Investigation Relevance of Methotrexate Polyglutamates in Biological Systems by High Performance Liquid Chromatography

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Methotrexate (MTX) is converted into methotrexate polyglutamates (MTX-PGs) in 24 hours post-administration. MTX-PGs may affect MTX efficacy and enhance its toxicity in therapies for psoriasis, cancer, rheumatoid arthritis and because of that routine MTX-PGs monitoring is recommended. We developed a HPLC method for the quantification of MTX-PGs in vivo. The MTXPGs were extracted from the biological matrix using and injected into the HPLC. MTXPGs, up to hepta order, were quantified in a single run. In 32 patients with psoriasis receiving weekly stable dose of MTX, no MTX-PG, was detected in RBC samples after MTX-PG enrichment prior to enzymatic conversion, the MTX-PG, peak occurred during enzymatic conversion. The method above can be helpful in actual practice for effective and effortless MTX dosing as well as for reduction of potential adverse reaction to a minimum.

Keywords: psoriasis, erythrocytes, methotrexate polyglutamates

Considering that ca. 95% of any methotrexate (MTX) dose is generally metabolised in 24 h post-administration, the monitoring based on plasma concentration in therapies with low MTX doses [1-3] may be of little relevance. Inside the cell, MTX undergoes the action of folylpolyglutamate synthase (FP-GS), thereby converted into methotrexate polyglutamates (MTX-PG) [4, 5].

With growing research data indicating that presence of MTX-PGs may affect MTX efficacy and enhance its toxicity in therapies for psoriasis [6, 7], cancer [8, 9], rheumatoid arthritis [10, 11] and other diseases [12-14], routine MTX-PGs monitoring has been recommended. The recommendation cannot however be actually implemented because of the scarcity of methods for quantifying MTX-PGs in vivo [15, 16].

Experimental part

Methods

Materials and equipment – were presented in our previous work, Negrei C. et al [17].

The general method was also presented in our previous work; in addition, to render calibration curves, determined amounts of MTX-PGs were added to a red blood cell hemolysates 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 and an MTX-PG,–MTX-PG, equimolar mixture. To fit calibration curves, linear regression was used with peak area/concentrations.

Packed RBC hemolysate was used, of which a 100μL volume was homogenised in water (150μL). Perchloric acid was next added to the homogenate (added 25μL of 700 mL/L), which was mixed in a vortex for 1 min and finally and centrifuged for 10 min at 4000 g. Of the resulting supernatant, 50 μL in total were directly fed into the HPLC system.

For HPLC separation of MTX-PG, mobile phase B was performed for 20 min, using a linear gradient from acetonitrile 0% to 20%, at a 1 mL/min flow rate.

At the end of the 20 min period, mobile phase B was returned to full mobile phase A, left for 10 min to re-equilibrate. Samples were injected every 20 min, one by one.

For quantification of the MTX-PG resulting photolytic product, the excitation wavelength was set at 274 nm and 470 nm emission wavelength.

Record of the fluorometric signal was performed between 10 and 25 min.

Spectral identification involved comparing the respective excitation spectra specific to the MTX product after RBC extract photolysis and the MTX post-photolytic product in water.

Relevance in biological systems

A study group was set up of 32 psoriasis patients diagnosed according to clinical criteria, with a ca. 2 months history of weekly administration of low, stable doses of MTX (10 mg, median; range, between 7.5 and 15 mg). After patient consent, samples of EDTA whole blood were...
drawn right before administration of the patients’ weekly MTX dose.

Samples were next centrifuged for 10 min at 3000 g, and the RBC plasma was separated from the buffy coat. The RBC samples were further washed using saline (2 volumes), in two separate sessions. Before testing, the packed RBCs obtained were laid in store at -80°C. Plasma FP-GS was used for conversion and total MTX-PG concentrations were measured. Values thus obtained were compared with values of total MTX-PGs, obtained by calculating the sum of all individual MTX-PGs, using linear regression.

The RBC of EDTA-whole blood samples (10 mL) obtained from patients given a stable 10 mg weekly MTX dose was first divided and placed in two different tubes. A stability study was carried out on respective MTX-PGs.

RBCs placed in one of the tubes were isolated. In contrast, the other, whole blood containing tube was placed in a coolant specimen transportation system. After 48 h, the blood kept in the transportation system was used for separation of RBCs.

Finally, a comparison was performed between measures of MTX-PG concentrations in RBC after storage for 48 h in conditions simulating typical transport of samples and measures of MTX-PG concentrations in RBC taken right after sample collection.

Results and discussions

The chromatogram displaying a calibration solution in water including MTX-PGs 1-7 (in amount of 25 nmol/L for each species) shows the separated MTX-PG species (fig. 1).

Identical excitation spectrum can be observed in MTX-PGs 1-7 specific to the post-column photolytic product (fig. 2).

As evident from chromatograms illustrating blank RBCs and MTX-PG-enriched RBCs, the possibility exists to separate the MTX-PG species 1-7 involved from endogenous components of the RBC (fig 3, and 4).

A linear relationship was shown by calibration curves between concentration and peak area, characterised by correlation coefficients >0.99 for MTX-PGs 1-7.

As regards the inter-day CV involving a control MTX-PG mixture supplemented to the RBC hemolysate, the respective value was within the 4.5–6% range, considering a 25 nmol/L concentration, whereas the 50 nmol/L concentration yielded a value of 3.5–5.6%.

Mean extraction recoveries recorded for each MTX-PG were as follows for MTX-PG1 to MTX-PG7: 65% (MTX-PG1), 67% (MTX-PG2), 65% (MTX-PG3), 64% (MTX-PG4), 74% (MTX-PG5), 70% (MTX-PG6) and 62% (MTX-PG7). For each polyglutamate, the limit for detection was set at signal-to-noise ratio x 3, i.e. 2 nmol/L. MTX-PG1/MTX-PG 1

Relevance in biological systems at low weekly MTX doses

Figure 5 shows chromatograms performed on RBCs collected from psoriasis patient on MTX therapy.

As shown in the figure, MTX-PG1 and MTX-PG2 could not be detected in the samples. The total concentrations of long-chain MTX-PGs were found by calculating the sum of MTX-PGs 3-7. The specificity of this analytical method becomes evident from the comparison involving spectra
of excitation related to the MTX-PG photolytic products between patient samples on one hand and water, on the other hand (fig. 6).

Whole blood samples were collected from 32 subjects and a stability study was performed on MTX-PGs. It could be observed that values of MTX-PGs concentrations and total values of long-chain MTX-PGs concentrations remained unchanged at 48 h after collection and subsequent storage using a coolant transportation system. This remained unchanged at 48 h after collection and subsequent storage using a coolant transportation system. This was possible due to slope and coefficient correlation > 0.90 occurring between values of MTX-PGs concentrations assessed.

No difference could be observed as regards the distribution of MTX-PGs species in the RBC samples after 48 h following placement in the coolant system, in comparison with distribution of the same right after collection of samples.

The actual possibility to convert every MTX-PGs into MTX increases method sensitivity and renders it applicable to both adult and paediatric patient samples, in spite of the limited volume of blood involved in the latter case.

The method however is fraught with one potential disadvantage, relating to its incapacity to perform separation and quantification of long-chain MTX-PGs, known for their enhanced effects on MTX targets [15, 19, 20]. That is why, a different approach was attempted, involving direct assessment of MTX-PGs species in their entirety.

An additional advantage of this method is its simplicity, because it only consists of mere RBC matrix de-proteinisation by H2O2, followed by direct extract feeding into the chromatographic system. If compared 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 [16].

The approach has been used on psoriasis patients on weekly, stable 10 mg MTX dose.

In order to ascertain the practical applicability of this method, MTX-PGs have been subject to a stability study conditions simulating specimen transportation to the laboratory from the collection site.

Data obtained confirm the stability of MTX-PGs and the preservation of the total RBC without alteration of either MTX-PG concentrations or MTX-PG species distribution pattern following storage for 48 h in a cooling system of transportation provided with a cold pack.

Since circulating erythrocytes are lacking in activity of FP-GS, MTX-PGs forms derive from medicine incorporation into erythrocytes precursors. RBC MTX-PG concentrations may be assumed to have representative value for other MTX-PG concentrations, present in tissue areas allowing less access [21, 22].

Conclusions

To conclude, taking into account the importance of MTX as first line treatment for patients with psoriasis, the method above can be helpful in actual practice for effective and effortless MTX dosing as well as for reduction of potential adverse reaction to a minimum in many biological systems.

References


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Fig.6. Chromatogram with separation of MTX-PGs