Qualitative and Quantitative Determination of Methotrexate Polyglutamates in Erythrocytes By High Performance Liquid Chromatography

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Methotrexate (MTX) is a structural folic acid analogue. It is one of the most easily tolerated antipsoriatic drugs with disease-modifying capacities and a prodrug which is activated to yield methotrexate polyglutamates (MTX–PG) by means of folypolyglutamate synthase (FP-GS). MTX-PGs concentration in the tissues is low and the quantification of individual MTX-PG species is difficult. A HPLC method for the quantification of these metabolites was developed. The MTXPGs were extracted from the biological matrix using 100 µl red blood cell hemolysate, by perchloric acid deproteinization step with direct injection of the extract into the HPLC. We used a C18 reversed-phase column, an acetonitrile buffer, and postcolumn photocatalytic oxidation of MTXPGs to fluorescent analytes. The complete conversion to MTX of the MTX-PG 2–7 pool, the incubation was performed in the dark, at 37 °C. The correlation coefficients was >0.995 for MTX-PG1 as well as for samples of MTX-PG 1 obtained following enzymatic conversion of MTX-PG 2–7. Intra- and interday imprecision were <10%. The advantage of this method lies in its simplicity and low volume of RBC required.

Keywords: HPLC, methotrexate, methotrexate polyglutamates

Methotrexate (MTX) - (2S)-2-[(4-{[(2,4-Diaminopteridin-6-yl)methyl]methyl}amino)benzoyl]amino]pentanedic acid - is structural folic acid analogue, with an amino group (-NH2), a methyl group (-CH3) and a fully oxidised pteridinic ring rendering it an inactive cofactor, N-[4-{{(2,4-diamino-6-pteridinil)methyl}methyl amino}benzoil]-L-glutamic acid (fig.1).

In agreement with MTX main mechanism of action, the blocking of the synthesis of nucleic acids precursor nucleotides, particularly during methylation, with tmytidilate synthase as a catalyst, from dUMP to dTMP, triggers discontinuation of DNA synthesis and inhibition of cell proliferation involved in inflammatory processes.

Successful use of MTX as nonbiologic antipsoriatic medicine is due to the diverse character of its action, affecting a wide range of pathogenic mechanisms. The molecular mechanism of most of such actions is associated with release of adenosine in the extracellular area, where it activates receptors on types of cells relevant for inflammation.

One of the most easily tolerated antipsoriatic drugs with disease-modifying capacities, MTX therapy is not free however from major downsides, among which extensive variability among patients in respect to clinical response, together with erratic occurrence of a wide range of adverse reactions [1, 2]. Such drawbacks make effective MTX dosing more difficult for patients.

There is various proof of MTX character as a prodrug, which is activated to yield methotrexate polyglutamates (MTX–PG) by means of folypolyglutamate synthase (FP-GS) - mediated successive addition to the parent drug of glutamic acid residues [3]. MTX properties are altered selectively by the respective γ-linked process, which also increases its maintenance as polyglutamates after MTX disappearance from circulation (i.e. plasma); that is why, dissimilar from other antifolates, MTX is only given once a week [5].

Active MTX-PGs intracellular pharmacokinetics has been difficult to characterise because of both intrinsic and extrinsic reasons. Intrinsically, MTX-PGs concentration in the tissues is several 100-fold lower, and, in addition, quantification of individual MTX-PG species poses particular analytical challenges [6-9].
To overcome this quantification issues, two HPLC-fluorometry methods have been developed, as follows:

- use of plasma FP-GS for MTX-PGs conversion into MTX,
- individual quantification of MTX-PGs in a single run.

**Experimental part**

**Materials and methods**

All reagents used were of chromatographic purity grade; the materials used were acquired from Sigma-Aldrich except acetonitrile:

- MTX;
- MTX-PGs - ammonium salts: MTX-PG$_1$–MTX-PG$_7$ (4-Amino-10-methylpteroylglutamic acid, 4-amino-10-methylpteroyldiglutamic acid, 4-amino-10-methylpteroyltriglutamic acid, 4-amino-10-methylpteroyltetroglutaric acid, 4-amino-10-methylpteroylpentaglutamic acid, 4-amino-10-methylpteroyhexaglutamic acid, 4-amino-10-methylpteroylheptaglutamic acid);
- HPLC-grade acetonitrile - Fisher Chemicals
- potassium phosphate;
- hydrogen peroxide (H$_2$O$_2$);
- plasma (lyophilised);
- mercaptoethanol.

A Surveyor Plus system liquid chromatograph, including quaternary pump, system controller, autoinjector, sample cooler set at 4 °C and fluorometric detector.

A 250 x 4.6 mm Terra MS C18 column (5-μm particle size) with guard column protection was used for chromatographic separation.

Post-column photooxidation in ultraviolet irradiation was used in H$_2$O$_2$ for MTX and MTX-PGs detection.

The photochemical reactor unit Aura Industries used was provided with a low-pressure, mercury ultraviolet lamp (254 nm) with one meter of 1/16-inch (o.d.) tubing (Teflon, 0.25 mm i.d.) in a knitted coil, assembled on-line between the analytical column and the fluorometric detector.

Mobile phases were as follows:

- Phase A: 10 mmol/L, pH 6.50 ammonium acetate, with 2 mL/L hydrogen peroxide;
- Phase B: acetonitrile.

A Hewlett-Packard Vector XA computer was used to obtain the chromatograms and perform their analysis.

A 0.1 mol/L potassium hydroxide dissolution was used for preparation of MTX and MTX-PGs. Dissolution was performed, followed by confirmation of each calibrator final concentration. This was achieved by use of a Perkin Elmer LS 50B model luminescence spectrometer provided with accessories for polarising, cuvette thermostatting and magnetic stirring in the cuvette. For all MTX-PGs, molar extinction coefficients were $\lambda$ = 256 nm.

The specified calibrators were brought to final concentration by water dilution, to be stored next at -80 °C.

To render calibration curves, determined amounts of MTX-PGs were added to a red blood cell hemolysate pool, isolated after sampling from healthy volunteers.

Next, plasma FP-GS was used for conversion of MTX-PGs, and the total MTX-PGs was quantified with calibrators made up of MTX-PG$_1$–MTX-PG$_7$ equimolar mixture. To fit calibration curves, linear regression was used with peak area/concentrations.

Quantification of total MTX-PGs was possible following conversion to MTX. This was performed in several steps, the first of which consisted of adding reconstituted plasma (100 μL) to RBC hemolysate (100 μL), obtained after RBC lysis in the freeze-thaw process. An Eppendorf tube was used for addition of plasma to the hemolysate.

Next, the sample was mixed thoroughly for 1 min and a buffer mixture (100 μL) of potassium phosphate (100 mmol/L) and mercaptoethanol (150 mmol/L) was added. Samples thereof were placed in an incubator and kept at 37 °C, in darkness conditions, for 12 h.

Incubation was followed by cooling, after which perchloric acid was added (25μL of 700 mm/L). The mixture was stirred for 10 s in a vortex and next centrifuged for 5 min.

Eighty μL of the mixture were fed into the HPLC system. For chromatographic separation, mobile phase B was performed for 15 min, using a linear gradient from acetonitrile 0% to 20%, at a 1 mL/min flow rate.

At the end of the 15 min period, mobile phase B was returned to full mobile phase A, left for 5 min to re-equilibrate. Samples were injected every 20 min, one by one.

For quantification of the MTX resulting photolytic product, the excitation wavelength was set at 400 nm and 470 nm emission wavelength. Record of the fluorometric signal was performed between 10 and 20 min.

To determine inter- and intra-day accuracy and precision, analysis of RBC hemolysates was performed after addition of low and high MTX-PG concentrations.

Inter-day assessment involved 5 enriched replicates on 5 days; intra-day assessment involved 10 such replicates.

To establish the performance of the method, the percentage difference was calculated between the concentrations as quantified from each enriched sample in relation with the target concentration. To establish recoveries, a comparison was performed between the peak height of the RBC enriched with MTX-PG and the peak heights of samples prepared in water at similar concentration.

**Results and discussions**

The resolution of MTX-PG$_1$ in the biological matrix was demonstrated by comparison of HPLC chromatograms for blank RBC samples with RBC samples after enrichment with MTX-PGs and subsequent conversion to MTX-PG$_1$ (fig. 2 and 3).
To complete the conversion to MTX of the MTX-PG₁, pool, incubation was performed in the dark, at 37 °C, for 10 h; the RBC concentration used was 1000 nmol/L (fig. 4).

The conversion to MTX-PG₁ to MTX-PG₂, was complete in 10 hours. Whereas no MTX-PG₁ was detected in RBC samples after MTX-PG₁ enrichment prior to enzymatic conversion, the MTX-PG₁ peak occurred during enzymatic conversion.

A linear relationship was shown by calibration curves between concentration and peak area, characterised by conversion.

Minor differences have also been observed among MTX-PG species regarding the respective extraction recoveries, which is reason for speculation that MTX moiety stability during de-proteinization may be affected by the number of glutamic residues number, in reverse as compared with the other authors method [15].

To conclude, an accurate, sensitive and speedy method has been developed for use in the quantification of MTX-PGs in RBC samples, suitable in MTX therapeutic practice for routine monitoring.

Conclusions

The advantages of this method lies in its simplicity and low volume (100 µl) of RBC required. In addition, the capacity to convert to MTX all MTX-PGs increases the method’s sensitivity and therefore its applicability to both adult and paediatric patient samples, in spite of the limited volume of blood involved in the latter case.

In comparison with the DHFR radioligand assay, this method has the major advantage of simultaneous MTX-PG quantification in a single run, eliminating the need to collect multiple fractions [15, 21-23]. In the newly devised chromatographic system, the MTX-PG species elution was set in decreasing order of glutamic residues number, in reverse as compared with the other authors method [15].

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The method involves deproteinization with H₂O₂ of the RBC matrix, followed by direct extract injection into the HPLC system.

Minor differences have also been observed among MTX-PG species regarding the respective extraction recoveries, which is reason for speculation that MTX moiety stability during de-proteinization may be affected by the number of glutamic residues.

Conclusion

Accurate quantification of these analytes is mainly deterred by their low concentrations (within the nmol/L range in the RBC) as well as by the complex character of the RBC matrix itself.

As a means to overcome such limitations, an alternative strategy has been devised, based on the MTX capacity to inhibit DHFR [15-17]. This is the so-called “DHFR inhibition enzymatic assay”, where MTX-PGs in RBC samples are extracted under heat conditions.

Based on the fact that MTX may be measured in plasma in the nmol/L range following post-column photo-oxidation to fluorescent products in neutral pH conditions when hydrogen peroxide is present [18-20], a new method has been developed for such situations.

The observed similarity of excitation spectra exhibited by all MTX-PGs after ultraviolet irradiation may be considered a firm indicator of photo-oxidation between the p-aminobenzoyl and the heterocyclic portion of the MTX molecule.

A strategy was set up involving conversion by plasma FP-GS of MTX-PGs back to MTX, using incubation with mercaptoethanol, at 37 °C. Efficient conversion of all MTX-PGs (up to 1000 nmol/l packed RBC samples), MTX glutamate included (a poor substrate for the enzyme), required 10 h of incubation.


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