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Short communication

Method for the determination of blood methotrexate by high performance liquid chromatography with online post-column electrochemical oxidation and fluorescence detection

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Abstract

Methotrexate (MTX) has been widely used at low dose for the treatment of different diseases including rheumatoid arthritis. MTX might be present in plasma in free form, and in blood cells in methotrexate polyglutamate (MTXPG). A rapid and sensitive HPLC method was developed for the determination of plasma MTX level, whole-blood MTX level, and whole-blood total MTX (MTX + MTXPG) level. To determine plasma MTX level or whole-blood MTX level, a 0.2-ml aliquot of plasma or whole blood (after a freeze–thaw cycle to break blood cells) was well mixed with 0.8 ml methanol and centrifuged. To determine whole-blood total MTX level, a 0.1-ml aliquot of whole blood (after a freeze–thaw cycle) was mixed with 80 μl ascorbic acid (114 mM) and incubated at 37 °C for 2 h to enzymatically convert the MTXPG to MTX. Then 20 μl NaOH solution (0.5 M) and 0.8 ml methanol were added and mixed well. After centrifugation, a 0.5-ml aliquot of the supernatant was evaporated to dryness and re-dissolved in 0.2 ml hydrochloric acid (10 mM). Methylene chloride (0.2 ml) was added and mixed well. After centrifugation, the top aqueous layer was injected to HPLC for analysis. After the MTX was eluted from the HPLC column, it was electrochemically oxidized and detected by a fluorescence detector. Recoveries of spiked MTX at ppb (ng/ml) level were between 87.9 and 118% with within-day relative standard deviation less than 5.2% and day-to-day relative standard deviation less than 9.8%. The limit of detection (LOD) and limit of quantitation (LOQ) of the described method were 1.2 and 2.6 ng/ml, respectively.

Keywords: Methotrexate; HPLC; Online electrochemical oxidation

1. Introduction

Methotrexate (MTX) is an analog of folic acid. It has been widely used at large dose for the treatment of acute lymphoblastic leukemia [1], and as an effective anti-inflammatory and immuno-suppressive drug, at low dose for the treatment of rheumatoid arthritis, juvenile idiopathic arthritis and other diseases [2–4]. MTX might be present in plasma in free form, and in blood cells in methotrexate polyglutamate (MTXPG). Monitoring blood MTX levels for the studies of the efficacy, dosing schedule, different individual responses related to genetic polymorphism, and adverse drug reactions of MTX requires rapid and sensitive analytical methods [5,6]. Different analytical methods have been reported for the determination of MTX and MTXPG in blood plasma and blood cells. Capillary electrophoresis (CE) analysis for MTXPG was used for the in vitro assay of the enzyme gamma-glutamyl hydrolase (EC 3.4.22.12) [7]. Capillary zone electrophoresis method was also reported for analyses of MTX and its metabolites in whole blood [8]. A more recent report described a sensitive CE method using immunoassay-laser induced fluorescence detection for the determination of MTX [9]. HPLC methods using electrochemical detection, or pre-column chemical oxidation with fluorescence detection for the determination of plasma MTX, or post-column photo-oxidation with fluorescence detection for the analyses of MTX and MTXPGs have been reported [10–13]. HPLC method with electrochemical detection was not sensitive enough for blood samples containing MTX at low ppb levels [10]. An HPLC method with pre-column chemical oxidation and online enrichment needed a special packed Cerium (IV) trihydroxy-

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hydroperoxide oxidation column and a special protein-coated pre-column for online sample enrichment [11], but this method was still not sensitive enough for blood samples containing MTX at low ppb levels.

In this article, a rapid and sensitive HPLC method is described for the quantitative determination of plasma MTX, whole-blood MTX and whole-blood total MTX (MTX + MTXPG). In the method, after MTX was eluted from the HPLC column, it was oxidized with an online coulometric electrochemical cell, and detected with a fluorescence detector. MTXPGs released from blood cells were enzymatically converted into MTX before HPLC analysis.

2. Experimental

2.1. Materials

N-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino] benzoyl]-l-glutamic acid (methotrexate, MTX), USP grade, was purchased from Sigma (St. Louis, MO, USA). 4-Amino-10-methylpteroylpenta-L-glutamic acid, ammonium salt (APA-Glu_5, or MTX-Glu_4), was purchased from Schircks Laboratories (Switzerland). Acetonitrile and methanol were HPLC grade and purchased from Merck KGaA (Darmstadt, Germany), and water was Milli-Q deionized water. HPLC grade ammonium acetate was purchased from TEDIA Company, Inc. (Fairfield, OH, USA). Ascorbic acid, methylene chloride and other reagents were analytical grade. Blood samples of rheumatoid arthritis patients who received MTX (10 mg) for therapy for the first time were obtained from the local hospital with permission and informed consent, and stored at −60 °C.

2.2. Standard solution and calibration standard curve

(a) MTX stock standard solution of 0.1 mg/ml was prepared by dissolving accurately weighed MTX in a volumetric flask with 0.01 M hydrochloric acid and stored at 4 °C. Stock standard solution was prepared monthly. (b) A series calibration working standards of MTX, 2, 5, 10, 25, 50, 100, and 200 ng/ml were prepared by diluting the MTX stock standard (0.1 mg/ml) with the HPLC mobile phase. The working standards were analyzed with HPLC and a linear regression calibration equation was generated. (c) For daily calibration, a three or four-point calibration curve including, e.g., 2, 5, 10, 25, and 100 ng/ml MTX working standards was prepared, depending on the sample MTX levels, to cover the MTX concentration range of the samples. (d) Matrix-matched calibration curves were prepared by spiking MTX standards at different levels to blank blood plasma or whole blood. A series of plasma or whole blood samples containing 0, 2.5, 5, 12.5, 25, 50, and 100 ng/ml were therefore prepared and analyzed by the described procedure.

2.3. Sample preparation procedure for plasma MTX or whole-blood MTX

An aliquot of 0.2 ml human blood plasma or whole blood (after a freeze–thaw cycle) was pipetted into a 1.5-ml eppendorf tube, to which 0.8 ml methanol was added while vortexing. The tube was vortexed for 1 min and centrifuged at 10,000 × *g* for 12 min. An aliquot of 0.5 ml of the supernatant was pipetted to a 5-ml glass test tube and evaporated in a dry heater at 45 °C to dryness with a stream of nitrogen. The residue was dissolved in 0.2 ml hydrochloric acid (10 mM). Methylene chloride (0.2 ml) was added and mixed well. After centrifugation, the top aqueous layer was injected to HPLC for analysis.

2.4. Sample preparation procedure for whole-blood total MTX (MTX + MTXPG)

An aliquot of 0.1 ml whole blood (after a freeze–thaw cycle) was mixed with 80 μl ascorbic acid (114 mM) and incubated at 37 °C for 2 h to enzymatically convert the MTXPG to MTX. After the incubation, 20 μl sodium hydroxide (NaOH) solution (0.5 M) and 0.8 ml methanol were added to the sample. After vortex and centrifugation, a 0.5-ml aliquot of the supernatant was evaporated to dryness with a stream of nitrogen and the residue was dissolved in 0.2 ml hydrochloric acid (10 mM). Methylene chloride (0.2 ml) was added and mixed well. After centrifugation, the top aqueous layer was injected to HPLC for analysis.

2.5. Liquid chromatographic analysis for MTX

The high performance liquid chromatographic (HPLC) system was an HP 1100 HPLC (Hewlett Packard, USA), which consisted of a pump, an auto-sampler, a column chamber, a fluorescence detector, and an HP ChemStation for LC system. The column chamber temperature was set at 40 °C and the sample injection volume was set at 20 μl. The excitation wavelength and emission wavelength of the fluorescence detector were set at 367 and 463 nm, respectively. A coulometric electrochemical cell (Model 5010, ESA, Inc., Bedford, MA, USA) was connected online between the HPLC column and the fluorescence detector. The electrochemical cell was controlled by the Coulochem II Multi-Electrode Detector (ESA, Inc.). The voltage of the electrochemical cell was set at E1 = 0 mV, E2 = 1000 mV. The mobile phase was acetonitrile–50 mM ammonium acetate in water (7:93, v/v) with a flow rate of 1 ml/min. The HPLC column was an HP Zorbax StableBondSB-C18, 5 μm, 150 mm × 4.6 mm column (Germany). The peak area was used for quantitative calculation.

2.6. Validation of the procedure

Control blood plasma and whole blood were spiked with MTX to prepare blood samples containing different levels of MTX. The spiked samples were analyzed with the described procedure within-day and in different days. Recoveries and relative standard deviations were calculated. Blood samples collected from dosed patients at different time points were also analyzed to evaluate the procedure in a real situation.

2.7. Confirmation of the identity of MTX produced by enzymatic conversion from MTXPG

Blood samples spiked with MTXPG were prepared with the described sample preparation procedure for whole-blood total MTX. When the electrochemical cell was turned off, fraction of chromatographic peak of the MTX (at the pre-determined retention time) was collected from several injections of human blood samples spiked with MTXPG. Solvent was evaporated under vacuum and residue was dissolved in a small volume of the HPLC mobile phase, ready for LC/ESI/MS/MS analysis. A Q Trap MS/MS Spectrometer (Applied Biosystem, Canada) equipped with an ESI ion source with negative ion mode was used. A Q1 scan (single MS) was done to identified the molecular ion (−453.2), and after fragmentation an MS spectrum of the molecular ion was obtained by a product ion scan. An MTX standard solution was used for comparison.

3. Results and discussion

3.1. Sample preparation procedure for plasma MTX and whole-blood MTX

To determine MTX in blood plasma or whole blood, samples were simply mixed with methanol and centrifuged to extract the MTX and to remove proteins. Different pH values of the extraction system were tried and neutral pH was found to give satisfactory recoveries. In the final step, methylene chloride was used to remove the lipids.

3.2. Sample preparation procedure for whole-blood total MTX (MTX + MTXPG)

In the blood, a small portion of the MTX enters the blood cells and is present in the blood cells as MTXPGs. Therefore, to determine whole-blood total MTX (MTX + MTXPG), blood cells were broken by a freeze–thaw cycle, and MTXPGs were released and mixed with the blood plasma. The endogenous polyglutamates hydrolase in the plasma catalyzed the conversion of the MTXPGs to MTX. The acidity created by the added ascorbic acid accelerated this conversion reaction. The similar procedure had been used to convert 5-methyltetrahydrofolate (5-MTHF) polyglutamates to 5-MTHF for the determination of whole-blood total 5-MTHF [14,15]. In order to decide a proper length of incubation time for the conversion reaction, blood samples collected from dosed patients at time point of 48 h were used and different incubation time at 37 °C was tested. As a compromise between the completeness of the conversion from MTXPGs to MTX and time efficiency, the incubation time of 2 h was selected and gave satisfactory results (Fig. 1). To obtain a satisfactory recovery of MTX, the sample needs to be neutralized with NaOH before extraction and protein precipitation with methanol. In the final step of the sample preparation procedure, a small volume of methylene chloride was used to remove the extra lipids.

In order to further investigate the efficiency of the enzymatic conversion of MTXPG to MTX, blank whole-blood samples were spiked with MTXPG (MTX-Glu_4) at two levels, i.e., 23.6 and 47.2 ng/ml, which are equivalent to MTX at 10 and 20 ng/ml. Blood samples spiked with MTXPG or MTX were analyzed following the above described procedure. The results indicated the enzymatic conversion rates were 89.3% (S.D. = 4.5, $n = 3$) and 94.3% (S.D. = 5.7, $n = 3$), respectively.

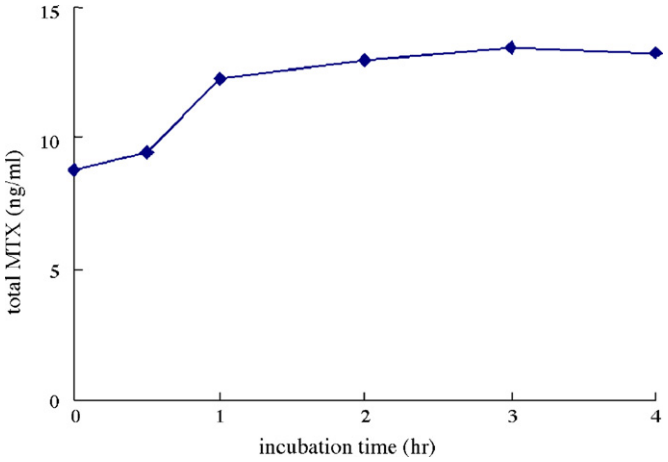

Fig. 1. The effect of incubation time on the whole-blood total MTX (MTX + MTXPG) content determined by the HPLC method described.

3.3. The HPLC analysis for MTX

After elution from the HPLC column, MTX passed through the coulometric electrochemical cell (set at +1000 mV) and was oxidized to form fluorescent products, which were detected by the fluorescence detector. The fluorescence signal from MTX oxidized products was stable, very reproducible and linear proportional to the quantity of MTX injected into the HPLC. Other oxidation methods by using chemical oxidant or photo-oxidation device had been reported [11–13]. The electrochemical detector including the electrochemical cell used in this study is commercially available and a widely used HPLC detector, and is very easy to set up. The signal from the electrochemical detector alone was not sensitive enough to detect low level of MTX in blood samples. This combination of an electrochemical cell as an online post-column oxidation device and a fluorescence detector gave satisfactory results in terms of reliability, sensitivity, reproducibility, linearity, easy to set up and easy to use. The addition of the online electrochemical cell between the HPLC column and the fluorescence detector did not deteriorate the quality of the chromatograms (Fig. 2).

3.4. Calibration and validation of the procedure

A seven-point calibration curve was prepared as described in Section 2.2 and its linearity was tested with a statistical software. The regression equation of the calibration curve is $y = a_1x + a_0$ with a linear regression coefficient (γ) of 0.9999, where x is the peak area count of the fluorescence signal from the MTX electrochemical-oxidized products, and y is the concentration (ng/ml) of the MTX standard working solutions used for preparing the calibration curve. The coefficients a_1 and a_0

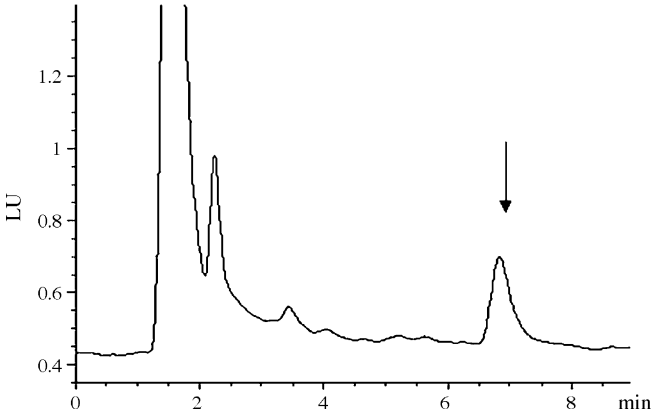

Fig. 2. The chromatogram of a whole-blood sample containing total MTX (MTX + MTXPG) of 86.2 ng/ml. MTX was eluted at 6.8 min.

were 3.956 and 0.180, respectively. For daily calibration, a three or four-point calibration curve was prepared to cover the MTX concentration range of the samples. To calculate MTX concentrations of the blood samples using the calibration curve, the dilution factor was 2 for plasma MTX or whole-blood MTX, and 4 for whole-blood total MTX according to the sample preparation procedures.

Control blood plasma and whole blood were spiked with MTX at different levels and analyzed with the described procedures. Results of the spiked recoveries and variations were summarized in Table 1. The data indicate that the analytical method for the determination of plasma MTX level, whole-blood MTX level, and whole-blood total MTX level (MTX + MTXPG) is accurate with good precision and reproducibility.

The matrix-matched calibration curves showed good linearity with a linear regression coefficient (γ) of 0.9997 for plasma and 0.9999 for whole blood. Using matrix-matched calibration curves to determine the spiked recoveries of samples showed satisfactory results. Plasma samples spiked with MTX at 5, 25, and 100 ng/ml, had recoveries of 104% (S.D. = 0.57, $n = 3$), 106% (S.D. = 3.1, $n = 3$), and 99.1% (S.D. = 0.89, $n = 3$), respectively. Whole blood samples spiked with MTX at 5, 25, and 100 ng/ml, had recoveries of 108% (S.D. = 2.5, $n = 3$), 101% (S.D. = 2.8, $n = 3$), and 99.0% (S.D. = 0.66, $n = 3$), respectively.

To estimate the limit of detection (LOD) and limit of quantitation (LOQ), replicates of control blood samples (blanks) were analyzed by the described procedure. LOD was calculated as mean blank response plus three times the standard deviation, and LOQ was calculated as mean blank response plus 10 times the standard deviation, according to the guidelines of the American Chemical Society [16]. The LOD and LOQ of the described method for MTX in blood samples were 1.2 and 2.6 ng/ml, respectively.

3.5. Confirmation of the identity of MTX produced by enzymatic conversion from MTXPG

MTXPG in the blood samples was enzymatically converted into free MTX and determined with the described HPLC method. In this research a tandem mass spectrometry method was used to confirm the identity of the MTX produced by enzymatic conversion from MTXPG in the blood samples. The negative charged molecular ion of MTX (−453.2) was identified by the Q1 mass analyzer, and after fragmentation in the Q2 collision cell, its fragments pattern was scanned by the Q3 mass analyzer. MTX produced by enzymatic conversion from MTXPG in the blood samples showed the same characteristic mass spectrum with the

Table 1
Spiked recoveries of the procedure

Spike level (ng/ml)	Recovery (%)	R.S.D. ($n = 5$) (%)
Blood plasma MTX		
Within-day		
5	110	5.2
25	107	1.6
100	109	1.3
Day-to-day		
5	115	3.4
25	108	3.2
100	106	3.2
Whole-blood MTX		
Within-day		
5	89.2	5.0
25	88.9	3.6
100	90.0	0.5
Day-to-day		
5	99.8	9.8
25	91.1	4.2
100	91.0	4.2
Whole-blood total MTX		
Within-day		
5	118	2.5
25	89.8	3.1
100	87.9	1.8
Day-to-day		
5	105	7.8
25	91.2	3.4
100	90.0	2.8

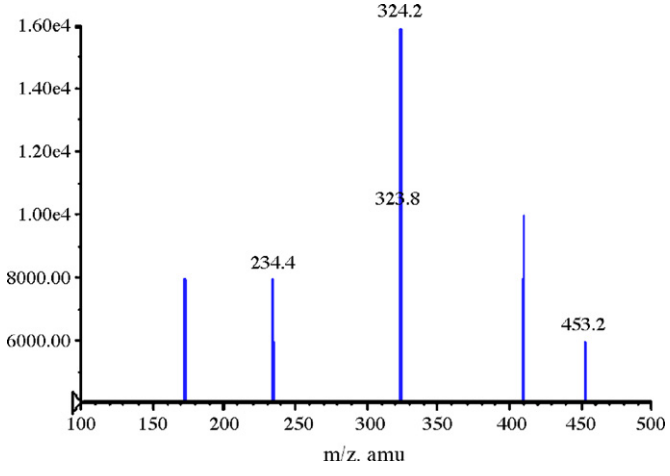

Fig. 3. The mass spectrum of the molecular ion (−453.2) of the MTX produced by enzymatic conversion from MTXPG in the blood samples showed the same characteristic mass spectrum with the MTX standard solution used for comparison.

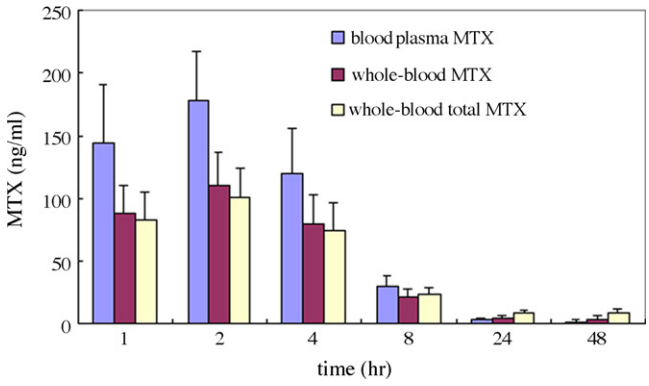

Fig. 4. Patients ($n = 6$) were dosed with MTX and blood samples were collected at different time points, and plasma MTX, whole-blood MTX, and whole-blood total MTX (MTX + MTXPG) of blood samples were determined by the HPLC method described.

MTX standard solution used for comparison. The characteristic mass spectrum of the fragmented molecular ion (−453.2) is depicted in Fig. 3.

3.6. Application of the MTX analytical method to rheumatoid arthritis patients' blood samples

The developed methodology was used to analyze plasma MTX, whole-blood MTX, and whole-blood total MTX (MTX + MTXPG) of blood samples collected from rheumatoid arthritis patients dosed with low dose (10 mg) MTX. Blood samples were collected from six patients at different time points as 1, 2, 4, 8, 24, and 48 h after orally taking the drug. The results were summarized in Fig. 4. At the time points of 1, 2, and 4 h after administration of MTX, concentrations of free MTX in plasma were relatively high, and the levels of MTXPG were relative low. The differences between the values of whole-blood total MTX (MTX + MTXPG) and values of whole-blood MTX at the points of 1, 2, and 4 h (Fig. 4) were mainly due to variations of determination. Concentration of MTX in plasma dropped rapidly and MTXPG was formed after MTX entered into blood cells. At the point of 48 h, the plasma MTX level was very low, the difference between whole-blood total MTX (MTX + MTXPG) and whole-blood MTX became significant. Considering MTX is used weekly for rheumatoid arthritis patients, it is therefore possible to indirectly determine the MTXPG by using this method. We were able to calculate the whole-blood MTXPG (48 h after dosing) by subtracting the value of whole-blood total MTX (MTX + MTXPG) by the value of whole-blood MTX of a blood sample. Among the six patients who received MTX 10 mg for the first time, 48 h later the whole-blood MTXPG levels were determined and calculated to be between 2.53 and 19.2 nmol/l. In a report by Dervieux et al. [13], they found patients receiving weekly low-dose MTX (10–25 mg weekly for 6–240 months) had total MTXPG concentration in erythrocyte 117 nmol/l (S.D. = 56). Their results were based on the volume of erythrocyte, and blood samples were from patients receiving MTX weekly for a relatively long period of time. Blood samples in our experiment were from the patients receiving MTX the first time for treatment, and the results were based on the total volume of whole blood rather than erythrocyte.

4. Conclusion

A rapid and straightforward HPLC method using online coulometric electrochemical oxidation and fluorescence detection was developed for the quantitative determination of plasma MTX level, whole-blood MTX level, and whole-blood total MTX (MTX + MTXPG) level. This method might be used to estimate the whole-blood MTXPG by subtracting the value of whole-blood total MTX (MTX + MTXPG) by the value of whole-blood MTX. The methodology had been tried with success on a preliminary clinical pharmaceutical study with rheumatoid arthritis patients.

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