Simple and Reliable HPLC Method for the Monitoring of Methotrexate in Osteosarcoma Patients

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Methotrexate (MTX) is a dihydrofolate reductase inhibitor that is used for the treatment of tumors and autoimmune diseases. Several automated binding assays are used in clinical practice and numerous chromatographic methods have been developed toward higher specificity and sensitivity. In the present study, phenyl cartridges were used for the solid-phase extraction (SPE) of MTX from human serum samples; subsequently, extracts were analyzed by reversed-phase high-performance liquid chromatography. Isocratic separation was implemented on a Kromasil-C18 column with a mobile phase consisting of 50 mM sodium acetate buffer (pH 3.6)–acetonitrile (89:11, v/v) and ultraviolet detection at 307 nm. MTX eluted in less than 12 min with no interference from impurities or 24 examined drugs. Detector response was linear in the range of 0.025–5.00 μM (coefficient of correlation > 0.99). Recovery from the serum was 93.1–98.2% and bias was < 8.3%. Intra-day and inter-day precision were < 7.8 and 12.6%, respectively (n = 6). The limit of quantitation was 0.01 μM and the limit of detection was 0.003 μM. The method was validated by using serum samples from osteosarcoma patients treated with high-dose MTX (8–12 g/m²). In conclusion, the combined use of a phenyl-functionalized sorbent for SPE and a Kromasil-C18 column, and specific detection at 307 nm, assured a selective, fast, robust and cost-effective method for the monitoring of MTX in osteosarcoma patients under high-dose MTX treatment, thus contributing to more efficient treatment.

Introduction

Methotrexate (MTX, 2,4-diamino-N10-methyl-folic acid) is an antimetabolite and an antifolate drug acting as a dihydrofolate reductase (DHFR) inhibitor. High-dose MTX (HDMTX) is well established for the treatment of solid tumors and leukemias (1, 2), whereas low-dose regimens are widely used in the treatment of autoimmune diseases (3, 4), and recently, as immunosuppressive agents in organ transplantation (5). MTX was introduced to clinics over six decades ago and is one of the most widely used and studied anticancer agents. Its administration, however, has the potential of severe side effects, including neurologic toxicity, renal failure due to tubular obstruction by crystal deposits of MTX and its primary metabolite, 7-hydroxy-methotrexate (7-OH-MTX), myelosuppression, and mucositis. The effectiveness of HDMTX therapy has been greatly enhanced by the observation that patients at high risk of serious toxicity may be detected by monitoring serum MTX concentrations. Therefore, the routine monitoring of drug serum concentrations is important in guiding leucovorin rescue and is considered to be imperative for both patient safety and evaluation of therapeutic concentrations of MTX.

Numerous analytical approaches, primarily based on different binding assays, have been employed for MTX monitoring. Early analytical methods, although highly sensitive, were labor-intensive, requiring the use of radioisotopes (6, 7), whereas others proved to be unreliable due to potential antibody cross-reactivity between MTX and its metabolites, thus raising doubt about their accuracy and specificity (8). Consequently, several chromatographic methods have been developed using different conditions for sample preparation, analyte extraction, separation and detection of MTX in biological samples. Among these methods, those with higher sensitivity have detected MTX by employing fluorescence and tandem mass spectrometry (MS-MS) (9–12). Because of continuing MTX use and because MS facilities are not always available as standard equipment in hospital laboratories, there is still a need for a sensitive, fast and inexpensive method broadly applicable to clinical routines for therapeutic drug monitoring (TDM) of MTX.

Therefore, the purpose of the present study was to develop a simple, rapid and highly specific and sensitive high-performance liquid chromatography (HPLC) method for the quantitation of MTX in serum samples; the method was validated in a pilot study of routine drug monitoring in cancer patients under HDMTX treatment. This method involved the isolation of MTX from a sample of less than 1 ml by solid-phase extraction (SPE) and subsequent HPLC–ultraviolet (UV) quantification of MTX. This study included the evaluation of several frequently co-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.

Experimental

Instrumentation and reagents

Chromatographic analysis of MTX and the internal standard (IS), 1,3,7-trimethyluric acid (137U) was performed by using a Marathon III pump (Rigas Labs, Greece) equipped with a FASMA model 500 UV-Vis detector (at 307 nm; Rigas Labs) and a Rheodyne 7125 injection valve (Rheodyne, Oak Harbor, WA) with a 100 μL loop. Separation was achieved by using a Kromasil 100 C18 column (5 μm particle size, 250 × 4.6 mm i.d.; MZ-Analysentechnik, Mainz, Germany) operated at 30 °C. The mobile phase, consisting of 50 mM sodium acetate buffer (pH 3.6)–acetonitrile, 89/11 (v/v), was filtered (0.2 μm pore size filter, Alltech, Deerfield, IL), degassed by an online degassing system (ERC, Inc., Japan) and delivered at a flow rate of 1 ml/min. Chromandspec software (Ampersand, Russia) was used for data processing.
acquisition and analysis of chromatograms. Representative chromatograms obtained from the analysis of blank serum, standard solution of MTX (2.00 mM) and the IS, blank serum spiked with MTX (0.50 μM) and the serum sample of a patient 24 h after MTX dosing (measured MTX concentration of 4.75 μM) are shown in Figure 1.

Methotrexate, [(+)-amethopterin], 137U and paracetamol (4-acetamidophenol) were purchased from Sigma (Steinheim, Germany). 7-OH-MTX was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Acetonitrile and methanol were HPLC-grade and were supplied by SDS (Val de Reuil, France). Hydrochloric acid was of analytical grade and was obtained from Riedel-de Haen (Seelze, Germany). Glacial acetic acid and sodium acetate anhydrous were purchased from Merck (Darmstadt, Germany). MTX controls (X Systems Methotrexate II Controls) were supplied by Abbott Diagnostics (Wiesbaden, Germany). HPLC-grade water was used throughout the analysis. SPE LC-Ph cartridges were purchased from Supelco (Bellefonte, PA).

**Methods**

**Preparation of standard solutions**

Stock solutions of MTX (100 mM) and the IS (95.20 mM) were prepared in 0.01 M NaOH and HPLC-grade water, respectively, and stored at –20°C. Matrix calibrators were prepared daily by the addition of properly diluted stock solution to drug-free serum.

**SPE procedure**

For the SPE procedure, a VacMaster (IST, Basel, Switzerland) SPE manifold was used. A sample of 800 μL of serum was mixed with 100 μL of 1 N HCl and 50 μL of IS solution and applied on Supelco LC-Ph 1 mL–100 mg cartridges preconditioned with 1 mL methanol followed by 1 mL of H2O. Low vacuum was applied to achieve a sample flow rate of 1 mL/min. Subsequently, the cartridges were washed with 1 mL of 5% methanol in 0.01 N HCl and dried under vacuum for 7 min. Finally, MTX and the IS were eluted with 2 mL of 100% methanol. The eluent was collected in conical glass tubes and evaporated to dryness by using a sample concentrator apparatus (Techne Dri-Block, UK) under a gentle stream of nitrogen at 45°C. The residue was redissolved in 200 μL of mobile phase and centrifuged for 2 min at 5,000 rpm; 100 μL were injected into the chromatographic column.

**Method validation**

**Specificity**

Specificity was determined by the analysis of pooled drug-free serum collected from six volunteers. 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: irbesartan, amoxicillin, atorvastatin, aztreonam, caffeine, caspofungin, cefprozil, clarithromycin, clavulanate, clopidogrel, doxycycline, folic acid, furosemide, gentamycin, ketoprofen, levofolinate, methylprednisolone, metoprolol, naproxen, omeprazole, paracetamol, piroxicam, salicylate and valaciclovir.

**Recovery**

The absolute recovery of methotrexate was determined by comparing the peak area of extracted spiked serum samples with that of the corresponding unextracted standards at concentrations of 0.05, 1.00 and 3.50 μM.

Figure 1. HPLC chromatograms: blank serum (A); standard solution of 2.00 μM MTX and the IS (B); blank serum spiked with 0.50 μM MTX and the IS (C); serum sample of a patient 24 h after MTX dosing spiked with the IS (D). HPLC conditions: isocratic elution with 50 mM, pH 3.6, mixture of acetate buffer–acetonitrile (89:11, v/v) and UV detection at 307 nm. Samples in Figures 1A, 1C and 1D were solid-phase extracted as described previously with final 4.25-fold preconcentration of the sample. Initial concentrations of the IS were: 0.00 μM (A); 23.80 μM (B); 5.60 μM (C) and (D).
Linearity of calibration curves
A calibration curve was constructed to confirm the linear relationship between MTX/IS peak areas and drug concentrations in the spiked samples. Matrix calibrators were prepared with drug concentrations of 0.025, 0.05, 0.10, 1.00, 2.00, 3.50 and 5.00 μM by spiking drug-free serum samples. The calibrators were extracted as described previously and analyzed in duplicate. A standard calibration curve was fit by linear regression analysis.

Precision and accuracy
Three quality control (QC) samples at low (0.05 μM), medium (1.00 μM) and high (3.00 μM) concentrations of MTX were prepared by diluting 50 and 500 μM MTX controls (Abbott Diagnostics) in drug-free serum. Intra-day precision and accuracy was performed with six replicates of each QC sample, whereas inter-day evaluation was assessed by the analysis of one replicate at each QC sample concentration on six different days (Table I). For each QC, concentration was recalculated from the equation of the linear regression curve. Accuracy, designated as a percentage of bias, was calculated by the percentage difference between the measured and target concentration from each sample relative to the target concentration [100 × (measured – target concentration)/target concentration]. Imprecision was defined by estimating the percent coefficient of variation (CV).

Limit of quantitation and limit of detection
The limit of quantitation (LOQ) of the method was determined as the amount of the extracted analyte with a signal-to-noise ratio (S/N) equal to 3.

Biological samples
Blood samples were obtained from patients diagnosed with osteosarcoma (Department of Clinical Oncology, University General Hospital of Larissa, Greece) who were receiving HDMTX as part of a treatment protocol approved by the University Hospital Scientific Review Board; all patients who participated in the study gave their written informed consent. During HDMTX treatment, patients received intensive hydration and urine alkalization; leucovorin rescue commenced 24 h following HDMTX treatment. Blood samples, obtained at 24 h, and at 48 and 72 h when necessary, at the end of MTX infusion, were collected by venipuncture into 5 mL BD SST II vacutainer collection tubes (BD, United Kingdom). Serum was prepared by centrifugation at 3,500 rpm for 5 min; serum samples were either analyzed immediately or stored at –20°C until analysis.

Results
Method validation
Figure 1 shows representative chromatograms of a blank human serum sample (Figure 1A), an aqueous standard solution of MTX (2.00 μM) and the IS (23.80 μM) (Figure 1B), a blank serum sample spiked with 0.50 μM of MTX and the IS (5.60 μM) (Figure 1C) and a patient’s serum sample collected 24 h following MTX administration, spiked with the IS (5.60 μM) (Figure 1D). MTX and the IS were detected at 11.19 ± 0.27 min and 6.96 ± 0.11 min, respectively (n = 40); no interference from endogenous compounds was observed (Figure 1A). Because 7-OH-MTX is a primary metabolite of MTX, the possibility of co-elution of these two compounds was examined. Under the previously described chromatographic conditions, the retention time of 7-OH-MTX was 43.63 min, far from the elution time of MTX (data not shown). Furthermore, of the compounds tested for possible chromatographic interference with MTX and/or the IS, those that exhibited UV absorption at 307 nm eluted within the time range of 5–13 min, suggesting possible overlap of chromatographic peaks; this possibility was ruled out because resolution factors of MTX or IS peaks were of satisfactory magnitude (Table II).

The recovery of MTX from spiked serum was 96.22 ± 4.82% (3.50 μM, n = 5), 93.07 ± 1.87% (1.00 μM, n = 5) and 98.20 ± 7.72% (0.05 μM, n = 5). The mean recovery of the IS was 81.18 ± 2.74% (n = 15). The calibration curve for MTX was linear over the range of 0.025–5.00 μM; it was described by the equation y = (1.062 ± 0.0089)x + (0.0099 ± 0.0184) and had an intercept that did not significantly differ from zero. The correlation coefficient (r²) was 0.9994.

The results of intra-day (n = 6) and inter-day (n = 6) method validation are presented in Table I. The method was reproducible, with CVs less than 15% for the low QC sample and less than 5.55% for the medium and high QC samples. The bias values, at all concentrations tested, ranged between 0.33 and 8.33%.

The LOQ was established at 0.01 μM (S/N = 10); the LOD was 0.003 μM.

Table II
Compounds Frequently Co-Administered with MTX and Detectable at UV 307 nm, Characterized by Retention Times Distinguishable from of MTX and IS

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Retention time (min)</th>
<th>Resolution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Versus MTX</td>
<td>Versus IS</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>11.19</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>10.50</td>
<td>2.00</td>
</tr>
<tr>
<td>Cefprozil (trans-cefprozil)</td>
<td>8.76</td>
<td>7.32</td>
</tr>
<tr>
<td>Cefprozil (cis-cefprozil)</td>
<td>6.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>6.30</td>
<td>3.81</td>
</tr>
<tr>
<td>Salicylate</td>
<td>6.27</td>
<td>3.81</td>
</tr>
<tr>
<td>Levofolinate</td>
<td>5.56</td>
<td>1.45</td>
</tr>
</tbody>
</table>

*The following compounds were also examined, though not detected, at UV 307 nm: amoxicillin, clavulanate, atorvastatin, aztreonam, caspofungin, clarithromycin, clopidogrel, doxycycline, furosemide, gentamycin, ibresartan, ketoprofen, methylprednisolone, metoprolol, naproxen, omeprazole, proxicam and valaciclovir.
Biological validation

The clinical applicability of the method was examined by determining the concentration of MTX in human plasma samples, obtained from four osteosarcoma patients (three males/one female, aged 17–56 years) who were under HDMTX (8–12 g/m²) treatment, by using the previously described procedure. The plasma concentration–time profiles of MTX in these patients are shown in Figure 2. MTX could readily be detected 72 h after the end of infusion.

Discussion

In cancer chemotherapy, the routine pharmacokinetic monitoring of serum MTX is essential, primarily in high-dose MTX therapy, because it minimizes the risk of toxicity and allows early modification of leucovorin rescue in oncologic patients. In the present study, a sensitive HPLC method was developed and validated for the quantification of MTX in serum samples.

The prevention of potentially life-threatening MTX-associated toxicities has long been based on pharmacokinetically guided leucovorin rescue (14, 15). However, wide inter-patient variability, owing to hepatic and renal interindividual differences, or interactions between MTX and co-administered drugs, may lead to unpredictable MTX disposition and may require patient-tailored MTX dosing and individualized leucovorin rescue (1, 15). Furthermore, it has been reported that no significant rescue will occur if leucovorin administration is delayed and that high doses of MTX require disproportionately elevated leucovorin doses, suggesting that both the timing and the dose of leucovorin have to be based on monitored MTX serum levels (16, 17). For these reasons, serial MTX sampling, usually up to 72 h after MTX infusion, has been routinely required (2, 17), although shorter sampling strategies have also been employed (1). Several nomograms, based on the disappearance of MTX from serum, have suggested that the cutoff points at which the drug is considered adequately cleared and that are associated with the end of leucovorin rescue may range from 0.90–1.00 µmol/L at 42–48 h (15, 18) to 0.20 µmol/L (1) and as low as 0.10 µmol/L at 72 h post-MTX (2, 17, 19). The present method accurately determines MTX concentrations as low as 0.01 µmol/L, which is 10 to 20-fold below the lowest MTX level that may present a risk for toxicity, thus ensuring effective and safe therapy for patients under HDMTX.

Therapeutic drug monitoring may be applied in the low-dose (5–30 mg/week) MTX therapy that is commonly used to treat autoimmune diseases, such as rheumatoid arthritis and psoriasis. Despite the favorable risk–benefit profile of the drug, autoimmune patients are more likely to discontinue MTX therapy because of adverse drug effects, rather than lack of response (4, 20–22). However, repeated sampling in chronically administered MTX, such as in autoimmune diseases, is difficult for practical reasons. A limited sampling strategy has been suggested, according to which MTX therapeutic values are estimated with sufficient accuracy and precision from a single plasma sample at 3 h post-dosing (23). Using this approach, serum concentrations of MTX have been reported to be as high as 0.25 µmol/L in rheumatoid arthritis (24) and psoriasis (25) patients, which is 25-fold above the LOQ of the present study. These data suggest that although MTX concentrations lower than 0.01 µmol/L, such as those observed 24 h after the administration of a single oral dose (26), cannot be determined, the present method is practically useful in monitoring MTX limited sampling and optimizing drug treatment in autoimmune diseases. Disease activity has been correlated with the biologically active polyglutamate MTX metabolites (4, 27, 28) rather than MTX serum concentration (29).

The HPLC method developed in the present study is simple, selective, accurate, precise and cost-effective. Possible interference between MTX and serum endogenous compounds and co-administered drugs has been precluded by proper sample pretreatment and chromatographic separation. Furthermore, isoocratic elution has been achieved by the use of an inexpensive HPLC system utilizing one HPLC pump. The developed SPE method of MTX isolation from serum is more efficient and column friendly than previously described sample pretreatment by protein precipitation only. In the latter treatment, part of the drug is obviously co-precipitated with proteins, leading to reduced recovery compared to the current method (30). Moreover, protein precipitation does not ensure sufficiently clean samples, leading to detrimental impacts on the lifetime of the HPLC column and the long-term quality of analysis. The high recovery (>93%) and selectivity of this method have been assured by the selective MTX isolation from serum due to the efficient and specific π–π interactions between functional phenyl groups of the selected solid phase and the aromatic system of the drug, and specific detection at 307 nm. In fact, there are few serum components that absorb at this wavelength. As a result of the attained selectivity, there are no endogenous compound peaks in serum blank chromatograms (Figure 1A). The application of either a C8 or C18 phase in biological samples resulted in substantially lower recovery of the analyte [79% in the present study, data not shown; ~86% in the study by Albertoni et al. (9); ~88% in the study by Turci et al. (31)]. Finally, a phenyl functionalized sorbent for the isolation of MTX from biological samples was only scarcely applied (32).

Conclusion

A simple, isocratic, reversed-phase HPLC method has been developed and validated for the determination of MTX in human
serum. The selectivity of the method is assured by the use of specific phenyl functionalized sorbent for SPE and specific detection at 307 nm. The present method combines the universality and robustness of UV detection with the high sensitivity, accuracy and precision of a relatively simple procedure, thus providing an important tool that allows routine application in clinical practice.

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