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Technology

Quantification of Methotrexate by Liquid Chromatography Ultraviolet Detection for Routine Monitoring of Plasma Levels

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Abstract. A high-performance liquid chromatographic (HPLC) technique with ultraviolet detection incorporating solid phase extraction (SPE) was developed to meet analytical and metrological requirements for routine serum level monitoring of methotrexate (MTX), with several parameters optimised such as temperature, flow rate, composition of the mobile phase and pH of the buffer solution. Two standard curves were constructed to cover the high and low levels of the calibrator range (0.02-600 µmol/litre). Reproducibility (precision) of the method for intra assay was 2.7; 2.10; 1.38% at the lowest level and 2.11; 3.4; 2.01% at the highest level and for inter assay was 2.8; 2.2; 2.94% at the lowest level and 2.4; 2.74; 2.68% at the highest level; recovery was between 90.47 and 98.53 percent. Response was found linear over the whole range of the calibrator set with a correlation coefficient of 0.999. The limit of quantification and the limit of detection were 0.02µmol/litre and 0.0063µmol/litre, respectively. The method is suitable for quantification of methotrexate with good accuracy and precision

Keywords: methotrexate, liquid chromatography, solid-phase extraction, plasma level monitoring

Introduction

Methotrexate (MTX) is a competitive inhibitor of dihydrofolate reductase (Balloy *et al*., 2007), a key enzyme of nucleic acid biosynthesis (Albertioni *et al*., 1995). Thus, it can block tumoral cell growth and is widely used as a cytostatic agent (Albertioni *et al*., 1995). In cancer treatment, high-dose MTX therapy is followed by leucovorin (folinic acid) rescue. Serious toxicity might be detected by monitoring serum methotrexate concentrations (Balloy *et al*., 2007; Aboleneen *et al*., 1996; Albertioni *et al*., 1995).

The aim of the study is to describe a simple, fast and precise method for determination of methotrexate in plasma for pharmacokinetic studies and for use in routine therapeutic drug monitoring in cases of high-dose intravenous infusion of this drug.

Materials and Methods

Chemicals and reagents. Methotrexate, hydroxymethotrexate and 8-chlorotheophylline (used as internal standard) were purchased from Sigma, potassium dihydrogen phosphate (KH_2PO_4) was purchased from Carlo Erba whereas methanol, and acetonitrile, HPLC grade, were obtained from Merck. All other chemicals and solvents used were of analytical grade. The reference of reversed phase cartridge for solid phase extraction (SPE) was Chromsystems Ref: 7008 lot 212.

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Instrumentation. The LC system, used for method development and validation, was obtained from Merck and consisted of L-6000 pump, L-5025 oven and L-4250 spectra absorbance detector.

Sample preparation. Stock solutions used in this work were MTX, 0.75 mg/ml (1651 µmol/litre) and OH-MTX 0.05 mg/ml (106.15 µmol/litre). They were serially diluted in 14% $CaCO_3$. Further stock solutions of MTX and OH-MTX were prepared in different concentrations as follow: MTX (0.02 to 0.8 µmol/litre and 5 to 500 µmol/litre) and OH-MTX (0.2 to 0.77 µmol/litre and 4.8 to 480 µmol/litre).

8-Chlorotheophylline was used as internal standard (IS) because it is a synthetic molecule that cannot be administered to patients. Stock solution of internal standard was prepared in water at a concentration of 200 µg/ml.

MTX, OH-MTX and IS stock solutions were stored at -30 °C.

Chromatographic conditions. The chromatographic column used was 250 x 4 mm (Lichrospher®) with 5 µm particle size and 1 cm long guard column. Mobile phase was phosphate buffer, pH = 6.1: methanol (80/20, v/v); flow rate of mobile phase was 1 ml/min. Column was maintained at 25 °C, detection wavelength was 297 nm and injection volume was 50 µl. These parameters were used at the beginning of the experiment and the chromatographic conditions were optimized for the required analysis.

Extraction procedure. The coextractive cleanup procedure was performed according to the method reported by Aboleneen *et al*. (1996) with slight modification.

Heparinised plasma (300 µl) containing 20 µl of internal standard, 40 µl of MTX and methanol, twice its volume, were mixed thoroughly. After centrifugation (10 min, 3000 g at 4 °C), the deproteinized supernatant mixture was transferred to the extraction cartridge. Cartridges were prepared with 9 ml methanol followed by 3 ml of 0.04 M phosphate buffer (pH 6), drawn through centrifugation or suction, discarding the effluent. The eluate, containing analyte and IS, was dried under nitrogen stream at 60 °C. The residue was reconstituted in 200 µl HCl 0.005 M. A 50 µl aliquot of the reconstituted sample was injected on to the HPLC column.

Patients and treatment. The study was approved by the local ethics committee and informed consent was obtained from all the children and their parents. The study group comprised of 34 children with acute lymphocytic leukaemia (ALL). They were enrolled at the time of diagnosis in the European Organization for Research and Treatment of Cancer (EORTC). MTX infusion (5 g/m^2) was administered at 24 h interval. For MTX monitoring, blood samples were collected from all the patients at intervals of 24 h, 48 h and 72 h from the time of injection/infusion and adjusted until MTX concentration was below 0.2 µmol/litre.

Results and Discussion

Optimized chromatographic conditions. For optimizing separation of the drugs, several mobile phase compositions were investigated. Initially, phosphate buffer pH=6.1: methanol (80/20, v/v) was used; however, MTX, OH-MTX and IS cannot be separated under this condition. Subsequently, phosphate buffer pH=4: methanol (80/20, v/v) was used, wherein all the drugs were separated but the retention time was as long as 25 min. When acetonitrile was added to the mobile phase, with adjustment ratio of phosphate buffer pH=4: acetonitrile: methanol (82:12:6, v/v/v), the retention time of three drugs decreased. This mobile phase was found the most suitable and gave a good baseline.

In the study of effects of different temperatures in the range of 25 °C to 70 °C and flow rate of 0.7 to 1.2 ml/min, good separation was obtained at 40 °C and flow rate of 1 ml/min (resolution > 1.5). Other chromatographic conditions included the ratio of mobile phase of phosphate buffer pH=4: acetonitrile: methanol to be 82:12:6, v/v/v, flow rate 1, ml/min and temperature, 40 °C. The retention time for MTX, OH-MTX and IS was 8.77, 10.5 and 12.88 min, respectively. Chromatograms are shown in Fig. 1.

Precision. For precision, six assays per concentration were made. Within- and between-day accuracy and precision values are given in Table 1.

The inter-assay and intra-assay variation of the measured concentration was assessed by the relative standard error (% RSD) which was calculated as the standard deviation of the measured concentrations divided by the mean and multiplied by 100. Batches, for which the recovery did not fall within 71-125%, and/or having RSD value greater than 5%, were reanalyzed. For assessing the accuracy of the method,

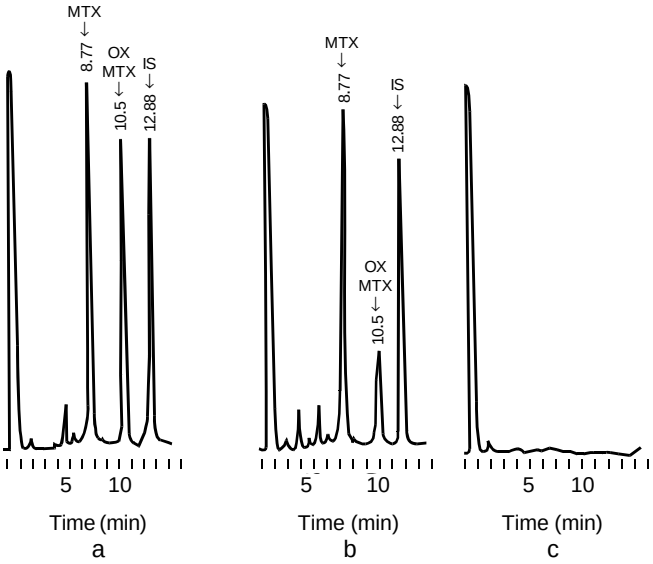

Fig. 1. Representative chromatograms of spiked plasma with MTX and OH MTX and IS (8-chlorotheophylline) (a), patient sample (b) and blank plasma (c)

Table 1. Precision data for methotrexate

Theoretical value (µmol/litre)	Measured value (mean ± SD) (µmol/litre)	RSD (%)	Accuracy (%)
Intra-assay (n = 6)			
0.02	0.018 ± 0.0005	2.77	90
0.4	0.38 ± 0.008	2.10	95
0.8	0.72 ± 0.010	1.38	90
5	4.55 ± 0.096	2.11	90
50	35.88 ± 1.22	3.4	71.76
500	531.83 + 10.72	2.01	106.36
Inter-assay (n = 6)			
0.02	0.025 ± 0.0007	2.8	125
0.4	0.36 ± 0.008	2.2	90
0.8	0.68 ± 0.02	2.94	85
5	4.91 ± 0.12	2.4	98.2
50	44.46 ± 1.22	2.74	88
500	526.25 + 14.12	2.68	105

recovery of methotrexate from plasma samples of known concentrations was compared with the solutions of methotrexate at the same concentrations. Mean recoveries were between 90.47% and 98.53% for inter- and intra-assay, respectively.

Linearity. Calibration curves for plasma samples were constructed within the concentration ranges of (0.02-500 µmol/litre) using linear least-square regression. The MTX calibrators were divided in two concentration ranges: the low levels (0.02; 0.4; 0.8 µmol/litre) and the high levels (5; 50; 500 µmol/litre). Correlation coefficient, slope and intercept for six standard curves constructed for each range, were 0.99, 0.85 and 0.34, respectively. The specificity was determined by comparing 15 plasma samples collected from patients who did not receive MTX. Each sample was analysed and any detectable signal corresponding to the retention times of MTX and IS was not found.

Limit of detection and limit of quantification. For estimating the limit of detection (LOD) and the limit of quantification (LOQ), 10 replicates of control plasma 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 (Vassault *et al*., 1999). The LOD and LOQ of the described method for MTX in plasma samples were 0.0063 and 0.02 µmol/litre, respectively.

Application. The method has been applied successfully for determination of MTX concentration in 234 dosages (Fig. 1). The average age was 18 years (8 to 21 years). Mean plasma concentration at 24 h, 48 h and 72 h intervals was 1.90; 0.18 and 0.03 µmol/litre, respectively. Statistic analysis showed no correlation between dose and plasmatic level of MTX at 24 h, 48 h and 72 h.

Considerable individual variability has been observed and regular monitoring is required in clinical use. Moreover, further investigations are necessary to assess the clinical utility of monitoring MTX concentrations and to identify the relation between the pharmacokinetic parameters of MTX and clinical efficacy and toxicity in Tunisian population.

The present study describes a highly sensitive, accurate, and reproduceable HPLC method for determination of MTX in human plasma. The procedure of sample preparation is rapid; it uses a small plasma volume (300 µl) and is less expensive.

In order to optimise a method, several parameters were modified. Solid-phase extraction (SPE) was essential for plasma samples; in fact, without SPE, a larger solvent front interferes with the two analyte peaks. This extraction was the preferred method by Albertioni *et al*. (1995). This extraction required, a procedure for denaturing proteins, which was achieved by the precipitation of proteins in the samples with methanol (Floridia *et al*., 1999). The other method uses acetonitrile, trichloroacetic acid or centrifugation (Fotoohi *et al*., 2005, Nadège *et al*., 2003; Albertioni *et al*., 1995).

The procedure was based on the internal standard method, as reported in several studies (Albertioni *et al*., 1995; Cociglio *et al*., 1995). The mobile phase reported in the present study uses phosphate buffer at pH=4: methanol: acetonitril in the ratio 82:12:6, v/v/v). This phase was described in other studies but in different proportions (Fotoohi *et al*., 2005, Turci *et al*., 2000, Vassault *et al*., 1999; Aboleneen *et al*., 1996).

Calibration curves were constructed using linear least-square regression. Determination was based on the internal standard method, (Fotoohi *et al*., 2005; Aboleneen *et al*., 1996). The coefficient correlation of the method was R = 0.999 and the overall RSD < 5%. Within- and between-day accuracy was 3.65 and 3%, respectively. In other studies, these values varied between 3 and 4% (Aboleneen *et al*., 1996; Albertioni *et al*., 1995; Cociglio *et al*., 1995). Values of quantification reported in the literature ranged from 0.03 µmol/litre to 0.08 µmol/litre. In the present assay, the limit of quantification was 0.02 µmol/litre and the limit of detection was 0.0063 µmol/litre.

Conclusion

A technique for rapid and sensitive assay of methotrexate in the serum was developed. This method was free from plasma matrix interferences and determined methotrexate concentrations at the lowest and the highest levels. The utility of the method was further demonstrated by measuring methotrexate levels in patient samples. The method can be used to estimate OH-MTX in plasma sample.

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