SPECTROFLUORIMETRIC METHOTREXATE ASSAY IN HUMAN PLASMA

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Abstract
Methotrexate (MTX) is an antimetabolite used at high doses in several neoplastic disease (acute lymphoblastic leukemia, osteosarcoma, etc.), but also, at low doses, in certain autoimmune diseases. As the treatment with MTX in neoplastic disease is always accompanied by the administration of leucovorin, acting as a “rescue” agent, the actual levels of MTX and leucovorin in patients has to be carefully monitored.

The paper presents a simple, yet sensitive, accurate and precise spectrofluorimetric method for MTX assay in human plasma using the oxidation reaction of the drug with KMnO₄ in acetate buffer. The method can be used to monitorise the MTX plasmatic level in patients treated for acute lymphoblastic leukemia or osteosarcoma.

Keywords: methotrexate, plasma levels, spectrofluorimetry

Introduction
Methotrexate ((2S)-2-[4-[(2,4-diaminopteridin-6-yl) methyl] methyl-amino]-benzoyl] amino] pentandioic acid – MTX) is an antimetabolite (antifolate) used in the treatment of certain neoplastic diseases and autoimmune disorders. MTX inhibits dihydrofolate reductase, an enzyme participating to the tetrahydrofolate synthesis, thus maintaining a low intracellular folate level. Therefore, by its action MTX inhibits the purine base synthesis, and consequently the synthesis of thymidylates, RNA, DNA, and proteins [4].

MTX is used in the treatment of various types of neoplastic diseases at high doses (over 20 mg/m², weekly). In order to prevent against a too low amount of existing folic acid at the cellular level, leucovorin (2-
[4-[(2-amino-5-formyl-4-oxo-5,6,7,8-tetrahydro-1H-pteridin-6-yl)methylamino] benzoyl]-aminopentanedioic acid) is used in conjunction with MTX in the treatment of neoplastic diseases, and the balance between the administered MTX and leucovorin has to be carefully monitorised during the treatment [12, 14]. Therefore, a simple and rapid method had to be developed for MTX assay in human plasma; the method has to be relevant for osteosarcoma and acute leukemia treated patients, as they get higher MTX doses than other kinds of cancer treated patients.

Several methods were described for MTX assay in biological fluids, mainly HPLC methods with pre-column or post-column derivatisation and fluorescence detection [3, 5, 7 – 9, 12, 13]. As these methods are rather difficult to be validated for biological samples [6, 10], we tried to investigate a spectrofluorimetric assay method for the quantification of MTX in human plasma.

The method is based on MTX acid hydrolysis at the amidic group by KMnO₄ in acetate buffer (pH=5), in the presence of ascorbic acid, followed by the analysis of the resulting fluorescent compound 2-amine-4-hydroxypteridin-6-carboxylic acid (Figure 1).

![Methotrexate oxidation reaction used in the assay](image)

**Figure 1**

Methotrexate oxidation reaction used in the assay

**Materials and methods**

**Patients**

We selected 45 patients diagnosed with acute leukemia treated with high doses of MTX at “Al. Trestioreanu” Oncology Institute. Informed consent was obtained from the patients prior to the enrolment in the study.

The patients received MTX by continuous perfusion for 4 hours in doses of 20 to 1000 mg/m², depending on the stage of the treatment, the stage of the disease and the general state of the patient. Blood samples were collected on heparin as an anticoagulant from the patients before the treatment, at the end of the treatment and at 24, 36 and 48 hrs. following the treatment. The plasma was separated from the blood samples and was further used for the MTX quantification.
**Reagents**
- Methotrexate hydrochloride Ph.Eur. grade was bought from Sigma and was used as a standard substance; a stock solution of 10 μg/mL MTX in 0.01 M NaOH solution was prepared and used in standard solutions;
- Working solution of 60 μg/mL ascorbic acid (from Merck) in bidistilled water was freshly prepared;
- Acetic acid / sodium acetate buffer, pH=5.0, was prepared conform to Ph.Eur. [2]
- Working solution of 1 mM KMnO₄ was freshly prepared in bidistilled water
- Human plasma from the “C.T.Nicolau” National Haematologic Institute, diluted 3:100 with bidistilled water was used for the settlement and validation of the method.

**Device and instrumental parameters:** all measurements were performed on a LS50B Perkin Elmer spectrophotometer working mainly in “Scan” and “Time drive” modes, FLWinlab 2.01 software version.

**Procedure**
The spectrofluorimetric MTX assay in human plasma is based on the oxidation of MTX with KMnO₄ in acetate buffer (pH=5) in the presence of the ascorbic acid to balance the oxidation procedure. The following protocol was applied: to a 2 mL sample (human plasma, containing or not different amounts of MTX), 0.6 mL of acetic acid/sodium acetate buffer, 0.3 mL of 1 mM KMnO₄ and 0.01 mL of ascorbic acid working solutions were added; the mixture was mildly shaken and kept in darkness for 5 minutes; for each MTX containing plasma sample an appropriate blank sample was prepared. The samples and the blank solutions were registered the emission spectrum (λ<sub>excitation</sub> = 380 nm, λ<sub>emission</sub> = 400 – 500 nm), than the emission intensity signal was measured at 457 nm. The blank sample emission intensity value was subtracted from the corresponding MTX-containing emission intensity value, and the result was further used for the calculations.

For each patient participating to the present study, a blood sample was collected and processed as described above before the beginning of the treatment.

**Results and discussion**

**Preliminary experiments**
In order to get the needed instrumental parameters, we first used a 1 μg/mL MTX solution in NaOH 0.01M, which was treated as described above. The excitation and the emission spectra recorded (Figure 2) provided for the emission peak from 457 nm two excitation wavelengths: 275 nm and
380 nm. As the first peak could give spectral interferences due to proteins, we chose the excitation wavelength of 380 nm.

We also investigated the kinetics of the oxidation process, by constantly measuring the emission intensity at 457 nm when exciting the sample at 380 nm, for 25 minutes. The results indicate that the reaction time needed to obtain the fluorescent adduct is 15 minutes.

![Figure 2](image)

**Figure 2**

Excitation and emission spectra after MTX oxidation with KMnO₄ in acetate buffer

**Validation of the method on MTX–spiked plasma samples**

We validated the method on human plasma samples spiked with known amounts of MTX. The validation was performed conform to reference [2], and had in view the following parameters: selectivity, accuracy, precision, recovery, standard curve, lower limit of detection and stability.

For the purpose we spiked normal human plasma samples containing no MTX, with known amounts of MTX solution to get concentrations ranging between 0.077 to 1.05 μg/mL, and we prepared these samples as described above. For each sample containing MTX, a blank sample was also prepared in the same conditions. We registered the emission spectra excited at 380 nm for all samples and blanks. No fluorescence signal was obtained for the blank samples in the region of
interest (457 nm). However, as the background signal was rather high, we decided to correct each spectrum by subtracting the corresponding blank from the sample spectrum. A collection of such corrected spectra is presented in Figure 3.

![Emission spectra corrected for the blank used for the calibration curve](image)

**Figure 3**
Emission spectra corrected for the blank used for the calibration curve

The validation of the method was performed using the corrected emission intensity measured at 457 nm.

The selectivity of the method is ensured by the fact that the fluorescent compound is due only to 2-amine-4-hydroxypteridin-6 carboxylic acid, as discussed before. Moreover, any chemical or spectral interference from the matrix is eliminated by correcting the emission spectra from their blanks.

The accuracy of the method was tested on 3 replicates performed for the concentrations 0.75 μg/mL, 0.45 μg/mL and 0.15 μg/mL; the mean value obtained was within the permitted range of 15% deviation from the actual value.

The precision of the method was determined at each concentration on 5 replicates, and the variation coefficient was less than 10% at each concentration tested.

The recovery of the method was also tested for 0.75 μg/mL, 0.45 μg/mL and 0.15 μg/mL, and led us to a 99.8% mean recovery coefficient, which is excellent, even for a method without extraction.
The linearity was tested within the concentration range 0.077 – 1.05 μg/mL and lead to a correlation coefficient of 0.994, with a lower limit of quantification (LLOQ) determined by the peak response method [1] was of 0.075 μg/mL.

The stability of the method was not tested, as the method is intended to be performed on fresh blood samples drawn from patients, in order to monitorise the metabolisation of the drug and to establish the leucovorin doses for each patient as well.

Results obtained on blood samples from patients
The method was tested on blood serum from patients treated with different doses of MTX. The results indicate a great variability in respect of the MTX plasma peak among patients (Figure 4) and even for the same patient in different series of treatment. The results indicate also a MTX elimination curve following a first order exponential decay.

![Figure 4](image)

MTX plasmatic levels in different patients, measured at different moments after administration

The obtained values also indicate the possibility to monitorise MTX levels in cancer-treated patients and to adjust the needed leucovorin doses.

Conclusions
We developed and validated a simple and sensitive spectrofluorimetric method for the quantitative MTX assay in human plasma using the oxidation of the drug with KMnO₄ in acetate buffer, pH=5.00, in the presence of ascorbic acid. The method is accurate and precise, linear within the range 0.077 – 1.05 μg/mL, with a lower limit of quantification of 0.075 μg/mL.

The method is useful for monitoring plasmatic levels in neoplastic patients treated with MTX to avoid unwanted response to the treatment.
References
2. *** European Pharmacopoeia, 6-th edition, 2009

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