature than to changes in the other conditions.

Application of the Analytical Method to Serum Samples

The optimized and calibrated method was used for analysis of MTX in serum samples from male wistar rats. After intraperitoneally administration of MTX to the rats the concentrations of the drug in serum samples were monitored by HPLC for 4 hours. Twenty three serum samples were tested in this study. Pharmacokinetic parameters e.g. C_{max}, T_{max} and AUC are presented at Table 2.

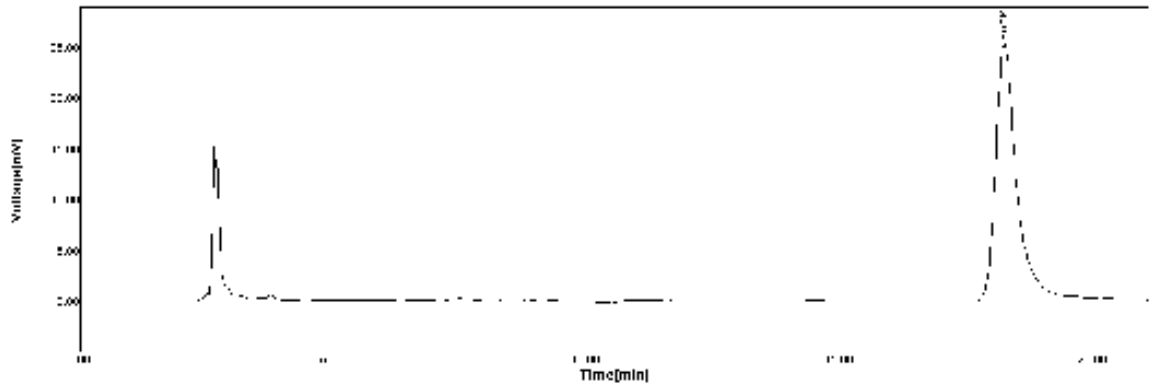
Discussion

In this work we developed an HPLC method for quantification and pharmacokinetic study of MTX in rat serum samples. The intra-day and inter-day precision, accuracy and recovery of the analytical method were calculated. The results presented in this report indicate that the method is reproducible. Finally, the calibrated assay was successfully applied for pharmacokinetic study of MTX in rat after intraperitoneally administration of drug. The results indicated that LC calibrated method could be used for quantification and pharmacokinetic studies of MTX in rat serum samples.

Acknowledgements

The autors gratefully acknowledge the Iranian Science Foundation for financial support.

a



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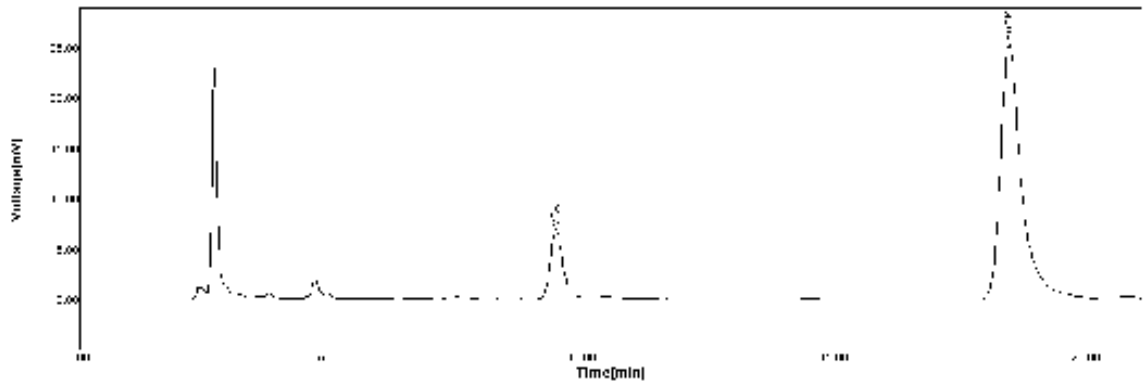

Fig. 1: Chromatograms obtained from a blank serum sample (a) and from a MTX Spiked Serum Sample (b)**,** Chromtographic condition: mobile phase, ACN 7%, methanol 11% and phosphate-TRIS buffer (0.1 M dihydrogen phosphate and 0.01 M TRIS; pH 5.7) 82%; Flow rate, 1 mL min^{-1}; Column, ODSA C18 (4.6 × 250 mm, 5µm); λ_{max}, 313nm; column temperature, 30 °C

Table 1: Inter-day precision and accuracy of determination of MTX in rat serum samples

Nominal concentration ($\mu g\ mL^{-1}$)	Observed mean concentration ($\mu g\ mL^{-1}$)	SD	RSD (%)	Accuracy (%)
0.25	0.013	0.001	11.643	103.100
0.5	0.025	0.001	6.866	91.103
1	0.055	0.003	5.221	97.420
2.5	0.132	0.001	1.255	102.421
5	0.271	0.007	2.696	104.685

Table 2: Intra-day precision and accuracy of determination of MTX in rat serum samples

Nominal concentration ($\mu g\ mL^{-1}$)	Observed mean concentration ($\mu g\ mL^{-1}$)	SD	RSD (%)	Accuracy (%)
0.25	0.013	0.000	6.264	108.007
0.5	0.026	0.001	4.392	102.613
1	0.058	0.003	6.402	109.434
2.5	0.126	0.004	3.428	94.042
5	0.260	0.005	2.239	96.238

Table 3: Pharmacokinetic parameters of MTX in rat given i.p. MTX (10 mg kg^{-1})

Parameters	Methotrexate (n = 4)
AUC (mg mL^{-1}/min)	3.976
C_{max} (mg mL^{-1})	1.5
T_{max} (min)	60

AUC = Area Under concentration-time Curve; Cmax: Maximum Concentration of MTX; Tmax: Time of maximum concentration; n: number of samples

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