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Simultaneous quantitation of methotrexate and its two main metabolites in biological fluids by a novel solid-phase extraction procedure using high-performance liquid chromatography

Freidoun Albertioni[a,*], Birgitta Pettersson[a], Olof Beck[a], Charlotte Rask[b], Peter Seideman[c], Curt Peterson[a]

[a]*Department of Clinical Pharmacology, Karolinska Hospital, S-171 76 Stockholm, Sweden*
[b]*Department of Pediatrics, University Hospital of Aarhus, Aarhus, Denmark*
[c]*Department of Internal Medicine, Division of Rheumatology, Karolinska Institute at Danderyds Hospital, Stockholm, Sweden*

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Abstract

We have developed an assay for the simultaneous determination of methotrexate (MTX) and its main metabolites, 7-hydroxymethotrexate (7-OHMTX) and 2,4-diamino-N^{10}-methylpteroic acid (DAMPA) in plasma, urine and saliva meeting the requirement of rapidity for routine use in high-dose MTX therapy and the requirement of sensitivity for its potential use in therapeutic drug monitoring in low-dose MTX therapy. Sample preparation is based on solid-phase extraction using C_8 Isolute cartridges. Chromatographic separation was achieved with a reversed-phase column (C_{18}), and quantitation by subsequent exposure to UV light of 254 nm, which converted MTX and its two metabolites by photolytic oxidation to fluorescent products. The recoveries of MTX, 7-OHMTX and DAMPA from plasma at 100 nmol/l were 85.8, 91.1 and 102.3%, respectively. The limits of detection for MTX, 7-OHMTX and DAMPA in plasma and saliva were 0.1 nmol/l. In urine the limit of detection was 10 nmol/l for all compounds. The limits of quantitation in plasma and saliva were 0.5 nmol/l for all compounds.

1. Introduction

Methotrexate (MTX, 2,4-diamino-N^{10}-methylfolic acid), a folic acid antagonist, acts as an inhibitor of dihydrofolate reductase (DHFR) leading to DNA damage and cell death [1,2]. The effectiveness of MTX in the treatment of various neoplastic and arthritic diseases is well established [3–5].

High-dose MTX (>300 mg/m^2) is always followed by leucovorin (folinic acid) rescue leading to an increase in the cellular concentration of reduced folates. Monitoring of plasma MTX is of importance for guidance of the leucovorin therapy in order to protect patients from severe bone marrow toxicity and mucositis [6]. Furthermore, it has been suggested that the therapeutic

* Corresponding author.

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effect of MTX is determined by a balance of exposure to MTX and leucovorin rescue [7].

The pharmacokinetic data concerning plasma metabolites of MTX, 7-hydroxymethotrexate (7-OHMTX) and 2,4-diamino-N^{10}-methylpteroic acid (DAMPA) have not yet been fully elucidated. Although the role of these metabolites in the therapeutic and toxic effects is still unclear, there is some evidence that they may contribute to clinical effects (7-OHMTX and DAMPA are 1/100 and 1/200 times, respectively, as effective as MTX as inhibitor of DHFR) [8–11]. The excessive formation of these metabolites may also influence the pharmacokinetics of MTX and/or leucovorin due to competition for transport systems across cell membranes [5].

Methods for MTX analysis have been reviewed elsewhere [12]. These methods include high-performance liquid chromatography (HPLC) with UV and fluorescence detection [13–19], radio immunoassay (RIA) [20], enzyme-inhibition assay, enzyme-multiplied immunoassay (EMIT) [21], and fluorescence polarization radioimmunoassay (FPIA) [22].

Automated assays based on immunological techniques are most often used for therapeutic drug monitoring (TDM) of high-dose MTX therapy due to their rapidity. However, these techniques have a risk of co-determination of metabolites due to crossreactivity of the antibodies. Cross-reactivity between 7-OHMTX and MTX has been reported to be 1% in enzyme-inhibition assays, 4% in EMIT and 1% in FPIA and the cross-reactivities between DAMPA and MTX for these techniques are 10, 100 and 42%, respectively [21,23].

Monitoring of plasma MTX at low-dose therapy (≤ 25 mg/m^2 per week) during treatment of arthritis, psoriasis or maintenance therapy of childhood leukemia has also been suggested in order to improve the therapy [24]. Furthermore, concurrent treatment with non-steroidal antiinflammatory drugs [25,26] and with other drugs [27–29] may lead to delayed elimination of MTX and its metabolites and subsequently increase the risk of toxicity. The automated techniques are usually not sensitive enough to allow determination of plasma MTX during the whole dose interval.

In order to meet the requirements of a rapid and specific assay we have developed an HPLC procedure with a solid-phase extraction procedure for the simultaneous determination of MTX and its two main metabolites in plasma, saliva and urine, using fluorescence detection as previously described [18,30,31].

2. Experimental

2.1. Chemicals and reagents

MTX, (+)-amethopterin, was purchased from Sigma (St. Louis, MO, USA) and 7-OHMTX was generously donated by Dr. W.E. Evans, St. Jude Children's Research Hospital (Memphis, TN, USA). 2,4-Diamino-N^{10}-methylpteroic acid (DAMPA) was enzymatically prepared from (+)-ametopterin by carboxypeptidase according to a procedure described previously [32]. Purification was performed by preparative HPLC. The final concentration of DAMPA was determined by UV spectroscopy using a molar extinction coefficient of 15 500 at 254 nm [33]. C_8 Isolute, 3 ml, solid-phase extraction (No 290-0020-B) cartridges were obtained from International Sorbent Technology (UK). Methanol was HPLC grade (JT Baker, Netherlands). Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) was from Merck (Darmstadt, Germany). Other chemicals used in this study were of analytical purity and obtained commercially.

2.2. Sample preparation

A sample aliquot (1 ml for plasma, 10 μl for urine, or 100 μl for saliva) was mixed with 2 ml of 0.1 *M* phosphoric acid. The solutions were loaded onto C_8 solid-phase extraction cartridges previously activated with 3 ml of methanol and 3 ml of 0.1 *M* phosphoric acid with an approximate flow-rate of 2.5 ml/min. The columns were prevented from drying out before applying the specimens. The cartridges were then washed

with 2 ml of 5% methanol in water and dried by aspiration of air. The cartridges were finally eluted with 2 ml of 2% trifluoroacetic acid in methanol. The eluates were collected in glass tubes and evaporated to dryness under a stream of nitrogen in a water bath (40°C). The residues were finally dissolved in 100 μl of water and 10-μl aliquots were injected. Standard samples were prepared by spiking blank plasma with known amounts of MTX, 7-OHMTX and DAMPA and used for construction of calibration curves.

2.3. Apparatus

The HPLC equipment consisted of a CM4000 pump (Milton Roy, LDC Division, USA), a CMA-240 autosampler (Carnegie Medicine, Stockholm, Sweden), a beam boost photochemical reactor (ICT HG, Frankfurt, Germany) equipped with a 254-nm lamp and a 10 m × 0.3 mm I.D. reaction coil and used at an operating temperature of 37°C, a Shimadzu 551 fluorescence detector (Shimadzu Corp.) with excitation and emission wavelengths of 350 nm and 435 nm, respectively. For the solid-phase extraction, a VacMaster (International Sorbent Technology, UK) was used.

2.4. Chromatographic system

The mobile phase consisted of 0.01 *M* phosphate buffer (pH 6.5), with 6% of acetonitrile and 0.2% of 30% hydrogen peroxide and was delivered at a flow-rate of 1 ml/min at a pressure of approximately 135 bar. The mobile phase was filtered (0.22-μm filter, Millipore, Ireland), degassed and allowed to stand overnight before use. A C_{18} column (80 × 4.6 mm I.D., 3 μm, Perkin-Elmer, Norwalk, CT, USA) was used.

The chromatographic data was collected and processed on a Macintosh Classic computer (Apple, Chicago, IL, USA) equipped with Chromac 3.1. software (Drew, London, UK). MTX, 7-OHMTX and DAMPA concentrations in patient samples were determined by comparison of their respective peak areas with those of standards prepared in blood plasma from drug free subjects. The capacity factor of each compound was calculated as $(t_r - t_0)/t_0$, where t_r is the retention time of the compound and t_0 is the retention time of the first distortion of the baseline.

2.5. Assay validation

The intra- and inter-assay variability of the MTX, 7-OHMTX and DAMPA determination were examined in spiked plasma and urine samples pooled from 5 drug-free volunteers. Linearity of detector response was determined at different concentrations in plasma (0.2–500 nmol/l) for each compound. The recovery of each compound was assessed at the level of 100 nmol/l by comparing the peak areas in extracts with those obtained from direct injections of non-extracted standards (dissolved in water) of the same concentration.

2.6. Sample collection

As a test of the feasibility of the method for assaying MTX and the metabolites in clinical samples, blood and urine were collected from two patients treated with oral low-dose MTX, one suffering from acute lymphoblastic leukemia (ALL) treated with 5 mg/m^2 oral MTX and the other suffering from rheumatoid arthritis treated with 10 mg/m^2 oral MTX. Plasma was obtained by centrifugation at 4°C. Saliva was collected from one patient suffering from ALL treated with high-dose MTX (5–8 g/m^2 according to the NOPHO-ALL protocol from 1992). The samples were stored at −20°C until analysis.

2.7. Assay specificity

Since MTX is frequently coadministered with other anticancer drugs, antibiotics and non-steroidal antiinflammatory drugs, we examined possible chromatographic interferences with MTX, 7-OHMTX and DAMPA. The following substances were injected without sample preparation onto the column: allopurinol, leucovorin, 2-fluoro-9-β-D-arabinofuranosyladenine (F-ara-A), 6-mercaptopurine (6-MP), azathioprine, 1-

β-D-arabinofuranosylcytosine (ara-C), ciprofloxacin, amoxicillin, trimethoprim, sulfamethoxazol, chloroquine, acetylsalicylic acid, paracetamol, naproxen and ibuprofen.

3. Results

Fig. 1 shows a representative chromatogram of an aqueous standard of MTX, 7-OHMTX and DAMPA. Good separation of MTX and its two metabolites was achieved in less than 25 min. The capacity factors (k') of MTX, 7-OHMTX and DAMPA were 8.4, 9.9, and 17.5, respectively. It should be pointed out that a slight modification of the percentage of acetonitrile in the mobile phase may be necessary after changing the chromatographic column in order to achieve optimal separation between the substances.

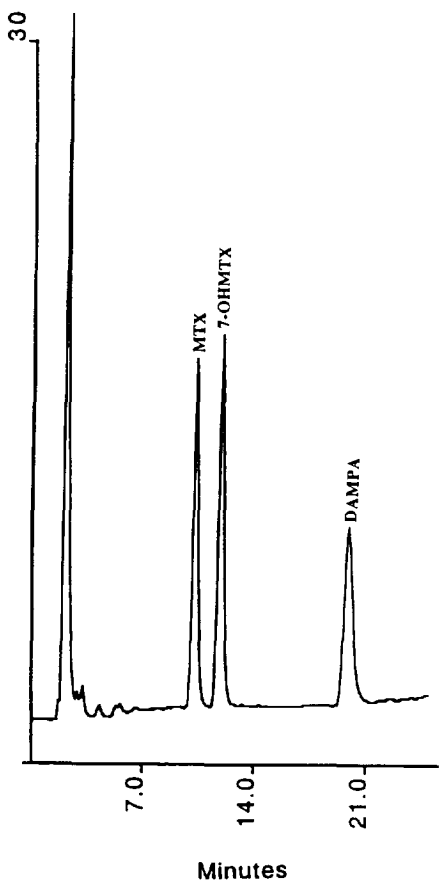

Fig. 1. Chromatogram of a mixture of MTX, 7-OHMTX and DAMPA in an aqueous solution. Five pmole of each substance were injected onto the HPLC system.

In order to optimize the photochemical reactions of MTX, 7-OHMTX and DAMPA on UV-irradiation, various post-column capillary coils were tested. The maximum response was found with an irradiation time of 60 s (10 m × 0.3 mm I.D., flow 1 ml) (Fig. 2). A decrease in the response was observed on prolonged irradiation, especially for 7-OHMTX.

A linear relationship between plasma concentration and peak area was obtained for all three substances within the concentration range of interest (0.2–500 nmol/l). The correlation coefficients were $r^2 > 0.996$ for low concentrations (range 0.2–62.5 nmol/l) and $r^2 > 0.997$ for the high concentrations (range 62.5–500 nmol/l) (data from individual curves not shown). Similar results were observed with urine and saliva. 7-OHMTX and DAMPA could not be detected in plasma, saliva or urine spiked with MTX only.

At a signal-to-noise ratio >3:1, the limits of detection of MTX, 7-OHMTX and DAMPA in plasma and saliva were 0.1 nmol/l. In urine the limit of detection was 10 nmol/l for all compounds. The limit of quantitation at a signal-to-noise ratio >10:1 was 0.5 nmol/l (C.V. < 15%) for all compounds.

Drug recovery was determined by comparing spiked plasma samples with an aqueous solution of MTX, 7-OHMTX and DAMPA. Mean re-

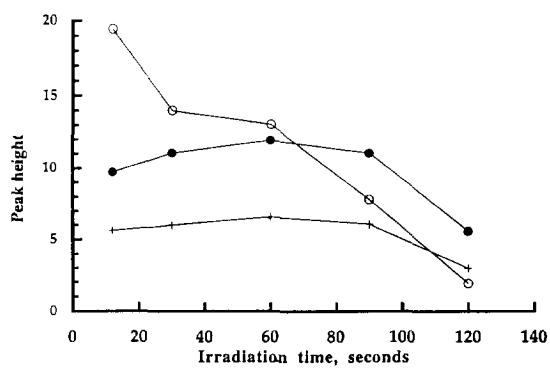

Fig. 2. Influence on fluorescence response of MTX (●), 7-OHMTX (○) and DAMPA (+) of the time of irradiation with UV light of 254 nm.

coveries of MTX, 7-OHMTX and DAMPA from plasma at 100 nmol/l were 85.8 ± 1.8, 91.1 ± 4.2 and 102.3 ± 7.2%, respectively. The intra- and inter-assay coefficients of variation for MTX, 7-OHMTX and DAMPA in plasma are shown in Table 1.

The results of the interference studies showed that only sulfamethoxazol interfered with MTX peak, but satisfactory separation could be achieved by a slight change in the mobile phase.

Chromatograms of plasma samples obtained from a patient receiving an oral MTX dose (5 mg/m^2), before and at 3, 24, and 168 h after dose intake, are shown in Fig. 3A–D. To further demonstrate the clinical applicability of the method, plasma and urine samples from a patient with ALL were analyzed according to the described procedure. The plasma pharmacokinetic profiles of MTX and 7-OHMTX after a single oral dose of 5 mg/m^2 are shown in Fig 4. MTX and 7-OHMTX could readily be detected during 1 week after oral MTX intake (Fig. 4). The maximum plasma concentration (C_{max}) of MTX was 926.5 nmol/l at 0.75 h after dosing, while the C_{max} of 7-OHMTX was obtained 7 h after administration and at a concentration of 216.9 nmol/l (observed values). The area under the plasma concentration–time curves (AUC), calculated from time zero to infinity were 3276.9 nM h and 4709.8 nM h for MTX and 7-OHMTX, respectively. The distribution and elimination half-lives of MTX were 1.5 and 128.2 h, respectively. The elimination half-life for 7-OHMTX was 66.5 h. In this patient, the concentration of DAMPA was 1.8 nmol/l at 1 h and less than 0.2 nmol/l at 168 h, respectively, after an oral MTX dose.

Typical chromatograms of plasma, urine and saliva from patients with rheumatoid arthritis and ALL are shown in Fig. 5A–C. No interferences from impurities in plasma, urine and saliva were detected in the chromatogram.

The concentration–time profiles for MTX and 7-OHMTX in saliva from a patient with ALL

Table 1
Intra- and inter-assay variability and precision of MTX, 7-OHMTX and DAMPA in blank human plasma

Compound	Concentration (nmol/l)			C.V. (%)	n
	Spiked	Analyzed	S.D.		
Intra-assay					
MTX	10.0	10.7	0.5	4.4	6
	100.0	95.6	6.5	6.9	5
	1500.0	1445.1	112.8	7.8	7
7-OHMTX	10.0	9.7	0.4	4.5	4
	100.0	105.9	13.3	12.5	5
	1500.0	1450.5	57.2	3.9	7
DAMPA	10.0	10.1	0.7	7.2	6
	100.0	97.1	8.8	9.1	5
	1500.0	1495.3	111.7	6.8	7
Inter-assay					
MTX	10.0	9.4	1.0	10.3	6
	100.0	94.1	6.4	6.8	9
	1500.0	1508.8	21.2	1.4	6
7-OHMTX	10.0	9.3	8.9	9.6	6
	100.0	99.2	8.1	8.2	9
	1500.0	1523.7	21.3	1.4	6
DAMPA	10.0	9.3	1.2	12.6	6
	100.0	100.7	8.1	8.0	9
	1500.0	1537.5	110.9	7.2	6

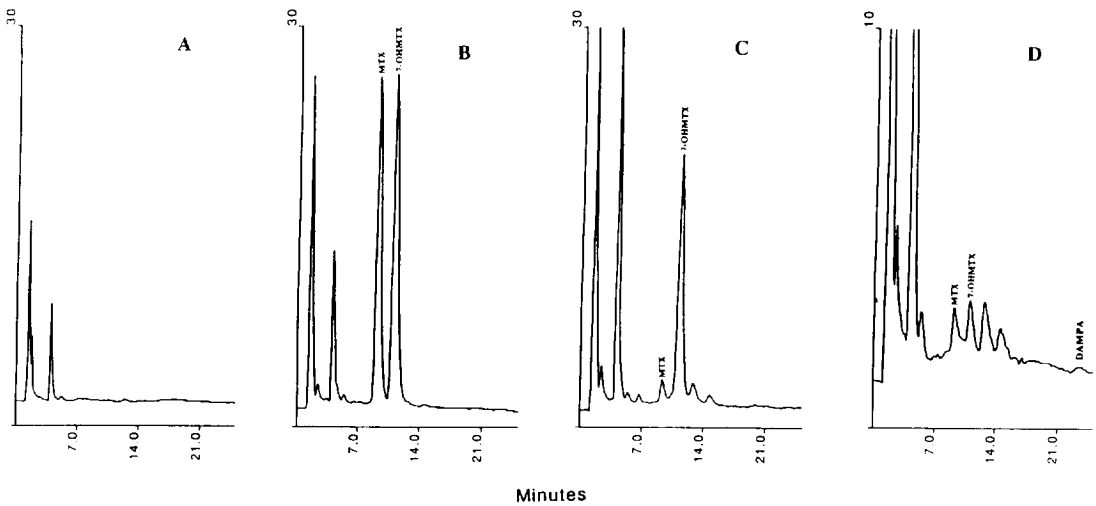

Fig. 3. Chromatograms from patient plasma samples. (A) Before drug administration, (B) 3 h (236.6 nmol/l of MTX and 175.0 nmol/l of 7-OHMTX), (C) 24 h (6.3 nmol/l of MTX and 58.1 nmol/l of 7-OHMTX), and (D) 168 h (2.6 nmol/l of MTX, 1.9 nmol/l of 7-OHMTX and less than 0.2 nmol/l of DAMPA) after a single oral (5 mg/m^2) administration of MTX.

treated with high-dose MTX (5 g/m^2) are shown in Fig. 6. In this patient, the 7-OHMTX/MTX ratio is higher than 7 at both 18 and 42 h after the end of infusion (Figs. 5C and 6).

4. Discussion

MTX has been used as an antineoplastic agent since the 1940s and in recent years it has received increased attention in the treatment of rheumatoid arthritis and psoriasis. High-dose MTX chemotherapy requires plasma drug monitoring as guidance for the leucovorin rescue. However, severe mucositis has been described in spite of normal plasma MTX decay and it has been postulated that this might be due to differences in metabolite concentrations [34].

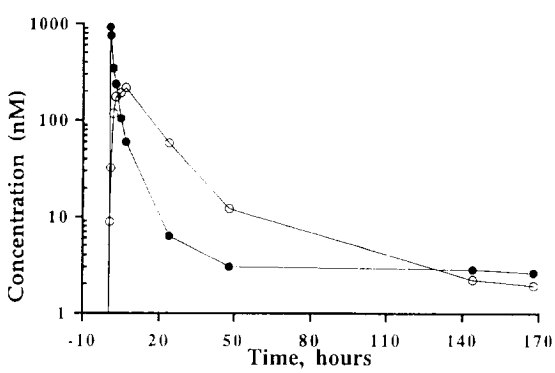

Fig. 4. Semilogarithmic plot of MTX (●) and 7-OHMTX (○) plasma concentrations versus time following a single oral dose of MTX (5 mg/m^2) administered to a patient with ALL.

For treatment of rheumatoid arthritis, low-dose MTX therapy (5–25 mg/m^2 per week) can be used. Most assays can, however, only detect plasma MTX during the first 24 h after low-dose therapy. Plasma concentrations of MTX, 7-OHMTX and DAMPA are lower than 10 nmol/l in less than 24 h in most patients after administration of a single oral low-dose [35–37]. In order to study the potential value of TDM in low-dose therapy, an assay with high sensitivity is needed. Our method allows the simultaneous monitoring of MTX and its two main metabolites (7-OHMTX and DAMPA) during the whole dose interval (one week) in oral low-dose therapy. Although the metabolites as such exert little cytotoxic effect they are not without interest since the possibility exists that they affect the intracellular effects of MTX through interaction with membrane transport and/or polyglutamation.

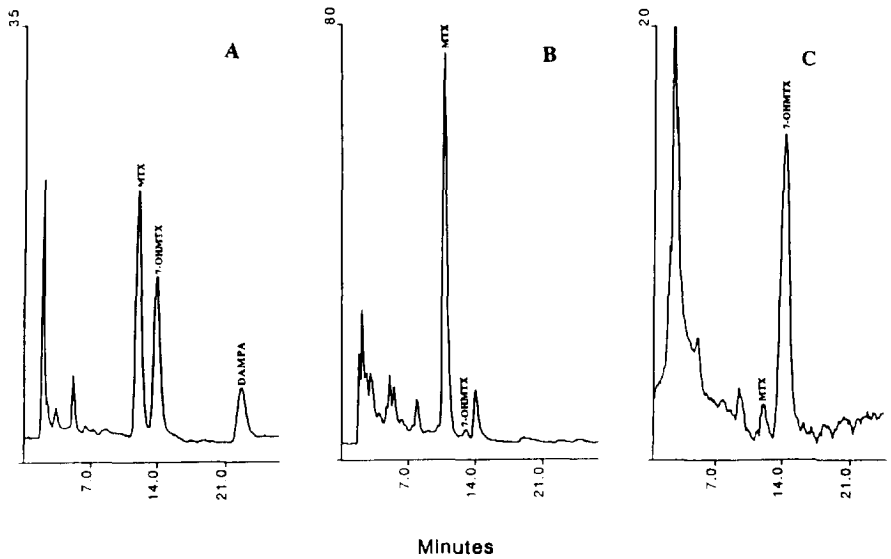

Fig. 5. Chromatograms from patients. (A) Plasma (264.6 nmol/l of MTX and 146.2 nmol/l of 7-OHMTX and 72.9 nmol/l of DAMPA), (B) urine (2111.2 nmol/l of MTX and 99.0 nmol/l of 7-OHMTX), (C) saliva (130.7 nmol/l of MTX and 1037.7 nmol/l of 7-OHMTX). The plasma and urine were collected 4 and 24 h after an oral low-dose intake, respectively. The mixed saliva was obtained 32 h post infusion from children with ALL treated with high-dose MTX (5 g/m^2, 24-h continuous infusion).

Oral toxicity is a major problem in the treatment of patients with high-dose MTX. During the past decade, there has been growing interest in the salivary excretion of the drugs and the correlation between free drug and salivary concentration with the aim to simplify sample collection. MTX in saliva has been shown to correlate well with its plasma concentrations in one study [38], but this could not be confirmed by others [39,40]. High salivary concentrations of 7-OHMTX may explain the low correlation between free plasma and salivary concentration of MTX (F. Albertioni et al., manuscript in preparation). However, concentrations of 7-OHMTX in saliva and their relation to oral toxicity have not yet been reported. Our assay allows the simultaneous determination of MTX and 7-OHMTX in saliva.

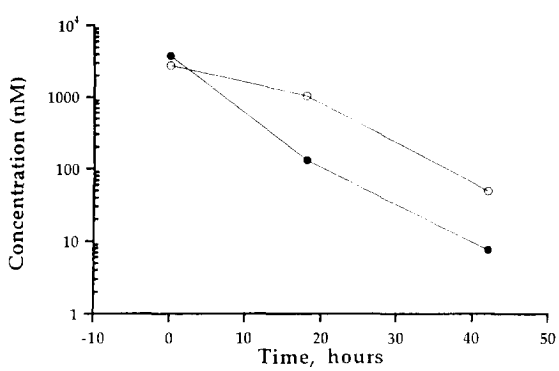

Fig. 6. The pharmacokinetic profile of MTX (●) and 7-OHMTX (○) in saliva of children with ALL treated with high-dose MTX (5 g/m^2, 24-h continuous infusion).

We adopted the post-column derivatization, giving highly fluorescence products (2,4-diaminopteridine-6-carboxyaldehyde and of the corresponding carboxylic acid) as described by Salamoun and co-workers [18,30] with some modification in combination with a solid-phase extraction procedure using C_8 Isolute cartridges as described in the Experimental section.

The present method has several advantages when compared with our previous method using anion-exchange Certify II cartridges [31]. First, recovery of DAMPA when extracted with C_8 Isolute is much higher (102%) than on Certify II ($<5\%$), which allows us to measure MTX, 7-OHMTX and DAMPA simultaneously in plasma, urine and saliva. Second, the extraction

procedure is less time-consuming with only two steps which can easily be automated. Finally, the use of C_8 Isolute is cost effective compared to Certify II (more than 4 times) and the C_8 Isolute cartridges can be reused for at least three times without loss of performance. This method is currently being used in our laboratory for pharmacokinetic studies of MTX and its metabolites in ALL of childhood and in patients with rheumatoid arthritis.

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References

[1] M.W. McBurney and G.F. Whitmore, *Cancer Res.*, 35 (1975) 586.
[2] W. Hryniuk and J.R. Bertino, *Ann. NY Acad. Sci.*, 186 (1971) 330.
[3] W.E. Evans, W.R. Crom, J.A. Sinkule, G.C. Yee, C.F. Stewart and P.R. Hutson, *Drug Metab. Rev.*, 14 (1983) 847.
[4] D.E. Furst and J.M. Kremer, *Arthritis Rheum.*, 31 (1988) 305.
[5] J.D. Borsi, E. Sagen, I. Romslo and P.J. Moe, *Pediatr. Hematol. Oncol.*, 7 (1990) 13.
[6] R.G. Stoller, K.R. Hande, S.A. Jacobs, S.A. Rosenberg and B.A. Chabner, *N. Engl. J. Med.*, 297 (1977) 630.
[7] J.D. Borsi, F. Wesenberg, T. Stokland and P.J. Moe, *Eur. J. Cancer*, 27 (1991) 1006.
[8] D.M. Valerino, *Res. Commun. Chem. Pathol. Pharmacol.*, 4 (1972) 529.
[9] D.M. Valerino, D.G. Johns, D.S. Zaharko and V.T. Oliverio, *Biochem. Pharmacol.*, 21 (1972) 821.
[10] D. Kessel, *Mol. Pharmacol.*, 5 (1969) 21.
[11] J. Lankelma, E. van der Kleijn and F. Ramaekers, *Cancer Lett.*, 9 (1980) 133.
[12] S. Eksborg and H. Ehrsson *J. Chromatogr.*, 340 (1985) 31.
[13] R. Durand, G. Fabre, J.P. Cano, J. Catalin, O.A. Ahmed and S. Just, *J. Appl. Toxicol.*, 3 (1983) 189.
[14] M.L. Chen, W.P. McGuire, T.E. Lad and W.L. Chiou, *Int. J. Clin. Pharmacol. Ther. Toxicol.*, 22 (1984) 1.
[15] H.N. Alkaysi, A.M. Gharaibeh and M.A. Salem, *Ther. Drug Monit.*, 12 (1990) 191.
[16] C.P. Collier, S.M. MacLeod and S.J. Soldin, *Ther. Drug Monit.*, 4 (1982) 371.
[17] N. So, D.P. Chandra, I.S. Alexander, V.J. Webster and H.D. O'Gorman, *J. Chromatogr.*, 337 (1985) 81.
[18] J. Salamoun, M. Smrz, F. Kiss and A. Salamounová, *J. Chromatogr.*, 419 (1987) 213.
[19] D.A. Cairnes and W.E. Evans, *J. Chromatogr.*, 231 (1982) 103.
[20] V. Raso and R. Schreiber, *Cancer Res.*, 35 (1975) 1407.
[21] R.G. Buice, W.E. Evans, J. Karas, C.A. Nicholas, P. Sidhu, A.B. Straughn, M.C. Meyer and W.R. Crom, *Clin. Chem.*, 26 (1980) 1902.
[22] L. Slördal, P.S. Prytz, I. Pettersen and J. Aarbakke, *Ther. Drug Monit.*, 8 (1986) 368.
[23] S.K. Howell, Y.M. Wang, R. Hosoya and W.W. Sutow, *Clin. Chem.*, 26 (1980) 734.
[24] P. Seideman, *Br. J. Rheumatol.*, 32 (1993) 751.
[25] C.F. Stewart, R.A. Fleming, B.F. Germain, M.J. Seleznick and W.E. Evans, *Arthritis Rheum.*, 34 (1991) 1514.
[26] D.E. Furst, R.A. Herman, R. Koehnke, N. Ericksen, L. Hash, C.E. Riggs, A. Porras and P.P. Veng, *J. Pharm. Sci.*, 79 (1990) 782.
[27] G. Ferrazzini, J. Klein, H. Sulh, D. Chung, E. Griesbrecht and G. Koren, *J. Pediatr.*, 117 (1990) 823.
[28] K.S. Basin, A. Escalante and T.D. Beardmore, *J. Rheumatol.*, 18 (1991) 609.
[29] R. Dean, J. Nachman and A.N. Lorenzana, *Am. J. Pediatr. Hematol. Oncol.*, 14 (1992) 88.
[30] J. Salamoun and J. Frantisek, *J. Chromatogr.*, 378 (1986) 173.
[31] O. Beck, P. Seideman, M. Wennberg and C. Peterson, *Ther. Drug Monit.*, 13 (1991) 528.
[32] J.L. McCullough, B.A. Chabner and J.R. Bertino, *J. Biol. Chem.*, 246 (1971) 7207.
[33] R.C. Donehower, K.R. Hande, J.C. Drake and B.A. Chabner, *Clin. Pharmacol. Ther.*, 26 (1979) 63.
[34] J.D. Borsi, C. Csáki, C. Paál, T. Ferencz and D. Schuler, *6th Pharmacology Workshop Satellite to XXV SIOP Meeting*, 1993, p. 112.
[35] F. Albertioni, B. Flaø, P. Seideman, O. Beck, O. Winje, C. Peterson and S. Eksborg, *Eur. J. Clin. Pharmacol.*, (1994) in press
[36] P. Seideman, O. Beck, S. Eksborg and M. Wennberg, *Br. J. Clin. Pharmacol.*, 35 (1993) 409.
[37] K.A. Skoglund, S. Söderhäll, O. Beck, C. Peterson, M. Wennberg, S. Hayder and O. Björk, *Med. Pediatr. Oncol.*, 22 (1994) 187.
[38] W.H. Steele, J.F. Stuart, B. Whiting, J.R. Lawrence, K.C. Calman, J.G. McVie and G.M. Baird, *Br. J. Clin. Pharmacol.*, 7 (1979) 207.
[39] H. Schrøeder, K.B. Jensen and M. Brandsborg, *Br. J. Clin. Pharmacol.*, 24 (1987) 537.
[40] A. Wolff, J.E. Moreira, Y. Marmary and P.C. Fox, *Arch. Oral Biol.*, 34 (1989) 109.