Cancer Chemother Pharmacol (1982) 9: 65–69

Cancer Chemotherapy and Pharmacology

Pharmacokinetics of Erythrocyte Methotrexate After High-Dose Methotrexate

Andreas Schalhorn[1], Hansjörg Sauer[1,2], Wolfgang Wilmanns[1,2], and Gabriele Stupp-Poutot[1]

[1] Medizinische Klinik III, Klinikum Großhadern, Ludwig-Maximilians-Universität, Marchioninistrasse 15, D-8000 München 70
[2] Department of Clinical Haematology, Institute of Haematology of the Gesellschaft für Strahlen- und Umweltforschung, Landwehrstrasse 61, D-8000 München 2, Federal Republic of Germany

Summary. *Erythrocyte methotrexate (MTX) concentrations were determined in 10 patients with metastatic osteogenic or soft tissue sarcoma after 52 cycles of high-dose methotrexate (HDMTX). In contrast to serum MTX, pharmacokinetics of erythrocyte MTX showed three distinct phases: A rapid decrease to a nadir 2–3 days after MTX was followed by a significant rise of erythrocyte MTX until days 10–14. Subsequently there was a third phase, with a definite decrease of erythrocyte MTX concentrations with half-lives of 30–40 days. Short-term repititions of HDMTX had considerable influence on the first two phases of the kinetics. Each curve surpassed that of the previous therapy, and erythrocyte MTX concentrations increased continuously to the values measured at the end of the HDMTX infusion.*

The following mechanism of MTX enrichment in erythrocytes is discussed: In erythro- and normoblasts MTX is converted to polyglutamate forms, which are retained inside the cell and are probably the reason for the relatively high sensitivity to MTX. Upon resumption of erythropoiesis the release of freshly prepared erythrocytes containing MTX and predominantly MTX polyglutamates causes the renewed increase in blood MTX levels.

Introduction

According to cell kinetic studies (P. Dörmer et al. 1981, unpublished work) and bone marrow differentials [26], high-dose methotrexate (HDMTX) impairs erythropoiesis to a much greater extent than granulocytopoiesis. This was confirmed by ferrokinetic studies during/after HDMTX [23]. At MTX serum concentrations between 4×10^{-8} *M* and 2.7×10^{-7} *M*, plasma iron turnover and ^{59}Fe utilization were depressed to considerably below the normal range in all cases investigated. In spite of normal MTX clearance and normal citrovorum factor rescue (CFR), erythropoiesis did not return to normal until the MTX serum levels had fallen to or below 4×10^{-8} *M* [23].

Significant erythrocyte MTX concentrations have been reported both after HDMTX and after long-term treatment with low-dose MTX [2, 11]. The purpose of this study was to investigate whether the pharmacokinetics of erythrocyte MTX might explain the high sensitivity of human erythropoiesis to HDMTX.

Materials and Methods

Patients and HDMTX Regimens. Comparative pharmacokinetics of serum and erythrocyte MTX have so far been estimated after 52 cycles of HDMTX in 10 adult patients with osteogenic sarcoma or metastatic soft tissue sarcoma. HDMTX was given according to Jaffe [14]: MTX was administered as a 6-h intravenous (IV) infusion at a conventional dosage of 8–12 g/m^2. Some 2–18 h after the completion of the MTX infusion, CFR was started at a dosage of 15 mg and repeated every 6 h until MTX serum concentrations had declined to 5×10^{-8} *M*. IV hydration and alkalinization was started 12 h prior to HDMTX, and special care was taken to ensure a 24 h urine output of at least 3,000 cm^3 and to maintain the urinary pH above 7.0 [18]. More detailed information on HDMTX/CFR has been published elsewhere [6, 7, 15, 22]. MTX serum clearance was normal in all cases, and apart from transient elevations of SGOT and SGPT in some cases, toxic side-effects were not observed.

MTX Serum Concentrations. MTX serum concentrations $\geq 3 \times 10^{-7}$ *M* were measured by an enzyme immunoassay [19, 24]. At lower concentrations MTX levels were assayed enzymatically according to the principles developed by Werkheiser [24, 28, 29] or by a radioimmunoassay using ^{125}I-MTX as competitive substrate for the MTX antibodies [17]. Using solutions with known quantities of hydroxy-MTX (HO-MTX), the assays showed cross-reactivities below 2%. Therefore, HO-MTX could be disregarded and the values taken to reflect the actual MTX serum concentrations.

MTX Erythrocyte Concentrations. Anticoagulated blood (2–4 ml) was immediately chilled, and erythrocytes were washed four times with four to five volumes of physiological saline at 0–4° C. Haemolysis was achieved by adding six volumes of distilled water. After centrifugation (30 min, 15,000 *g*) the haemoglobin content of the supernatant was determined by the methaemoglobin cyanide method [12]. The haemolysate was heated for 5 min in boiling water. The MTX concentration of the supernatant was measured with a radioimmunoassay [17]. This assay, using ^{125}I-MTX as competitive substrate for the MTX antibodies, includes MTX and its polyglutamate forms. Molar concentrations of the total erythrocyte MTX were calculated with regard to the haemoglobin content and haematocrit of the blood specimen and the haemoglobin of the haemolysate. The results given below reflect total erythrocyte MTX, i.e., unchanged MTX and its polyglutamate forms. First results of chromatographic separations of MTX and MTX polyglutamates on Sephadex G 15 will be described briefly in the discussion sector of this paper.

Reprint requests should be addressed to A. Schalhorn

0344-5704/82/0009/0065/$ 01.00

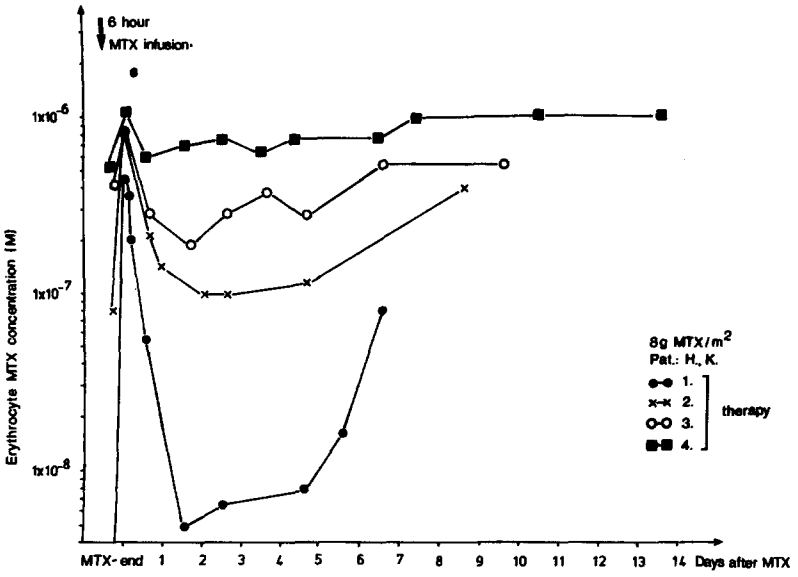

Fig. 1. Erythrocyte MTX concentrations after Nos. 1–4 of repeated HDMTX infusions. In this patient (H., K.) with metastatic osteogenic sarcoma the intervals between the therapies were short, only 7–10 days

Table 1. Erythrocyte MTX at the start of repeated HDMTX infusions with short intervals of 8–11 days in patients with different metastatic sarcomas

Patient	Diagnose	Dosage of MTX (g/m^2)	Period after preceding th. (days)	MTX conc. ($10^{-7}M$)	Period after preceding th. (days)	MTX conc. ($10^{-7}M$)	Period after preceding th. (days)	MTX conc. ($10^{-7}M$)
F. B.	Soft tissue sarcoma	6–7.5	8	1.60	13	2.60		
A. G.	Soft tissue sarcoma	8	8	0.17	7	0.99	8	1.46
K. H.	Osteogenic sarcoma	8	8	0.77	9	4.04	10	5.39
H. M.	Osteogenic sarcoma	8	14	0.56	7	9.40	7	12.2
A. R.	Osteogenic sarcoma	8–12	9	0.30	11	2.60	7	2.45

Results

During HDMTX, erythrocyte MTX concentrations remained extremely low. At the end of an MTX infusion erythrocyte MTX levels of $4-14 \times 10^{-7}$ *M* accounted for only about 1/1000th of the corresponding serum levels of $2-14 \times 10^{-4}$ *M*. In contrast to serum MTX, the pharmacokinetics of erythrocyte MTX are influenced considerably by the number and intervals of HDMTX. As a result, the characteristics of erythrocyte kinetics could not be calculated simply by using the erythrocyte MTX levels after all HDMTX treatments, but were studied for two typical courses with different intervals between the MTX infusions.

HDMTX Every 7–10 Days

Figure 1 shows the kinetics of erythrocyte MTX in a patient with pulmonary metastasis of an osteogenic sarcoma after one to four cycles of HDMTX. HDMTX was repeated every 7–10 days. Two days after the first HDMTX cycle the erythrocyte MTX concentration had fallen rapidly from 1×10^{-6} *M* at the end of the infusion to 5.6×10^{-9} *M*. This was followed by a sharp increase to 8.1×10^{-8} *M* on day 8. Similar kinetics were observed after each HDMTX treatment, minimum erythrocyte MTX levels being measured 2–3 days after MTX infusion, followed by an increase to maximum levels on days 10–14. With HDMTX courses repeated at short intervals, however, the decrease of erythrocyte MTX was less pronounced. After the second and third courses of therapy, erythrocyte MTX fell to 9.9×10^{-8} *M* and 1.9×10^{-7} *M*, respectively, whereas after the fourth course of therapy it fell to only 6.2×10^{-7} *M*. Although the relative increase of erythrocyte MTX diminished with each repetition of HDMTX, the erythrocyte MTX levels were invariably higher than those in the preceding course of therapy (Fig. 1). The maximum erythrocyte concentrations 10–14 days after HDMTX increased from 8.1×10^{-9} *M* (1st course of therapy) to 4×10^{-7} *M* (2nd course of therapy), to 5.35×10^{-7} *M* (3rd course of therapy), and finally to 1.06×10^{-6} *M* after the fourth course of therapy. As shown in Table 1, significant and continuously increasing erythrocyte MTX concentrations have so far been found in all patients studied just before the start of the second to fourth HDMTX infusions.

HDMTX Every 4–6 Weeks

Even after extension of the intervals between HDMTX infusions to 4–6 weeks, the typical biphasic behaviour of erythrocyte MTX kinetics was still observed. Now, however, the erythrocyte MTX curves did not differ significantly. This was shown in a patient with metastatic soft tissue sarcoma after the 18th–22nd courses of HDMTX/CFR therapy (Fig. 2):

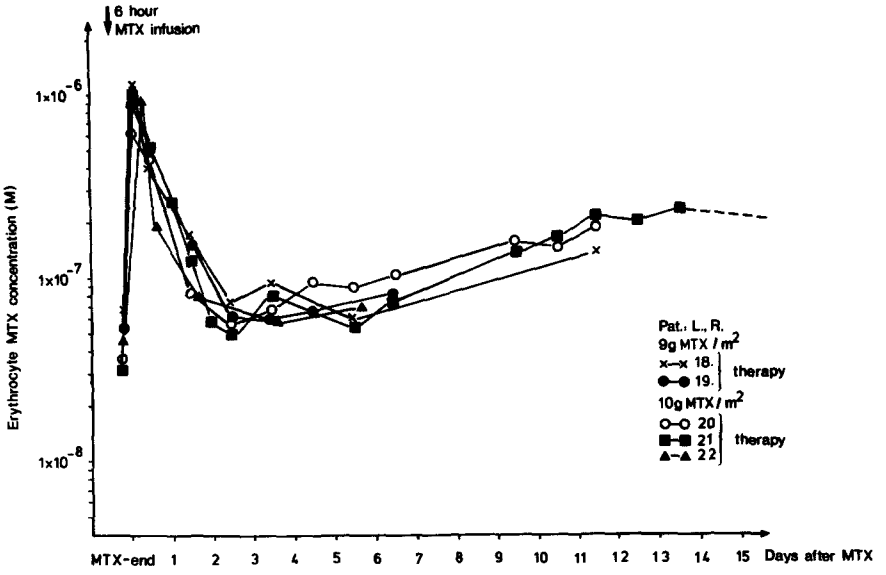

Fig. 2. Erythrocyte MTX concentrations after the 18th–22nd HDMTX infusions. In this patient (L., R.) with metastatic soft tissue sarcoma the intervals between the infusions were rather long, varying between 6 and 7 weeks

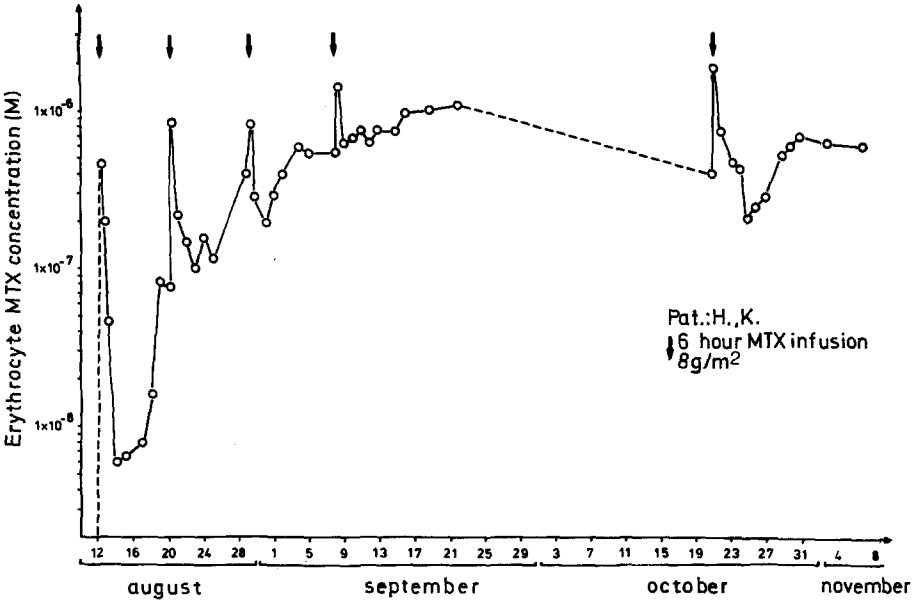

Fig. 3. Time course of erythrocyte MTX during and after repeated HDMTX infusions. In this patient (H., K.), HDMTX was started because of metastatic osteogenic sarcoma, and was repeated each time after a 7–10-day interval. After Adriblastin (22.9.), the 5th HDMTX infusion followed only 7 weeks after the 4th therapy

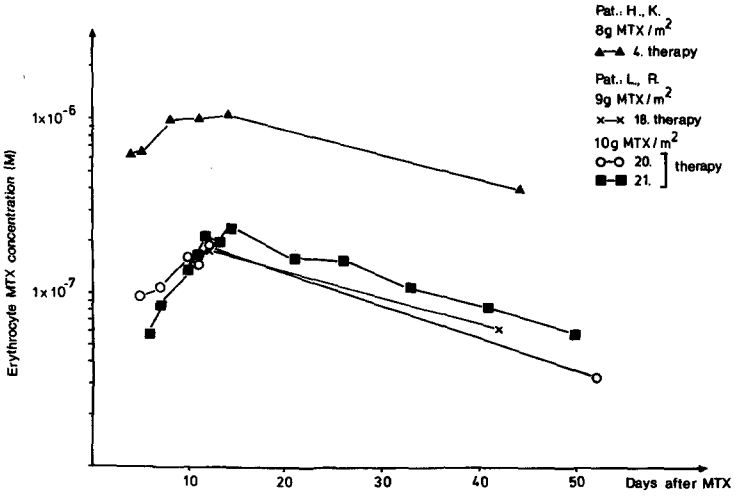

Fig. 4. Half-lives of 'late' erythrocyte MTX. For details, see the text

Erythrocyte MTX declined at an almost parallel rate from 6×10^{-7} to 1×10^{-6} *M* at the end of the infusion to $5-8 \times 10^{-8}$ *M* on days 2–3, and increased similarly to about 1×10^{-7} *M* on days 11–12 after HDMTX.

Long-Term Treatment with HDMTX

Figure 3 shows the time course of erythrocyte MTX after five cycles of HDMTX in the above-mentioned patient with a metastatic osteogenic sarcoma. Due to the recovery 2–3 days after each HDMTX course, total erythrocyte MTX increased continuously to almost complete saturation 14 days after the fourth therapy. The MTX infusions produced only a small and transient peak superimposed upon the increasing erythrocyte curve. Because of a subsequent Adriblastin treatment the fifth HDMTX could not be started until after an interval of 5 weeks. In the meantime, erythrocyte MTX dropped from 1.06×10^{-6} *M* to 3.99×10^{-7} *M*, the concentration determined at the beginning of the third HDMTX therapy. The kinetics of erythrocyte MTX after the fifth HDMTX infusion were thus quite similar to those after the third course of therapy.

Half-Life of the 'Late' Erythrocyte MTX

Only intervals of 6 weeks or more between the treatment cycles or discontinuation of the HDMTX therapy enabled us to study the late erythrocyte MTX kinetics without the influence of further MTX infusions. After reaching maximum values 10–14 days after HDMTX, the erythrocyte levels of MTX declined slowly. The half-life of this late erythrocyte MTX, studied so far after five HDMTX cycles in three different patients, is between 30 and 40 days (Fig. 4).

Comparison of Erythrocyte and Serum MTX

The recovery of erythrocyte MTX 2–3 days after each HDMTX infusion contrasted with the continuing decrease of serum MTX. Erythrocyte MTX always exceeded the serum MTX concentrations. The point of intersection depended upon the number and intervals of the HDMTX cycles.

After the first therapy the erythrocyte and serum MTX curves did not intersect for 132 h. With repetition of HDMTX every 7–10 days, however, the curves intersected earlier. After the fourth therapy, for example, erythrocyte MTX exceeded the serum MTX just 36 h after the HDMTX therapy. In several cases with intervals of 4–6 weeks between the HDMTX courses, the MTX curves intersected after 72 h.

Discussion

After HDMTX therapy, in each case a phase of rapid serum MTX clearance with half-lives between 2 and 2.5 h is followed by a slower disappearance with half-lives between 9 and 12 h [6, 7, 15, 20, 27]. Our investigations showed that the kinetics of erythrocyte MTX differ considerably from these well-known facts: The very low erythrocyte MTX concentrations of about 1/1,000th of the corresponding serum levels fall rapidly to a nadir 2–3 days after HDMTX. When erythropoiesis resumes at MTX serum concentrations of 4×10^{-8} *M* [24], erythrocyte MTX begins to increase again. This second phase is terminated on days 10–14 with maximum erythrocyte MTX levels nearly equal to the peak values at the end of HDMTX infusion. Only after the third phase of erythrocyte MTX kinetics can the half-life of the late erythrocyte MTX be determined. Especially in cases with repeated HDMTX treatment at short intervals of 7–10 days, phase 2 of erythrocyte MTX kinetics i.e., the recovery of erythrocyte MTX, causes overlapping with the values of the following therapy cycle. This is why the kinetic behaviour of erythrocyte MTX changed with the number and interval of treatments, and why it was not possible to calculate exact constants for the pharmacokinetics of erythrocyte MTX.

In mammalian cells, especially in hepatocytes and erythrocytes, polyglutamate derivatives of reduced folates are the predominant species [1, 2, 3, 5]. These compounds, with four to seven glutamates bound in γ-linkage to the glutamate moiety of the folate, are as effective as the monoglutamates in enzymatic reactions involving reduced folates [4, 8, 9, 16]. Like physiological folates, MTX can be converted to polyglutamates, which remain potent inhibitors of dihydrofolate reductase [4, 10, 13]. MTX polyglutamates are detected in human cell lines after incubation in vitro [21, 25] and in human liver [2, 11] and erythrocytes [11] during different forms of MTX therapy. Separation of MTX polyglutamates from MTX by gel filtration on Sephadex G 15 enabled us to demonstrate increasing erythrocyte MTX polyglutamate concentrations in two patients with repeated HDMTX infusions. In one patient MTX polyglutamates contributed 33% of total erythrocyte MTX on day 7 after the second HDMTX therapy. In the other patient MTX polyglutamates increased to 52% and 59% on day 7 after the second and third therapy courses, respectively. According to studies with human hepatocytes [1], intact polyglutamates of MTX can leave the cell only very slowly or after cleavage to MTX. This seems also to be true for erythrocytes. Regular measurements of erythrocyte MTX and its polyglutamates during phase 3 of the kinetics of erythrocyte MTX showed a great difference in the clearance of MTX and MTX polyglutamates. In the two patients studied the half-life of the polyglutamate form was three and four times longer than that of MTX. Further investigations are in progress to confirm these results.

Our results suggest the following mechanism for the recovery of erythrocyte MTX beginning 2–3 days after each HDMTX infusion: During/after the infusion, MTX is taken up by erythro- and normoblasts. Depending on the concentration and exposure time, these cells at least partially convert the free MTX to MTX polyglutamates, which are retained inside the cells for long periods. At serum MTX concentrations of 4×10^{-8} *M*, the regular CFR [22] neutralizes the MTX-induced inhibition of erythropoiesis [23]. The bone marrow releases freshly produced erythrocytes containing MTX chiefly in the polyglutamate form, and causes the recovery of MTX during the second phase of erythrocyte MTX kinetics. As our assay also includes old erythrocytes with very low MTX concentrations, real MTX levels in reticulocytes and especially in normo- and erythroblasts are very much higher. Although determinations of MTX in human erythro- and normoblasts are still lacking, it seems most likely that MTX and MTX polyglutamate accumulation in these precursor cells are the reason for the high MTX sensitivity of erythropoiesis.

References

1. Balinska M, Galivan J, Coward JK (1981) Efflux of methotrexate and its polyglutamate derivatives from hepatic cells in vitro. Cancer Res 41: 2751
2. Baugh CM, Krumdieck CL, Nair MG (1973) Polyglutamate metabolites of methotrexate. Biochem Biophys Res Commun 52: 27
3. Bernstein LH, Gutstein S, Weiner S, Efron G (1970) The absorption and malabsorption of folic acid and its polyglutamates. JAMA 48: 27

4. Bertino JR (1979) Toward improved selectivity in cancer chemotherapy: the Richard and Linda Rosenthal Foundation award lecture. Cancer Res 39: 293
5. Blakley RL (1969) The biochemistry of folic acid and related pteridines. North Holland, Amsterdam
6. Bleyer WA (1977) Methotrexate: clinical pharmacology, current status, and therapeutic guidelines. Cancer Treat Rev 4: 87
7. Bleyer WA (1978) The clinical pharmacology of methotrexate. Cancer 41: 36
8. Coward JK, Parameswaran KN, Cashmore AR, Bertino JR (1974) 7,8-Dihydropteroyl oligo-*y*-*l*-glutamates: synthesis and kinetic studies with purified dihydrofolate reductase from mammalian sources. Biochemistry 13: 3899
9. Coward JK, Chello PL, Cashmore AR, Parameswaran KN, Angelis LM de, Bertino JR (1975) 5-Methyl-5,6,7,8-tetrahydropteroyl oligo-*y*-*l*-glutamates: synthesis and kinetic studies with methionine synthetase from bovine brain. Biochemistry 14: 1548
10. Galivan J (1979) Transport and metabolism of methotrexate in normal and resistant cultured rat hepatoma cells. Cancer Res 39: 735
11. Hendel J (1978) Intracellular metabolites of methotrexate: In: High-dose methotrexate – pharmacology toxicity, and chemotherapy. Chemioterapia Oncologia 13 [Suppl Dec]
12. International Committee for Standardization in Haematology (1967) Recommendations for haemoglobinometry in human blood. Brit J Haematol 13 (Suppl): 71
13. Jacobs SA, Adamson RH, Chabner BA, Derr JC, Johns DG (1975) Stoichiometric inhibition of mammalian dihydrofolate reductase by the y-glutamyl metabolite of methotrexate 4-amino,4-deoxy-N10-methylpteroylglutamyl-glutamate. Biochem Biophys Res Comm 63: 692
14. Jaffe N, Frei E, Traggis D, Cassady JR, Watts H, Filler RM (1977) High-dose methotrexate with citrovorum factor in osteogenic sarcoma. Cancer Treat Rep 61: 675
15. Janka GE, Wiesner H, Biedlingmaier F, Haas RJ (1979) Hochdosierte Methotrexattherapie beim osteogenen Sarkom: Plasmapharmakokinetik zur Vorhersage der Toxizität. Klin Wochenschr 57: 411
16. Kisliuk RL, Gaumant Y, Baugh CM (1974) Polyglutamyl derivatives of folates as substrates and inhibitors of thymidylate synthetase. J Biol Chem 249: 4100
17. Methotrexate (^{125}J) radioimmunoassay kit instruction manual (1978) Diagnostic Biochemistry, San Diego
18. Pitman SW, Frei III E (1977) Weekly methotrexate-calcium leucovorin rescue: effect of alkalinization in nephrotoxicity; pharmacokinetics in the CNS; and use in CNS Non-Hodgkin's lymphoma. Cancer Treat Rep 61: 695
19. Poceri-Morton de B, Chang J, Specker M, Bastiani R (1978) Performance evaluation of the EMIT methotrexate assay. Syva Palo Alto, California
20. Pratt CB, Roberts D, Shanks E, Warmath EL (1975) Response, toxicity, and pharmacokinetics of high-dose methotrexate (NSC-740) with citrovorum factor (NSC-3590) rescue for children with osteosarcoma and other malignant tumors. Cancer Chemother Rep part 3, 6: 13
21. Rosenblatt SD, Whitehead VM, Dupont MM, Vuchich MJ, Vera N (1978) Synthesis of methotrexate polyglutamates in cultured human cells. Molec Pharmacol 14: 210
22. Sauer H, Schalhorn A (1980) Rationale Grundlage und Praxis des Citrovorumfaktor(Leukovorin)-Schutzes nach hochdosierter Methotrexat-Therapie. Onkologie 3: 64
23. Schalhorn A, Sauer H, Wilmanns W, Stupp-Poutot G (1981) Ferrokinetics after high-dose methotrexate therapy. Klin Wochenschr 59: 1109
24. Schalhorn A, Wilmanns W, Stupp-Poutot G (1980) Methotrexat-Nachweis im menschlichen Serum – Vergleich zwischen einer enzymatischen und einer enzymimmunologischen Nachweismethode. Onkologie 4: 193
25. Schilsky RL, Bailey BD, Chabner BA (1980) Methotrexate polyglutamate synthesis by cultured human breast cancer cells. Proc Natl Acad Sci USA 77: 2919
26. Schreml W, Lohrmann HP (1979) Effect of high-dose methotrexate with citrovorum factor on human granulocytopoiesis. Cancer Res 39: 4195
27. Stoller RG, Hande KR, Jacobs SA, Rosenberg SA, Chabner BA (1977) Use of plasma pharmacokinetics to predict and prevent methotrexate toxicity. New Engl J Med 297: 630
28. Werkheiser WC, Zakrzewski SF, Nichol CA (1962) Assay for 4-amino folic acid analogues by inhibition of folic acid reductase. J Pharmacol Exp Ther 127: 162
29. Wilmanns W (1974) Dihydrofolatreduktase. In: Bergmeyer: Methoden der enzymatischen Analyse; 3rd ed, vol 1. Verlag Chemie, Weinheim/Bergstraße

Received March 17/Accepted April 26, 1982