THE ROLE OF 7-HYDROXYMETHOTREXATE DURING METHOTREXATE ANTI-CANCER THERAPY

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SUMMARY

Plasma concentrations of 7-hydroxymethotrexate were measured in 10 individuals receiving methotrexate, 8 of them per infusion. After the end of infusion, the plasma concentration of formed 7-hydroxymethotrexate fell less rapidly than that of methotrexate. At 24 h after infusion, plasma concentration ratios of 7-hydroxymethotrexate ranged from 30 to 1 (n = 8). The presence of 7-hydroxymethotrexate was shown to influence the transport of methotrexate into Ehrlich ascites tumor cells and human KB cells, cultured in vitro. These findings suggest that monitoring of 7-OHMTX is important in MTX treatment.

INTRODUCTION

The use of methotrexate (MTX) against malignant neoplastic disorders and several of its pharmacological properties has been reviewed by Bleyer [2] and Shen and Azarnoff [13]. Different metabolic products of MTX have been found in animals [1,6,7,15]. Jacobs [5] reported the occurrence in man of 7-hydroxymethotrexate (7-OHMTX) as a major metabolite after administration of high doses of MTX (more than 50 mg/kg), suggests that production of 7-OHMTX occurs exclusively at high doses of MTX.

Quantitative measurement of 7-OHMTX has been carried out using DEAE cellulose chromatography. High pressure liquid chromatography (HPLC) is a

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more convenient tool for this purpose, resulting in better resolution of chromatographic peaks with inherent lower detection limits. Determination of plasma concentrations of MTX and 7-OHMTX after high-dose MTX treatment has been reported by Watson et al. [17]. The detection limit was $10^{-7}$ M. These investigators used an extraction step for sample clean-up; however, extraction of MTX and 7-OHMTX is rather tedious due to the polar character of both compounds. The method used here had a detection limit of $2 \times 10^{-5}$ M, which allows measurement of 7-OHMTX after relatively low doses of MTX, and sample clean-up involved only a deproteinization step. Two-column HPLC has been chosen for this purpose [8,9]. By this method, plasma concentrations of 7-OHMTX in different ranges for different MTX dosage schedules have been measured in 10 individuals.

MATERIALS AND METHODS

Analytical procedure

Deproteinization of plasma samples using trichloroacetic acid is followed by centrifugation. As much as 2 ml of the clear supernatant could be injected into a reverse phase column (RP8, Merck). After elution with 10 ml of tap water, 7-OHMTX is concentrated in the top part of the column. 7-OHMTX is subsequently backflushed using a phosphate buffer (0.05 M, pH 4.9)/methanol eluent (4:1, v/v) and eluted over a chemically bonded anion-exchange resin (Partisil SAX, Whatman). UV absorption of 7-OHMTX is monitored at 306 nm. Chromatographic conditions and the equipment used were identical to those described before for the determination of methotrexate [8]. A typical chromatogram is shown in Fig. 1.

The percentage recovery of 7-hydroxymethotrexate and methotrexate

Fig. 1. Chromatogram of methotrexate and metabolite: injection volume 1 ml; plasma metabolite concentration $1.7 \times 10^{-1}$ M. For other chromatographic conditions: see text.
when these substances are added to normal human plasma were 65.1 and 70.2, respectively.

Identification of the metabolite peak

Because no standard sample of 7-hydroxymethotrexate was available, the metabolite peak (Fig. 1) was isolated and structure of the compound identified as that of 7-OHMTX by NMR spectroscopy and emission controlled field desorption mass spectrometry [12].

Preparation of the metabolite

A rabbit liver (80 g) was homogenized in 1.15 M KCl. The total volume of the homogenate was 120 ml. Per 100 ml, 50 mg MTX was dissolved in 100 ml medium as described by Johns [6] and incubated with 20 ml of the homogenate at 37°C with agitation. After 1 h another portion of 20 ml of the homogenate was added. Total incubation time was 4 h. The reaction was stopped by adding 100 ml of trichloroacetic acid in water (10%, w/w) while vortexing. After centrifugation, the clear supernatant was fractionated on a column packed with silanized silica (Si60, Merck) and rinsed with water. The metabolite was seen as a yellow band at the beginning of the column. This band was eluted by 0.1 N HCl in methanol and collected. The methanol was evaporated and the residue neutralized to pH 7.0 and brought onto an Amberlite anion-exchange column (type IRA 47-C, BDH Chemicals). The band containing the metabolite was eluted with 0.5 N HCl and collected again. Elution on the reverse phase column was repeated to concentrate the metabolite-containing fraction and neutralized. The metabolite contained less than 1% MTX.

Further details of the identification and preparation procedures are available on request.

Cell cultures

Ehrlich ascites tumor cells were grown in suspension according to Van Venrooy et al. [16]. The final suspension contained 10⁶ cells/ml. KB cells were grown in suspension culture as described by Doerfler [3] and were used in a final concentration of 3 × 10⁶ cells/ml. For the stability of folate analogues, the medium contained 0.1% ascorbic acid (w/w) during the experiment. During the incubation, samples of 5 ml were taken in triplicate from the mixture at the times indicated and collected in 2 ml Eppendorf vials. After centrifugation for 5–10 s at 10,000 g, the supernatant was removed and the cells were washed twice with ice-cold isotonic buffer (triode solution as described by Paul [11]). Finally, the cells were dissolved in 0.5 ml of a solution of 0.5% deoxycholate in water.

Chemicals

All chemicals used were of analytical grade. HMTX was obtained from Amersham. MTX and leucovorin were both used as the commercially available products from Lederle and 5-methyltetrahydrofolic acid was obtained from Sigma.
RESULTS

Plasma concentrations after bolus injections of 7-OHMTX and MTX

Shown in Fig. 2 are concentrations after an i.v. injection of 2.2 mg 7-OHMTX in a volunteer, indicating a plasma half-life of 9 h. The plasma half-life of MTX in a period of 15 h after the end of infusion or after bolus injection has been reported to be less than 4 h [2].

Figure 3 shows the 7-OHMTX formation after i.v. bolus injection of 25 mg of MTX in another volunteer. In this case, equal concentrations are shown after 10 h for both MTX and 7-OHMTX.

Plasma concentrations after infusions of MTX

In 8 patients, plasma concentrations of MTX and 7-OHMTX have been measured during and after MTX infusion. From the first to the second infusion, a stepwise rise in the steady state concentration was observed. This level was maintained during the third infusion. The plasma concentrations are represented in Table 1.

In Fig. 4, an example of plasma concentrations of MTX and 7-OHMTX of a patient are given for three subsequent infusions with an interval of 3 days. After the end of infusion, 7-OHMTX appeared to be excreted slower than MTX, resulting in higher plasma concentrations for 7-OHMTX. In the patients of Table 1 at 24 h after the end of infusion, plasma concentration ratios of 7-OHMTX to MTX ranged from 30 to 1.

![Graph](image.png)

Fig. 2. Plasma concentrations of 7-OHMTX after i.v. bolus injection of 2.2 mg of 7-OHMTX in a volunteer.
METHOTREXATE IN MAN

25 mg i.v.

Fig. 3. Plasma concentrations of MTX and 7-OHMTX after an i.v. bolus injection of 25 mg MTX in a volunteer, showing the formation of 7-OHMTX. The half-life time of 2 phases of plasma disappearance has been indicated for MTX.

Influence of 7-OHMTX on the intracellular concentration of MTX

7-OHMTX is reported to be less active as an inhibitor of mammalian dihydrofolate reductase [5]. The relatively high plasma concentration of 7-OHMTX after the end of infusion might affect the transport of MTX into the cell. The presence of leucovorin and 5-methyltetrahydrofolate acid, which are both present in plasma during leucovorin rescue therapies [10], were shown to have a similar effect. In order to investigate whether 7-OHMTX influences the MTX amount in cells, KB cells and Ehrlich ascites tumor cells were incubated with $^3$HMTX and after 50 min, 7-OHMTX was added to the medium. The intracellular amount of radioactivity is plotted vs. time in Fig. 5. A significant decrease in the amount of intracellular MTX is shown. Repeating this experiment with Ehrlich ascites cells produced the same result. This finding can be explained as a continuation of passive transport out of the cell, while the transport of MTX into the cell is decreased. Assuming the steady state level of $^3$HMTX, reached after the addition of excess
<table>
<thead>
<tr>
<th>Dose of MTX</th>
<th>Range of plasma concentrations during MTX infusion</th>
<th>Interval between 2 sequential infusions (days)</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTX</td>
<td>1st infusion</td>
<td>2nd and 3rd infusion</td>
</tr>
<tr>
<td>25 mg/day × 7 days</td>
<td>2–4 × 10^{-9} M^a</td>
<td>6–8 × 10^{-6} M^a</td>
<td>1–3 × 10^{-7} M^a</td>
</tr>
<tr>
<td>240 mg/m^2 × 1 day</td>
<td>3–7 × 10^{-6} M^a</td>
<td>6–9 × 10^{-7} M^a</td>
<td>1–4 × 10^{-4} M^a</td>
</tr>
<tr>
<td>950 mg/6 h</td>
<td>3 × 10^{-6} M^b</td>
<td>6 × 10^{-4} M^b</td>
<td></td>
</tr>
</tbody>
</table>

^a Steady state concentration.

^b Maximum concentration.
Fig. 4. Plasma concentrations of MTX and 7-OHMTX in a patient treated for head and neck cancer. Interval between infusions 3 days, weight and length of the patient: 91 kg and 177 cm.
Fig. 5 (a) Fractional amount of intracellular \(^{3}\text{H}\text{MTX}\) in KB tumor cells, which were grown in suspension. At \(t = 0\) min \(^{3}\text{H}\text{MTX}\) was added to the medium (conc. \(10^{-5}\) M), at \(t = 50\) min, 7-OHMTX was added to one half of the suspension, (conc. \(2 \times 10^{-5}\) M) the other half was used as a control. The standard deviation has been given (\(n = 3\)). (b) The effect of 7-OHMTX, 5-methyltetrahydrofolic acid and leucovorin in equimolar concentrations (\(2 \times 10^{-5}\) M) on intracellular concentration of \(^{3}\text{H}\text{MTX}\) in KB cells.
7-OHMTX to the medium, represents the tight binding of MTX to dihydrofolate reductase [14], the dihydrofolate reductase binding equivalence is 0.7 pmol of MTX/10^6 cells. This is in the same order of magnitude as that measured for other cell lines [14]. The effect of an equal concentration of leucovorin and 5-methyltetrahydrofolic acid was the same (Fig. 5).

**DISCUSSION**

The measurement of plasma concentrations of 7-OHMTX during MTX dose schedules, other than the high-dose treatment, appeared to be possible by the relatively low detection limit of the two-column method. These lower concentrations were not reported before. It was even suggested that formation occurs exclusively at high-dose treatment with MTX [5].

In our study, we chose for multiple sampling of relatively few patients in order to follow the concentrations of 7-OHMTX carefully. Therefore, the reported data obtained by the described method do not pretend to be a pharmacokinetic study with a statistical evaluation of the measured parameters. However, in all patients who received MTX infusions (in total, 8), much higher ratios of 7-OHMTX to MTX were found in plasma after the end of infusion.

This report emphasizes the influence of transport of MTX into the cell by the presence of relatively high concentrations of 7-OHMTX as was shown with 2 cultured cell lines. This may cause an increase in rescue of tissues from MTX. On the other hand, the transport of leucovorin and 5-methyltetrahydrofolic acid, which are present during leucovorin rescue, into the cell may also be decreased by the presence of 7-OHMTX. The overall effect would be tissue dependent. The stepwise rise in plasma concentrations of 7-OHMTX was observed in all 7 patients during three sequential MTX infusions. This can be explained as a stimulation of the hydroxylating enzyme system.

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**REFERENCES**