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Determination of methotrexate and its metabolite 7-hydroxymethotrexate by direct injection of human plasma into a column-switching liquid chromatographic system using post-column photochemical reaction with fluorimetric detection

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Abstract

A simple, sensitive and fully automated column-switching system by direct injection of plasma samples for determination of methotrexate and its metabolite 7-hydroxymethotrexate was developed. The system utilized a C_8 alkyl-diol silica precolumn coupled with a LiChrospher RP-18 analytical column, followed by a photoreactor and fluorimetric detection. The photo-oxidative irradiation was accomplished at UV 254 nm in the presence of 0.1% hydrogen peroxide in the eluent. Studies showed that the fluorimetric response was influenced by the reaction time, the degree of the reactor's transparency and the choice of the working wavelengths. By optimizing the content of acetonitrile in the eluent, methotrexate can be separated from 7-hydroxymethotrexate completely. The method validation revealed quantitative recoveries (≥94%) with coefficients of variation ≤4.4%. The limits of detection and quantitation for determination of methotrexate were 0.20 and 0.36 ng, respectively, corresponding to 2.0 and 3.6 ng/ml for an injection volume of 100 μl. It was possible to enhance the sensitivity further by injecting larger plasma volumes, up to 500 μl.

Keywords: Methotrexate; 7-Hydroxymethotrexate; 4-Amino-10-methylfolic acid

1. Introduction

Methotrexate (4-amino-10-methylfolic acid) (MTX) in high doses (>3 g/m^2) has been widely used as a cytostatic agent in oncology since 1953 [1]. Recently, low-dose MTX therapy (5–10 mg/m^2) was found to be very effective for rheumatoid arthritis [2,3]. Both pharmacokinetic studies and biological monitoring require the development of more sensitive but less laborious and, ideally, fully-automated methods for determination of MTX and its metabolite. All published chromatographic methods for determining MTX and its metabolites in plasma or serum with a detection limit of ≤10 ng/ml, can be classified into two types: (1) conventional LC methods with sample pretreatment steps such as liquid–solid phase extraction or (2) deproteinization [4–7] and direct injection techniques [8–11]. Direct injection techniques are generally preferable, since problems involved in off-line sample pretreatments, such as time consuming procedures, errors and risk for low recoveries, can be

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readily avoided. However, published methods with direct injection techniques, including a replaceable guard column inserted before the analytical column [8,9] and a column-switching system [11], could only endure the injection of ≤100 μl plasma (≤5 ml in total volume) because of the use of conventional reversed-phase material as precolumns. Micellar liquid chromatography [10], another kind of direct injection technique, also limited the injection volume, i.e. 20 μl, and experienced deteriorated chromatographic efficiency. With these limitations, improving the detection limit by increasing the injection volume and developing a fully automated system was beyond reach.

In terms of sensitivity, the fluorescence polarization immunoassay (TDx) also has a low detection limit (5 ng/ml). But it enables determination of MTX only within a narrow linear range (10-fold) and suffers from a poor selectivity (cross-reaction with 7-hydroxymethotrexate (7-OH-MTX)) [5,12,13].

Alkyl-diol silica (ADS) packing, a type of restricted-access media, has been found to be one of the suitable precolumn materials for direct injection of biological fluids regarding its life span (500-μl injection, ≥50 ml of plasma in total volume) [14–16] and the on-line clean-up effect as demonstrated in a system employing UV detection at 210 nm [14]. Therefore, ADS C_8 as the precolumn and LiChrospher RP-18 as the analytical column were chosen, coupled via a six-port switching valve in a back-flush mode, as described earlier [17]. Furthermore, taking the advantage of using more sensitive and selective fluorimetric detection as described by Salamoun et al. [9,18], a more advanced method could be created.

In this paper, a sensitive method for determination of MTX and 7-OH-MTX in plasma is described combining the column-switching system [17] for the direct and repeated injection of plasma with the post-column photochemical reaction followed by fluorimetric detection. Conversion of non-fluorescent MTX and its metabolite 7-OH-MTX into fluorescent products was achieved in a post-column mode by photo-oxidizing the analytes at 254 nm in the presence of hydrogen peroxide [9,18]. Superior to systems using conventional silica packings [9,11,18], the peak performance and the back pressure on both precolumn and analytical column of our system were constant during the whole study period. Besides, the system is simpler and more compact compared with Okuda's system dealing with three pumps plus gradient desorption in a heart-cut mode [11].

The optimization of the photochemical reaction, the chromatographic selectivity and the method validation are discussed in this paper.

2. Experimental

2.1. Reagents and materials

Hydrogen peroxide (30 wt.% solution in water) of analytical grade was obtained from Merck (Darmstadt, Germany). Methotrexate (MTX) was purchased from Sigma (St. Louis, MO, USA). 7-Hydroxymethotrexate (7-OH-MTX) was received as a gift from the German Cancer Research Centre (Heidelberg, Germany). Other reagents and materials were the same as described previously [17].

2.2. Preparation of standards and plasma samples

Similar to MTX, the stock and standard solutions of 7-OH-MTX were kept in plastic micro test-tubes to avoid adsorptions to glassware and were covered with aluminium foil to protect from light [17]. Blank human plasma was obtained from the Blood Bank (University Hospital, Munich, Germany) and patient plasma samples were received from the Institute of Clinical Chemistry (University Hospital, Munich, Germany). Both were stored at −20°C until analysis. Spiked plasma, made by mixing the working standard with the blank plasma (<10% dilution factors), and patient plasma were prepared daily and were centrifuged for 5 min at 4000 *g* prior to injection.

2.3. Chromatographic instrumentation and conditions

The column-switching system was similar to that described previously [17], except that the UV detector part was replaced by a photoreactor (PR) (Knauer, Berlin, Germany) and a F-1050 (or F-1080) fluorescence detector (FD) (Merck). The photochemical reaction was accomplished in the photoreactor irradiating at 254 nm in the presence of 0.1%

hydrogen peroxide. The reactor coil was a tefzel capillary tubing (108 cm in length, 0.5 mm I.D. and 1.6 mm O.D.) coiled around the UV lamp tube and inserted between the outlet of the analytical column and the inlet of the detector. The fluorimetric detection was set at 370 nm (excitation) and 417 nm (emission), respectively. A 100-μl volume was injected into the system unless otherwise stated.

The mobile phases (1 and 2) were the same as in the previous system [17]. However, the photochemical reagent, i.e. 0.1% (v/v) hydrogen peroxide, was added to the second mobile phase under a helium flow for 3–5 min. It was necessary to prepare the second mobile phase daily and to store in a brown bottle.

3. Validation parameters

3.1. Quantitation

Peak areas were used for the quantitation. Calibration curves were constructed by plotting the peak areas against the concentrations of the analytes.

3.2. Limit of detection/quantitation

According to the ACS Committee on Environmental Improvement [19], the limit of detection/quantitation is defined as:

$$S_t - S_b \geq K_d \sigma$$

where S_t is the observed signal (gross signal), S_b is the field blank, K_d is the criterion and σ is the variability of the blank. For the limit of detection (LOD), the minimum value of $K_d = 3$; whereas for the limit of quantitation (LOQ), $K_d = 10$. The mobile phase 2 was used as the blank injected into the analytical column 10 times sequentially. S_b and σ were calculated by setting the detector at a high sensitivity at the region where the MTX peak was expected. The LOD and LOQ, expressed in concentration units, were derived from the calibration function.

4. Results and discussion

4.1. Optimization of the photochemical reaction

It was presumed that MTX and its metabolite 7-OH-MTX underwent the course of photo-oxidation in analogy to folic acid (Fig. 1) [9,18,20]. The amount of oxidizer, the irradiation time and the quality of the reactor determined the outcome of the photoreaction. Only MTX was focused on in studies on the photochemical reaction, since the relationship for the fluorescent response between the parent compound and its metabolite 7-OH-MTX has been investigated [18]. No native fluorescence for either of the analytes was observed (see below), the peak

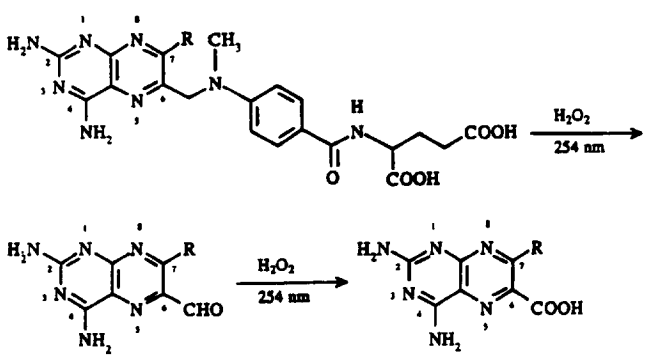

Fig. 1. Photo-oxidation of MTX and 7-OH-MTX.

height for MTX was, therefore, used as the parameter in the optimization of the derivatization.

4.1.1. Concentration of hydrogen peroxide

Initially, a T-coupling was placed between the reactor and the outlet of the analytical column to deliver the oxidizer by using an extra pump. However, it was difficult to generate a constant peak performance because of a variable reaction yield at a lower flow-rate (≤0.3 ml/min) and broadening of the peak at a higher flow-rate (≥0.5 ml/min). Furthermore, it was found that neither the retention time nor the chromatographic performance related to the direct injection technique, was affected by the addition of hydrogen peroxide in the mobile phase used for the analytical column. Since an increase of the oxidizer from 0.1% to 0.5% in the eluent only enhanced the detection response to a small extent (~5%), 0.1% hydrogen peroxide was chosen for keeping the concentration of mobile phase additives as low as possible to avoid stability problems of the chromatographic system. The 0.1% hydrogen peroxide was added to the mobile phase directly, under a helium flow to remove the dissolved oxygen.

4.1.2. Irradiation time

The irradiation time was varied by means of changing the reactor length; 1 cm of capillary reactor corresponded to 0.12 s of irradiation time. Fig. 2 shows the effects of reaction time on the yield of the photochemical reaction by injecting the standard MTX into the analytical column. The peak height increases initially with increasing reaction time and at 12.6 s the peak height reaches the apex corresponding to 108 cm of effective reactor length. Overextension of the reaction time may bring about changes in the chemical properties of the fluorophore, resulting in either wavelength shifts or diminishing fluorescent intensity. This may explain the decreasing response observed at the higher irradiation time. Longer capillaries had no influence on bandbroadening within the studied range.

4.1.3. Material of reactor

Since the fluorescent derivatization depends upon the irradiation with light at 254 nm, a more transparent reactor apparently gives higher energy. A comparison was made between the materials of teflon and tefzel. The fluorescent intensity increased about 25% by using the reactor made of tefzel, which then was used for the remaining experiments.

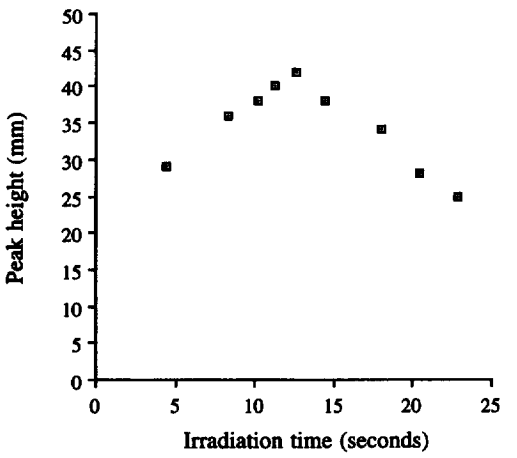

Fig. 2. Influence of irradiation time on the fluorimetric response of MTX. Single column LC system; column, LiChrospher RP-18, 5 μm, 125×4 mm I.D.; mobile phase, 0.1% H_2O_2 in 5 m*M* tetrabutylammonium with 18% acetonitrile + phosphate buffer pH 7.4 (I=0.05); fluorimetric detection, excitation 370 nm, emission 415 nm; reactor, teflon material, 37–195 cm in length and 0.5 mm I.D.

4.2. Choice of wavelengths

The choice of wavelengths is the main determinant for the sensitivity and selectivity of the method. The detection response increased about 20% by increasing the emission wavelength from 415 to 417 nm, keeping the excitation wavelength at 370 nm. An even more pronounced effect (74%) was obtained by changing the excitation wavelength from 370 to 282 nm with emission wavelength at 417 nm. Unfortunately, the background fluorescence observed after the direct injection of plasma limited the use of 282 nm as the excitation wavelength. The higher wavelength had to be used for excitation (370 nm), and the emission at 417 nm was measured.

4.3. Separation of MTX and 7-OH-MTX

The retention behaviour of MTX has been studied previously [17]. The metabolite 7-OH-MTX seems to be more hydrophobic than the parent compound [4], due to the formation of intramolecular hydrogen

bonding (see the structure in Fig. 1) and is stronger retained by the given mobile phase. Increasing the organic modifier decreases the separation factor, as shown in Fig. 3. Complete separation ($R_s \geq 1.5$) was attained with a mobile phase containing 15% acetonitrile. The choice of the content of the organic modifier in the mobile phase depends upon the analytes of interest. If only the parent compound needs to be analyzed, 18% of acetonitrile in the mobile phase may be used in order to gain a shorter assay time. Representative chromatograms demonstrating the separation of MTX and 7-OH-MTX in a standard solution and a patient plasma sample, respectively, with 15% acetonitrile in the mobile phase are shown in Fig. 4. The clean chromatogram from the patient sample emphasises the merit of fluorimetric detection on selectivity of the method.

4.4. Stability of fluorimetric response

As described earlier [17], it seemed that no degradation process occurred for MTX under the conditions used with UV detection. However, varied chromatographic performance of both MTX and 7-OH-MTX, with either patient plasma samples or standard solutions, was noticed under fluorimetric detection. Table 1 shows that peak areas of MTX and 7-OH-MTX decreased after storage in plasma

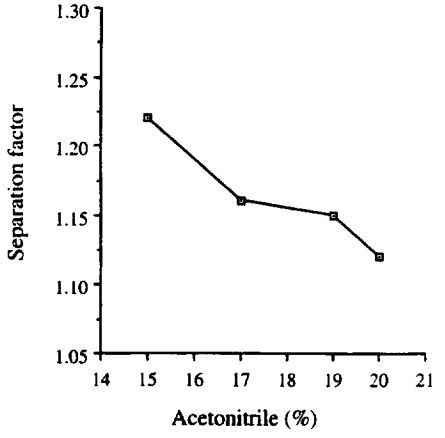

Fig. 3. Dependence of separation of MTX and 7-OH-MTX on the content of the organic modifier. Mobile phase: 0.1% H_2O_2 in 5 m*M* tetrabutylammonium with acetonitrile + phosphate buffer pH 7.4 ($I = 0.05$). Separation factor $(\alpha) = k'_{7\text{-OH-MTX}}/k'_{\text{MTX}}$. For conditions see Fig. 2.

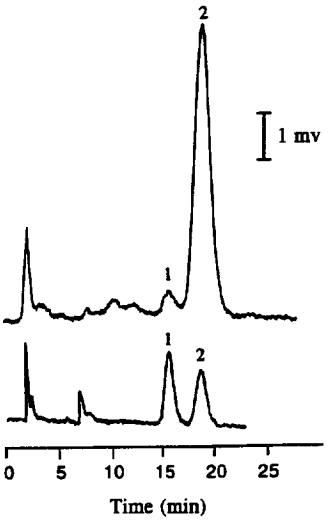

Fig. 4. Separation of MTX and 7-OH-MTX. (Upper) Patient plasma sample with 8.2 ng/ml of MTX and 79 ng/ml of 7-OH-MTX. (Lower) Standard solution of 19 ng/ml of MTX and 11 ng/ml of 7-OH-MTX. Peaks: 1 = MTX, 2 = 7-OH-MTX. Mobile phase: 0.1% H_2O_2 in 5 m*M* tetrabutylammonium with 15% acetonitrile + phosphate buffer pH 7.4 ($I = 0.05$); other conditions as described in Section 2.3.

for ≥4 days in the plasma matrix, although samples were carefully wrapped with aluminium foil and kept at −20°C. The metabolite was affected more than the parent compound; no extra peaks appeared, however, on the plasma chromatograms. This may be explained by a quenching effect developed in the complex plasma matrix during storage. Such problems can be avoided by preparing the standard solutions daily and analyzing the patient samples directly. Herein, the direct injection technique reveals its advantage in dealing with samples with such problems. Storage of samples at lower temperature, i.e. −70°C, may be a remedy which has to be studied. A degradation product was found from the

Table 1
Influence on peak areas of MTX and 7-OH-MTX after storage of plasma samples

Patient No.	Storage days	Reduced peak area(%)[a]	
		MTX	7-OH-MTX
1	4	<1	6
2	12	60	80

[a] Relative values calculated from the primary injection.

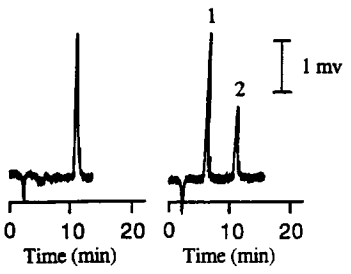

Fig. 5. Degradation of the standard MTX in the course of storage. (Left) Freshly prepared solution of MTX (45 ng/ml); (Right) the same solution stored 8 days. Peaks: 1=unknown, 2=MTX. Fluorimetric detection: F-1080; reactor: teflon material, 105 cm × 0.5 mm I.D.; other conditions as described in Section 2.3.

standard solution of MTX after storage for 8 days at −20°C, see Fig. 5. The reason for the instability is unknown. However, the presence of dissolved oxygen in the standard solution during the storage may cause MTX to decompose. A similar observation was reported by Salamoun [18].

Table 2
Calibration curves[a] of MTX

	Concentration (ng/ml)	Equation	Correlation coefficient
Eluent	4.09–1305	$y=0.088+0.402x$	0.996
Plasma	4.09–1305	$y=-0.180+0.417x$	1.000

[a] Eight different concentrations with triplicate injections were studied.

4.5. Method validation

Only MTX was involved in the following studies with the eluent containing 18% acetonitrile as the organic modifier.

4.5.1. Linear range and calibration

No deviation from linearity for MTX was obtained in the range from 4.5 ng/ml to 2.27 μg/ml of MTX with $r=0.998$. Representative calibration curves in two different media are given in Table 2.

4.5.2. Reproducibility

Table 3 summarizes the studies on recovery and precision of MTX assays. Quantitative recoveries calculated from the standard curve in plasma for intra- and inter-day with C.V.≤4.4% illustrate the reliability of the method.

4.5.3. Sensitivity of the method

The values of LOD and LOQ for MTX obtained by this method were 0.20 and 0.36 ng, respectively, referring to the injection volume of 100 μl. The results shown on both calibration curve and recovery at the spiked level of 4.54 ng/ml of MTX (see Tables 2 and 3) agree with the level of LOQ. The possibility of further improving sensitivity by increasing the injection volume up to 500 μl was tested. Fig. 6 indicates that the sensitivity of MTX was enhanced about five-fold applying a five times larger injection volume. About ten times higher sensitivity was gained by using fluorimetric detection instead of UV absorption [17].

Table 3
The intra- and inter-day precisions and recoveries of MTX in plasma samples

Spiked concentration (ng/ml)	Found concentration (ng/ml)	Recovery (%)	C.V. (%)	Number	Days
Intra-day					
4.54	4.36	96.1	3.9	8	
90.8	91.6	100.8	1.5	8	
908	941.0	103.6	1.3	6	
Inter-day					
4.54	4.27	94.1	4.4	16	10
90.8	93.8	103.4	1.4	14	8
908	938.1	103.3	1.8	12	8

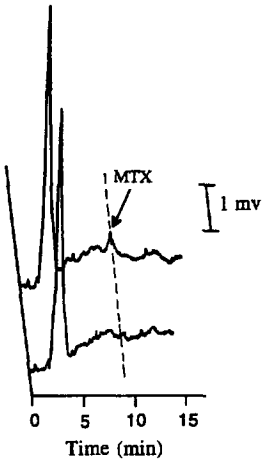

Fig. 6. Chromatogram of MTX with 500-μl injection. (Upper) Spiked plasma with 0.75 ng/ml of MTX; (Lower) blank plasma. Conditions as described in Section 2.3.

In addition, when the blank and spiked plasma samples (960 ng/ml of MTX and 800 ng/ml of 7-OH MTX) were monitored with the reactor lamp off, twin blank chromatograms were obtained, illustrating the non-fluorescent properties of both analytes.

5. Conclusions

A column-switching system for direct determination of MTX and 7-OH-MTX in plasma, using photochemical reaction with fluorimetric detection, is demonstrated. The method has the following features:

1. The direct injection of plasma by means of a column-switching system using a precolumn of restricted-access media is a fast and reliable technique for determination of MTX and its metabolite in plasma.
2. Post-column fluorescent derivatization is an effective way to increase the sensitivity and selectivity of the method.
3. Adding the oxidizer (H_2O_2) into the eluent avoids possible interferences due to the excess reagent and extra band-broadening caused by an additional pump.
4. The method validation demonstrates a broad linear range ($\geq$500 times) and high recovery $\geq$94% (C.V.$\leq$4.4%) with low limits of detection (0.20 ng) and quantitation (0.36 ng). However, there is a need for further studies on the influence of storage of the biological material on the fluorescence yield of the photochemical reaction.

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References

[1] W.A. Blayer, Cancer, 41 (1978) 36.
[2] D.E. Furst and J.M. Kremer, Arthritis Rheum., 31 (1988) 305.
[3] M.E. Weinblatt, D.E. Trentham, P.A. Fraser, D.E. Holdsworth, K.R. Falchuk, B.N. Weissman and J.S. Coblyn, Arthritis Rheum., 31 (1988) 305.
[4] U.R. Tjaden and E.A. De Bruijn, J. Chromatogr., 531 (1990) 235.
[5] Y.Y.Z. Farid, I. Watson and M.J. Stewart, J. Pharm. Biomed. Anal., 1 (1983) 55.
[6] F. Palmisano, T.R.I. Cataldi and P.G. Zambonin, J. Chromatogr., 344 (1985) 249.
[7] N. So, D.P. Chandra, I.S. Alexander, V.J. Webster and D.W. O'Gorman Hughes, J. Chromatogr., 337 (1985) 81.
[8] H. Breithaupt, E. Küenzlen and G. Goebel, Anal. Biochem., 121 (1982) 103.
[9] J. Salamoun and J. Frantisek, J. Chromatogr., 378 (1986) 173.
[10] F. Palmisano, A. Guerrieri, P.G. Zambonin and T.R.I. Cataldi, Anal. Chem., 61 (1989) 946.
[11] T. Okuda, M. Motohashi, I. Aoki and T. Yashiki, J. Chromatogr. B, 662 (1994) 79.
[12] M. Roach, P. Gozel and R. Zare, J. Chromatogr., 426 (1988) 129.
[13] L. Slordal, P.S. Prytz, I. Pettersen and J. Aarbakke, Ther. Drug Monit., 8 (1986) 368.
[14] Z. Yu and D. Westerlund, J. Chromatogr. A, 725 (1996) 149.

[15] K.-S. Boos, A. Rudolphi, S. Vielhauer, A. Wolfort, D. Lubda and F. Eisenbeiß, Fresenius J. Anal. Chem., 352 (1995) 684.
[16] S. Vielhauer, A. Rudolphi, K.-S. Boos and D. Seidel, J. Chromatogr. B, 666 (1994) 315.
[17] Z. Yu and D. Westerlund, J. Chromatogr. A, 742 (1996) 113.
[18] J. Salamoun, M. Smrz, F. Kiss and A. Salamounova, J. Chromatogr., 419 (1987) 213.
[19] ACS Committee on Environmental Improvement, Anal. Chem., 52 (1980) 2242.
[20] R.L. Blakley, The Biochemistry of Folic Acid and Related Pteridines, North-Holland, Amsterdam, 1969.