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Capillary electrophoretic drug monitoring of methotrexate and leucovorin and their metabolites[1]

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Abstract

High-dose methotrexate is an important element of treatment protocols in childhood acute lymphocytic leukemia or osteosarcoma. A capillary electrophoretic method has been developed to measure peak levels and the metabolite pattern in patients with delayed methotrexate elimination. It serves to determine plasma levels of methotrexate, leucovorin and their metabolites, 7-hydroxymethotrexate, 2,4-diamino-N^{10}-methylpteroic acid and 5-methyltetrahydrofolic acid. For the determination of high concentrations (>10 μM) protein precipitation by acetonitrile will suffice for sample preparation. All other samples undergo solid-phase extraction and upgrading on C_{18} columns. Aminopterin, a therapeutic antecedent of methotrexate, serves as internal standard. Detection is done on a UV detector with a 300 nm filter. Five hundred μl of serum are needed to determine 0.2 μM of a specified substance (0.5 μM 5-methylterahydrofolic acid) with good precision and accuracy. For peak levels, 20 μl of capillary serum are sufficient.

Keywords: Methotrexate; Leucovorin

1. Introduction

For more than 40 years now the cytotoxic agent methotrexate has been employed in the therapy of various malignancies [1–3]. Methotrexate (MTX, Fig. 1) inhibits the dihydrofolic acid reductase (DHFR) and consequently leads to a deficiency in reduced folates which supply the organism with one-carbon-units for the biosynthesis of nucleic acids and some amino acids (e.g., methionine) [4–6]. In the cell, MTX, like the naturally occurring folates is converted by folypolyglutamyl transferase into polyglutamates (PGns, with up to seven glutamine units) [7,8]. Those polyglutamates stay in the cell for a longer time (higher negative charge and molecular mass) [9–12]) and inhibit additional enzymes such as thymidilate synthase (TS) [13] and aminoimidazole-ribonucleotide-transformylase (AICAR) [14]. In the liver, MTX undergoes metabolic degradation to 7-hydroxymethotrexate (7-OH-MTX, Fig. 2) by the action of unspecific aldehyde oxidases [15,16]. 7-OH-MTX is an active metabolite which, however, exhibits only 1/100–1/200 of the original MTX activity regarding the inhibition of DHFR [17]. Enterohepatic circulation by the action of bacteria from the intestinal flora supplies another MTX

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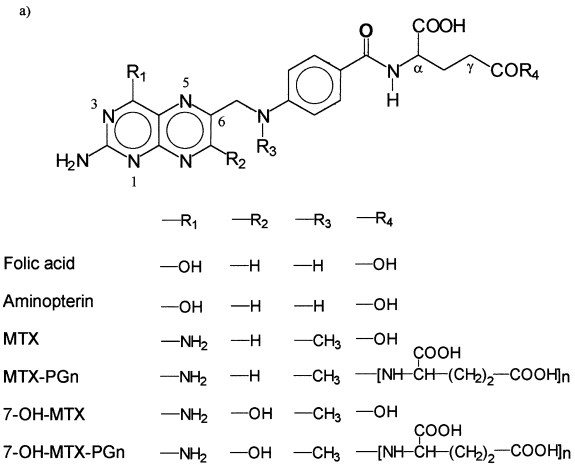
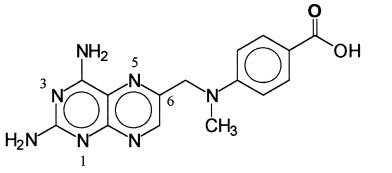




Dampa = 2,4-Diamino-N 10-methylpteroic acid

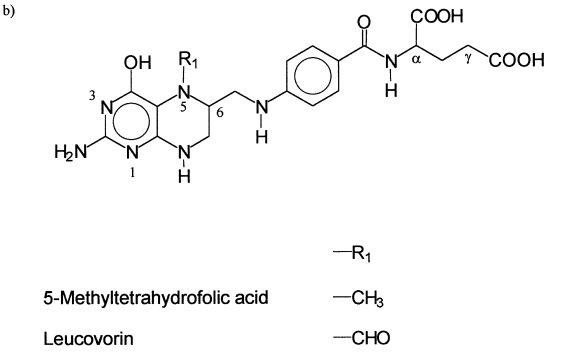
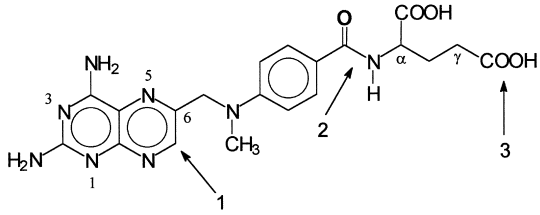


Fig. 1. (a) Structures of folic acid and the antifolates methotrexate (MTX), 7-hydroxymethotrexate (7-OH-MTX), 2,4-diamino-N^{10}-methylpteroic acid (Dampa), aminopterin (AMP) and some polyglutamates (PGns). (b) Structures of the folates leucovorin (LV) and 5-methyltetrahydrofolic acid (5-MTHF).



1 Hydroxylation: 7-OH-MTX
2 Cleavage: Dampa
3 Polyglutamation: MTX-PGn, 7-OH-PGn

Fig. 2. Metabolism of methotrexate.

metabolite, 2,4-diamino-N^{10}-methylpteroic acid (Dampa) [18]. Clinically, this metabolite which has only 1/200 of MTX activity and is produced at a rate of only 4% [18], is of minor importance. Both metabolites, however, interfere with the determination of MTX by enzyme immunoassays such as the EMIT methotrexate test which is routinely used in many hospitals [19,20]. Pronounced crossreactions with this test may lead to false results [21]. As a consequence, such tests are inappropriate when carboxy peptidase G2 is used for rescue in high-dose MTX therapy. The enzyme splits MTX and polyglutamates to form Dampa (Fig. 2). High-dose methotrexate (>500 mg/m^2) combined with leucovorin has been found to improve outcome in acute lymphatic leukemia (ALL), lymphoma and osteosarcoma [22–25]. The application-related impact of the metabolites regarding efficacy and toxicity of high-dose MTX therapy has not been clarified yet. 7-OH-MTX for example competes with MTX regarding both the transport into the cell by the reduced folate carrier (RFC) and the polyglutamation as well as the elimination [26,27]. Desensitization of the cell towards MTX due to 7-OH-MTX is also discussed. Studies have been hampered by large inter- and intra-individual variation of 7-OH-MTX formation [16,28,29]. The capillary electrophoresis (CE) method described in this paper has, on the one hand, been developed to study the metabolite pattern in those patients with delayed elimination or signs of toxicity for specific features which might allow an early diagnosis and treatment. Peak levels of MTX after high-dose infusions are discussed to be important prognostic variables with both 1 g/m^2/24 h infusion (ALL) as well as 12 g/m^2/4 h infusion (osteosarcoma). For the treatment of ALL, for exam-

ple, many protocols (ALL-BFM 95, POG 8698) employ repeated infusions of 1–5 g MTX/m^2/24 h. The peak level obtained after the end of the infusion is considered to be relevant with regard to treatment outcome (patients with MTX>16 μ*M* [22] or >11 μ*M* [30] show a lower rate of relapse). On the other hand, routine monitoring of the MTX peak levels was introduced for osteosarcoma patients to determine their relevance for the patients outcome (12 g/m^2/4 h). The described method is used to examine clarify prospectively if it is a peak level of e.g., 1000 μ*M* or the area under the curve (AUC) or the extent of production of the main metabolite 7-OH-MTX which bears prognostic significance with regard to the patients' outcome. The high-performance liquid chromatography (HPLC) methods reported in the literature entail long retention times [31,32], exceedingly large sample volumes [29,33–36] or the need for special equipment [37–39] all of which are disadvantageous. The method presented here, however, is very fast with analysis times below 5 min, and requires only 20 μl of serum to determine up to 10 μ*M* of substance. Moreover, the method would allow to perform routine drug monitoring of aminopterine, a long disregarded therapeutic antecedent of MTX, which currently seems to undergo a revival regarding clinical application [40].

2. Experimental

2.1. Chemicals and materials

Methotrexate, 2,4-diamino-N^{10}-methylpteroic acid, leucovorin, 5-methyltetrahydrofolic acid and aminopterin were supplied by Sigma (Munich, Germany). 7-Hydroxymethotrexate was purchased from Dr. B. Schirks Labs. (Jona, Switzerland). All other chemicals, except methanol and acetonitrile which were obtained from Baker (Deventer, The Netherlands), were from Merck (Darmstadt, Germany). Buffers used for electrophoresis were filtered through Millipore membranes (0.45 μm) every day. Stock solutions were prepared in 10 m*M* NaOH and stored at −20°C. All stock solutions except 5-MTHF, which was stable for at least four months, remained stable for about 12 months. All chemicals used in this study were of analytical grade and commercially available.

2.2. Instrumentation and separation

A Beckman P/ACE 5510 system (Beckman Instruments, Palo Alto, CA, USA) was used for the analysis. The electrophoretic separation was accomplished using a 90 m*M* phosphate buffer, pH 5.9, with 8 mg/ml hydroxypropyl-β-cyclodextrin (HP-β-CD) and a 30/37 cm×50 μm I.D. fused-silica capillary with an extended light path (Hewlett-Packard). The applied voltage was 540 V/cm resulting in a typical current of 80 μA. Detection was performed with a UV detector at 300 nm. Before each injection (pressure, 5 s) the capillary was rinsed with 0.1 *M* NaOH and running buffer each for 1 min, after each run it was rinsed with double distilled water for 2 min.

2.3. Preparation of standard solutions

Stock solutions were prepared at concentrations of 10 m*M* from MTX and 7-OH-MTX and 50 μ*M* from all other substances, the solutions were then divided and stored at −20°C. 5-MTHF remains stable for four months, all other substances for 12 months. Diluting with water serves to prepare calibration solutions at the concentrations of the calibration points. The internal standard is adjusted to a concentration of 0.1 mg/ml. Samples for quality control are separately adjusted and also determined at each analysis.

2.4. Sample preparation for determination of all substances (folates and antifolates), 500 μl of serum is required

Blood samples were collected in serum tubes and centrifuged at 3000 *g* for 10 min. The serum (minimum 500 μl) was separated and about 5 mg ascorbic acid/ml serum was added to protect oxidizing of 5-MTHF. The samples were then frozen until analysis. After adding 25 μl of a solution of aminopterin (0.1 mg/ml) to each sample (500 μl) it was mixed with 1.25 ml of buffer A, a simple dilution of 0.1 *M* citric acid and 0.2 *M* disodium hydrogenphosphate, pH 5.0, with double distilled water, was admixed. The solutions were loaded onto C_{18} cartridges (Bakerbond) which had been activated with 3 ml methanol and 3 ml of buffer B (two-fold

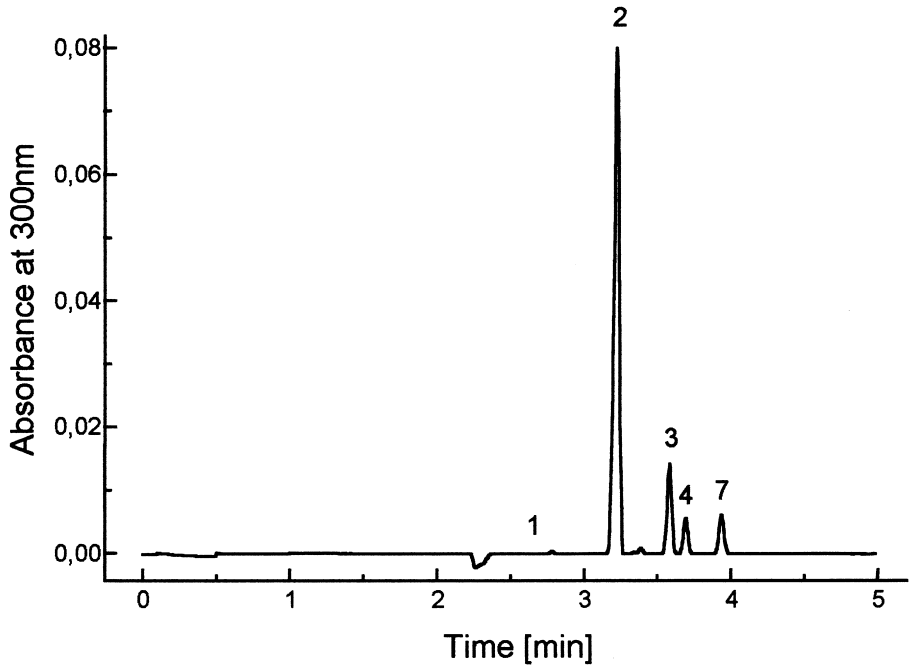


Fig. 3. Electropherogram obtained from a patient at the end of an infusion of 12 g/m^2/4 h MTX [found: MTX (2) 1343 μ*M* and 7-OH-MTX (4) 67.3 μ*M*, folic acid (7) not determined].

dilution of buffer A). The cartridges were then rinsed with 1.5 ml of buffer B and 1.5 ml double distilled water, and dried by air aspiration. Finally, the analytes were extracted twice with 0.5 ml of methanol. The eluate was evaporated at 35°C under a stream of nitrogen, and the residue was redissolved in 25 μl of 22.5 m*M* running buffer.

2.5. Sample preparation for determination of peak levels of MTX and 7-OH-MTX (down to 10 μM), 20 μl of serum is required

Blood samples were collected in capillary serum tubes and centrifuged at 3000 *g* for 10 min. The serum was separated and frozen at −20°C until analysis. A sample aliquot (20 μl) was mixed with 10 μl internal standard (1 mg/ml aminopterin) and 20 μl buffer A, and was vortex mixed for 1 min. To each sample 100 μl of cold acetonitrile was added, the sample was then vortex mixed for 1 min and ultrasound-treated for 1 min. Subsequently, all samples were centrifuged at 15°C, 21 000 rpm, for 15 min. The supernatant was then ready to be injected into the CE system.

Standard samples were prepared by spiking drug-free serum with known amounts of MTX, 7-OH-MTX, DAMPA, 5-MTHF and LV.

3. Results and discussion

3.1. Method development

A capillary with extended detection window (bubble cell) was used to lower the detection limit of the UV detection. Depending on the particular batch of capillaries commercially obtained a 2–3-fold improvement was achieved while there was no loss in separation capacity. Under current experimental conditions a regular 90 m*M* phosphate buffer would not reliably separate all substances. This could, however, be achieved by adding 8 mg/ml of HP-β-CD. Although HP-β-CD is a chiral selector, it does not lead to the separation of leucovorin, a mixture of stereoisomers, (only 6-*S*-LV is ascribed clinical

efficacy, because only 6-*S*-LV is metabolized to 6-*S*-5-MTHF, while 6-*R*-LV is eliminated unchanged). This admixture also allows to employ the internal standard aminopterin, which structurally differs by only one amino group from methotrexate (Fig. 1). An internal standard is needed since the sample application by means of pressure is subject to variations and not reliably constant. Furthermore, errors in sample preparation are thus compensated for. At a pH of 5.9 all substances are charged and are detected after the electroosmotic flow (EOF) which is apparent after about 2.3 min (Fig. 3). Variations in migration times hamper the analysis and interpretation of the results with many CE methods. Fig. 4 shows the migration times of the various agents on 15 consecutive days. At 1.2% for DAMPA, the substance eluted first, and 2.2% for leucovorin which appears last, the standard deviations of the migration times show a very good range. Analysing the relationship between migration times and EOF or the first eluted substance, DAMPA, standard deviations are found to be further reduced. Aided by the low count of interfering peaks in the blank plasma, the peaks in the electropherogram can thus in principle be easily identified (Fig. 5).

In order to remove proteins and other interfering substances from samples before folates and antifolates are determined, those samples undergo solid-phase extraction (SPE), since protein precipitation by heat or trichloroacetic acid leads to splitting of the folates [19,30]. Belz et al. [31] reported large substance loss when protein precipitation was done by means of perchloric acid, even in the presence of ascorbic acid. This effect is avoided by SPE. With a starting volume of 500 μl serum, SPE also serves to ensure upgrading of the samples after resumption in 25 μl of running buffer by a factor of 20. Thus, one arrives at the following theoretical limits of quantifi-

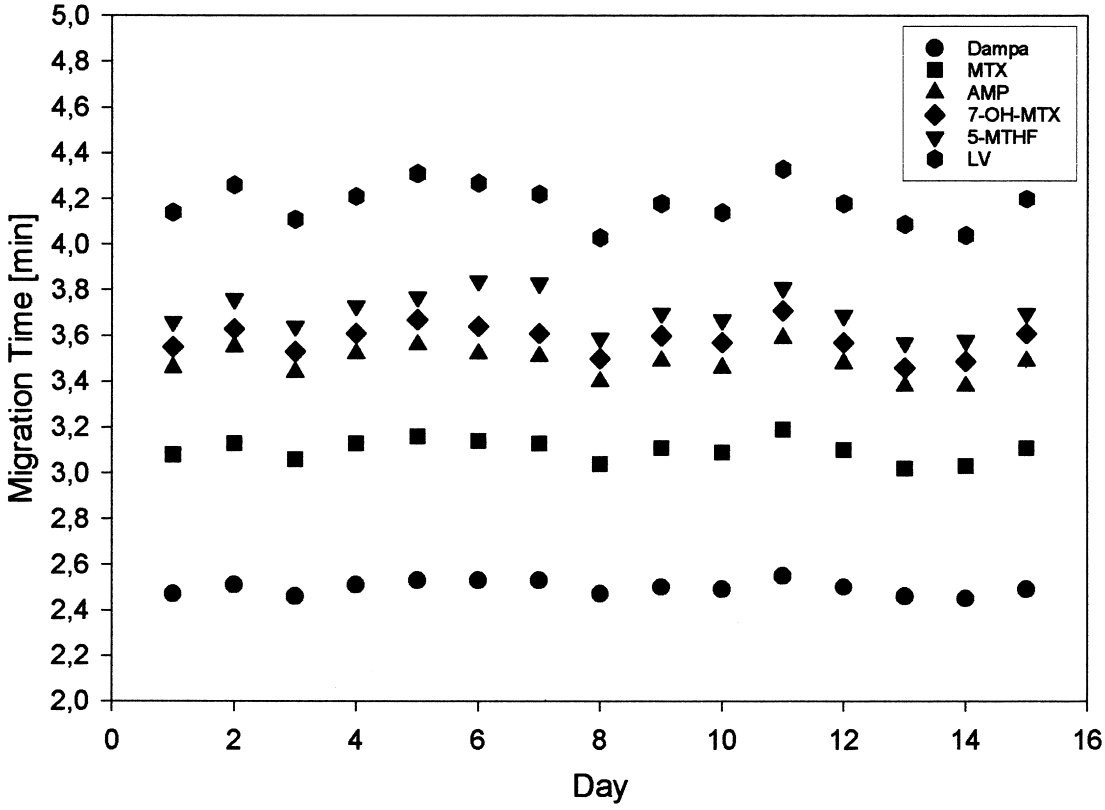


Fig. 4. Migration behaviour of Dampa, MTX, AMP, 7-OH-MTX, 5-MTHF and LV on 15 consecutive days (for CE conditions see Section 2.2).

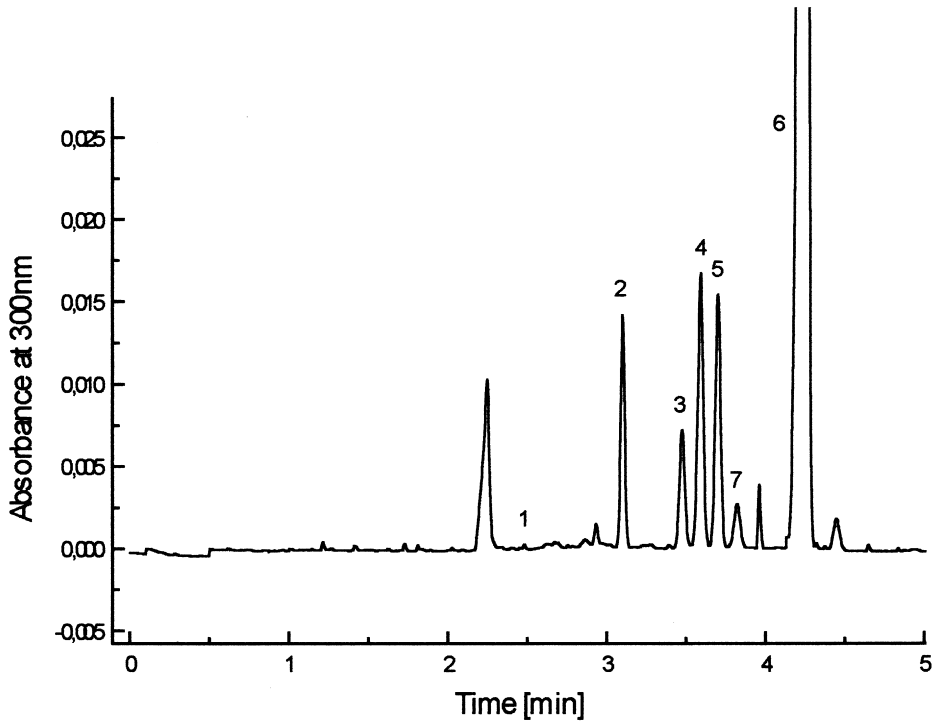


Fig. 5. Plasma sample of a patient (5 g/m^2/24 h MTX) with delayed elimination of MTX 60 h after the start of infusion [Dampa (1), MTX (2), I.S. (3), 7-OH-MTX (4), 5-MTHF (5), LV (6), folic acid (7)].

cation (min. peak height 200): DAMPA 0.07, MTX 0.038, 7-OH-MTX 0.068, 5-MTHF 0.091, LV 0.088 μ*M*. Resuming the residue in a 22.5 m*M* running buffer dilution will ensure slight sample stacking as well as an improved peak definition and peak symmetry. Thus, reducing the required sample volume to 100 μl, which would allow capillary blood sampling, is well within reach.

3.2. Quantitation

The concentrations of individual substances are done by calculating the ratio between the peak heights of the substance in question and those of the internal standard. Calibration curves are obtained by spiking blank plasma with the respective concentrations of the substances (for peak levels 10–1800 μ*M* for MTX and 10–300 μ*M* for 7-OH-MTX and for the rest 0.2–20 μ*M*).

3.3. Linearity and reproducibility

A linear relationship for the antifolates MTX, 7-OH-MTX and DAMPA was found over a calibration range of 0.2–20 μ*M*, and for LV for a range of 0.2–100 μ*M*. For concentrations above 10 μ*M* the calibration was done from 10–1800 μ*M* for MTX, and 10–200 μ*M* for 7-OH-MTX. The inter- and intra-day accuracy and precision was assessed by preparing and measuring four different levels of sample concentration six times (Tables 1 and 2) on six consecutive days. The correlation coefficients for all substances were above 0.99, and the accuracy and precision data are all within the range required for bioanalytical test methods [41]. The absolute recovery of all substances from human plasma after SPE was examined at four different concentrations: 0.2, 0.5, 3 and 7 μ*M*. A mean recovery of 92.7% MTX, 89.8% Dampa, 87.2% 7-OH-MTX, 85.8% 5-MTHF, and 84.4% LV was found. The limit of

Table 1
Accuracy and precision of the assay with solid-phase extraction

	MTX	7-OH-MTX	Dampa	5-MTHF	LV
Within-day reproducibility (determined from one standard curve on a given day)					
Added (μM) ($n=6$)	7.0000	7.0000	7.0000	15.0000	7.0000
Found (μM)					
Mean	7.0020	6.9265	6.9270	14.9512	6.9358
S.D.	0.1348	0.1811	0.1829	0.5098	0.1679
C.V.	1.93	2.61	2.64	3.41	2.42
Accuracy	100.03	98.95	98.96	99.67	99.08
Added (μM) ($n=6$)	3.0000	3.0000	3.0000	7.0000	3.0000
Found (μM)					
Mean	2.9730	3.0384	2.9414	6.9011	2.9650
S.D.	0.0512	0.1454	0.1104	0.2620	0.1690
C.V.	1.72	4.79	3.75	3.80	5.70
Accuracy	99.10	101.28	98.05	98.59	98.83
Added (μM) ($n=6$)	0.5000	0.5000	0.5000	3.0000	0.5000
Found (μM)					
Mean	0.5061	0.4981	0.5031	3.0451	0.4901
S.D.	0.0361	0.0232	0.0351	0.2040	0.0299
C.V.	7.13	4.66	6.98	6.70	6.10
Accuracy	101.22	99.62	100.62	101.50	98.02
Added (μM) ($n=6$)	0.2000	0.2000	0.2000	1.0000	0.2000
Found (μM)					
Mean	0.1981	0.1987	0.2090	0.9910	0.1910
S.D.	0.0182	0.0191	0.0190	0.0912	0.0206
C.V.	9.19	9.61	9.09	9.20	10.79
Accuracy	99.05	99.35	104.50	99.10	95.50
Day-to-day reproducibility (determined from single standard curves on different days)					
Added (μM) ($n=6$)	7.0000	7.0000	7.0000	15.0000	7.0000
Found (μM)					
Mean	7.0050	6.8695	6.9141	14.9006	6.9122
S.D.	0.1308	0.1724	0.1797	0.4948	0.1635
C.V.	1.87	2.51	2.6	3.32	2.37
Accuracy	100.07	98.14	98.77	99.34	98.75
Added (μM) ($n=6$)	3.0000	3.0000	3.0000	7.0000	3.0000
Found (μM)					
Mean	2.9420	3.0862	2.9242	6.8656	2.9595
S.D.	0.0476	0.1437	0.1139	0.2605	0.2026
C.V.	1.62	4.66	3.89	3.79	6.84
Accuracy	98.07	102.87	97.47	98.08	98.65
Added (μM) ($n=6$)	0.5000	0.5000	0.5000	3.0000	0.5000
Found (μM)					
Mean	0.5076	0.4961	0.5065	3.0562	0.4822
S.D.	0.0333	0.0204	0.0328	0.1975	0.0266
C.V.	6.55	4.12	6.48	6.46	5.51
Accuracy	101.52	99.22	101.30	101.87	96.44
Added (μM) ($n=6$)	0.2000	0.2000	0.2000	1.0000	0.2000
Found (μM)					
Mean	0.1985	0.1989	0.2055	1.0189	0.2080
S.D.	0.0195	0.0198	0.0201	0.0960	0.0211
C.V.	9.82	9.95	9.78	9.42	10.14
Accuracy	99.25	99.45	102.75	101.89	104.00

Table 2
Accuracy and precision of the assay with deproteinization

	MTX	7-OH-MTX
Within-day reproducibility (determined from one standard curve on a given day)		
Added (μ*M*) (*n*=6)	200.00	20.00
Found (μ*M*)		
Mean	197.21	21.76
S.D.	8.40	1.91
C.V.	4.26	8.78
Accuracy	98.61	108.80
Added (μ*M*) (*n*=6)	500.00	50.00
Found (μ*M*)		
Mean	492.92	49.57
S.D.	17.67	1.51
C.V.	3.58	3.05
Accuracy	98.58	99.14
Added (μ*M*) (*n*=6)	1100.00	100.00
Found (μ*M*)		
Mean	1110.23	98.89
S.D.	27.89	2.97
C.V.	2.51	3.00
Accuracy	100.93	98.89
Added (μ*M*) (*n*=6)	1500.00	150.00
Found (μ*M*)		
Mean	1492.34	148.21
S.D.	26.49	4.93
C.V.	1.78	3.33
Accuracy	99.49	98.81
Day-to-day reproducibility (determined from single standard curves on different days)		
Added (μ*M*) (*n*=6)	200.00	20.00
Found (μ*M*)		
Mean	198.62	19.51
S.D.	4.58	1.33
C.V.	2.31	6.82
Accuracy	99.31	97.55
Added (μ*M*) (*n*=6)	500.00	50.00
Found (μ*M*)		
Mean	491.04	49.71
S.D.	11.87	1.36
C.V.	2.42	2.74
Accuracy	98.21	99.42
Added (μ*M*) (*n*=6)	1100.00	100.00
Found (μ*M*)		
Mean	1109.00	101.06
S.D.	31.55	2.29
C.V.	2.84	2.27
Accuracy	100.82	101.06
Added (μ*M*) (*n*=6)	1500.00	150.00
Found (μ*M*)		
Mean	1496.76	151.62
S.D.	18.31	4.61
C.V.	1.22	3.04
Accuracy	99.78	101.08

detection at a signal-to-noise ratio of 3/1 was 0.005 μ*M* for MTX, 0.015 μ*M* for Dampa, 0.02 μ*M* for 7-OH-MTX, 0.05 μ*M* for 5-MTHF and 0.02 μ*M* for LV. For the determination of peak levels of MTX and 7-OH-MTX the absolute recoveries were 99.7% and 99.4%, respectively.

3.4. Clinical applications

High-dose methotrexate (>500 mg/m^2), combined with leucovorin as a rescue agent, has considerably improved treatment outcome in many malignancies (e.g., acute lymphatic leukemia, non-Hodgkin lymphoma or osteosarcoma) [22,42,43]. The optimal dosage and schedule in the treatment of e.g., ALL or NHL have remained controversial. Discussions focus on the MTX dose (1 g/m^2/24 h up to 5 g/m^2/24 h) and infusion time as well as the dose of the rescue agent and the time and duration of leucovorin application.

With the method presented here both methotrexate and its metabolites Dampa, 7-OH-MTX and 5-MTHF, can be measured, and it is thus of considerable advantage compared to the routinely used enzymatic test methods which determine MTX only. The metabolites, especially Dampa and to a lesser degree 7-OH-MTX, crossreact with the enzymatic test methods and may thus affect the test results [21].

Since 20 μl of serum suffice to determine peak levels after high-dose methotrexate infusions, samples may be obtained by capillary withdrawal and the strain on the patient is kept to a minimum.

The prognostic impact of MTX peak levels has been discussed with regard to infusions of 1 g/m^2/24 h [30] in the therapy of ALL as well as infusions of 12 g/m^2/4 h [23,44] in osteosarcoma patients. Several authors have reported a positive correlation between MTX peak levels and treatment outcome [23,24,45]. The CE method presented here, which is technically uncomplicated and requires only small sample volumes (20 μl) is a tool which allows fast testing for MTX and its metabolites in future prospective studies.

4. Conclusions

A fast and sensitive CE method to determine methotrexate and leucovorin as well as their metabo-

lites 7-OH-MTX, DAMPA and 5-MTHF from plasma has been developed. The method has been applied in the drug monitoring of those substances in patients undergoing various high-dose MTX treatment protocols (ALL-BFM 95, NHL-BFM 95, COSS 96). The advantage of short analysis times and faster sample preparation as well as smaller sample volumes when determining concentrations above 10 μ*M* are counterbalanced by a higher limit of detection, compared to some HPLC methods, of 0.2 μ*M*. This limit, however, is totally satisfactory for the type of studies described here.

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