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Simultaneous determination of methotrexate and its eight metabolites in human whole blood by capillary zone electrophoresis

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Abstract

A simple and selective capillary electrophoretic method was established for the simultaneous determination of methotrexate (MTX), 7-hydroxymethotrexate (7-OHMTX), 2,4-diamino-N^{10}-methylpteroic acid (DAMPA), and polyglutamate derivatives [MTX–$(Glu)_n$, $n=2–7$] in whole blood. After extraction, those analytes were separated by fused-silica capillary and a running buffer containing glycine (1.2 *M*, pH 9.3). The quantitative ranges were 10–50 μ*M* for each analyte. The intra- and inter-day RSD and RE values were all less than 6 and 11%, respectively. The limits of detection ($S/N=3$, injection 5 s) were found to be 1 μ*M* for MTX, 7-OHMTX, MTX–$(Glu)_2$, MTX–$(Glu)_3$, and MTX–$(Glu)_4$; 3 μ*M* for MTX–$(Glu)_5$ and MTX–$(Glu)_6$; 5 μ*M* for MTX–$(Glu)_7$, and 8 μ*M* for DAMPA. All recoveries were greater than 94%. This method was applied to blood MTX monitoring in a patient with acute lymphoblastic leukemia.

Keywords: Validation; Methotrexate; Hydroxymethotrexate; Diaminomethylpteroic acid; Methotrexate polyglutamates; Protriptyline

1. Introduction

Methotrexate (MTX, Fig. 1) is a folic acid antagonist that binds to the active catalytic site of dihydrofolate reductase (DHFR), and interferes with the formation of DNA, RNA, and protein [1,2]. MTX is widely used in the chemotherapy of various neoplastic diseases, such as acute leukemia and osteogenic sarcoma [3]. Intracellular formation of polyglutamate derivatives [MTX–$(Glu)_n$, $n=2–7$, Fig. 1] appears to be important in the therapeutic action of MTX [4]. The polyglutamates are retained by cells longer than MTX and have increased inhibitory effects on enzymes involved in folate metabolism, making them important determinants of the duration of action of MTX [5–7]. As a consequence, polyglutamylation results in a massive enhancement of cytotoxicity [8]. MTX is mainly metabolized to 7-hydroxymethotrexate (7-OHMTX) and to some extent 2,4-diamino-N^{10}-methylpteroic acid (DAMPA). Such a route of metabolism involves a major detoxification pathway [9]. Although these metabolites have much lower affinities for dihydro-

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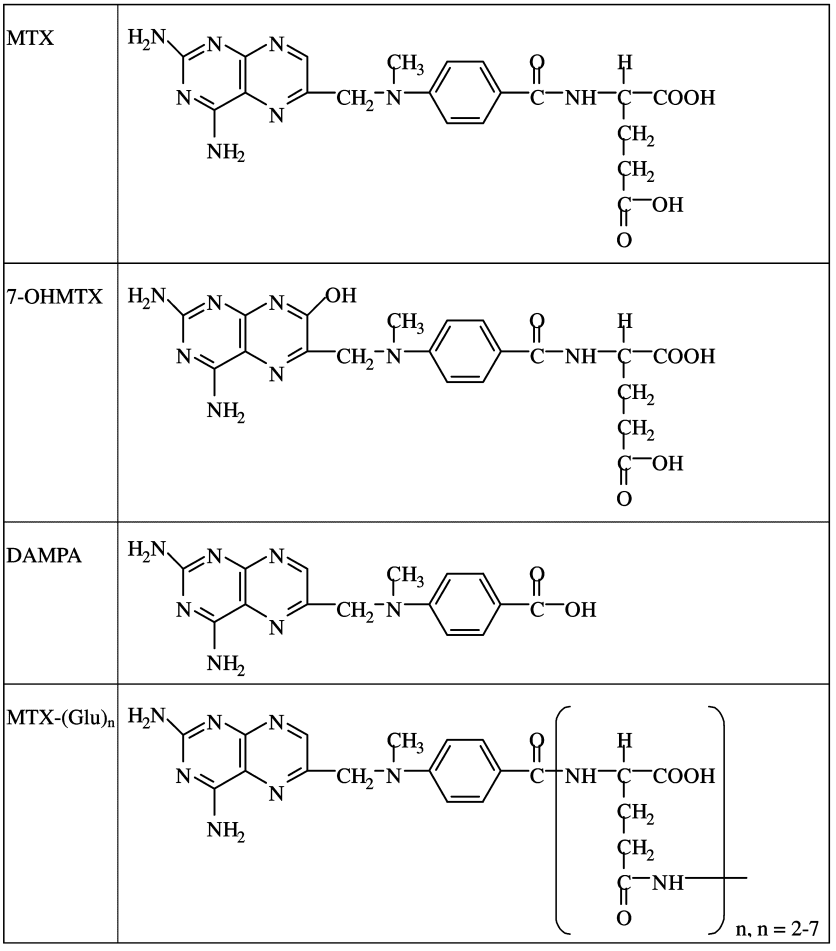

Fig. 1. Structures of MTX, 7-OHMTX, DAMPA and MTX–$(Glu)_n$.

folate reductase, they could influence the determination of MTX because of close structural similarities.

In Rubino's review, more than 70 papers describing chromatographic assays for MTX and its metabolites have been published in the literature between 1975 and 2000 [10]. MTX can also be measured by a number of different analytical techniques, such as immunoenzymatic method [11] and capillary electrophoresis (CE) [12–15]. To date, none of the methods simultaneously determined MTX and its eight metabolites (7-OHMTX, DAMPA and MTX–$(Glu)_n$, n = 2–7).

MTX is one of the very few antineoplastic drugs for which therapeutic drug concentration monitoring is currently employed in clinical practice. In addition, the enzyme activity should be reliably determined. The aim of this study was to establish a simple and rapid CE method for the simultaneous determination of MTX and its eight metabolites. The present paper describes the development and validation of a CE method that takes into account the running buffer. Application of the assay to monitor blood MTX concentration is described. Further evaluation between therapeutic drug monitoring and clinical efficiency is under investigation.

2. Experimental

2.1. Chemicals and reagents

MTX, protriptyline (PTP), ammonium sulfate and perchloric acid (70%) were from Sigma (St. Louis, MO, USA). 7-OHMTX, DAMPA and MTX–$(Glu)_n$, $n=2–7$ were purchased from Sckircks lab. (Jona, Switzerland). Glycine (Morecambe, Lancaster, UK), ethyl acetate and isopropanol (Tedia, OH, USA) and sodium hydroxide (Merck, Darmstadt, Germany) were used without further treatment. Milli-Q water (Millipore, Bedford, MA, USA) was used for the preparation of buffer and related aqueous solutions. The stock solutions of each analyte were prepared in 0.1 *M* NaOH at 1 m*M* and suitably diluted to working solutions (10–50 μ*M*) for method optimization and validation. All the stock solutions were stored under −70 °C. PTP was prepared in water and used as internal standard (I.S.).

2.2. CE system

Method development was performed on a Beckman P/ACE MDQ system (Fullerton, CA, USA). The analysis was carried out in an untreated fused-silica capillary, 40.2 cm (30 cm to detector)×50 μm I.D. The background electrolyte (BGE) was glycine buffer (1.2 *M*, pH 9.3). The detector wavelength was set at 300 nm. The sample was injected by pressure [0.5 p.s.i. (1 p.s.i. = 6894.76 Pa) for 5 s] at the anodic end of the capillary. All separations were achieved at 25 °C by 20 kV and computer-controlled using P/ACE system MDQ software. The capillary was conditioned with 0.1 *M* sodium hydroxide (20 min), water (10 min) and BGE (20 min) at 25 °C before start-up. The wash sequence between runs was by 0.1 *M* sodium hydroxide (1 min), water (1 min) followed by BGE (2 min).

2.3. Preparation of whole blood standard solutions

Drug-free whole blood was donated from the healthy volunteers and stored at 4 °C. Aliquots (0.2–1.0 ml) of the stock solution of each analyte (1 m*M*) were transferred to a 20-ml volumetric flask and diluted with drug-free whole blood to prepare calibration solutions at the concentrations (10–50 μ*M*). The solutions were divided and stored at −70 °C.

2.4. Extraction procedures of whole blood sample

The whole blood samples from patients were collected and frozen at −70 °C until analysis. Modifying the treatment procedure [16], the whole blood samples (500 μl each) were added to 30 μl of

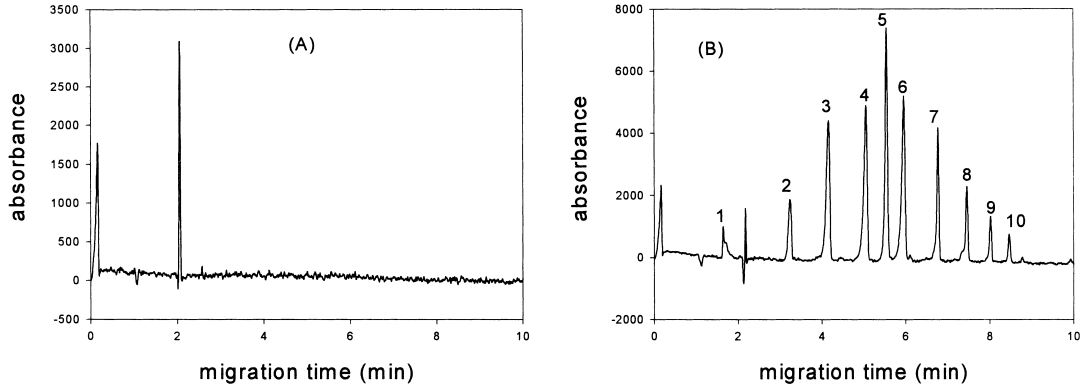

Fig. 2. Electropherograms of MTX and its metabolites in whole blood after sample extraction. (A) blank (B) analytes spiked. Peaks: 1 = PTP; 2 = DAMPA; 3 = MTX; 4 = MTX–$(Glu)_2$; 5 = 7-OHMTX; 6 = MTX–$(Glu)_3$; 7 = MTX–$(Glu)_4$; 8 = MTX–$(Glu)_5$; 9 = MTX–$(Glu)_6$; 10 = MTX–$(Glu)_7$. CZE conditions: buffer, 1.2 *M* glycine (pH 9.3); applied voltage, 20 kV (detector at cathode side); uncoated fused-silica capillary, 30 cm (effective length)×50 μm I.D.; sample injection, 5 s by pressure; wavelength, 300 nm.

perchloric acid, then vortexed for 1 min and centrifuged at 12 000 rpm for 5 min. The denatured supernatant was transferred to a tube containing 500 mg of $(NH_4)_2SO_4$, then 1 ml of extraction solvent (ethyl acetate–isopropanol, 10:1, v/v) was added. After vortexing (1 min) and centrifuging (3000 rpm, 5 min), the organic layer was evaporated under a gentle stream of nitrogen. The residue was reconstituted with 30 μl of 0.1 *M* NaOH and 20 μl of 1 m*M* PTP before CE analysis.

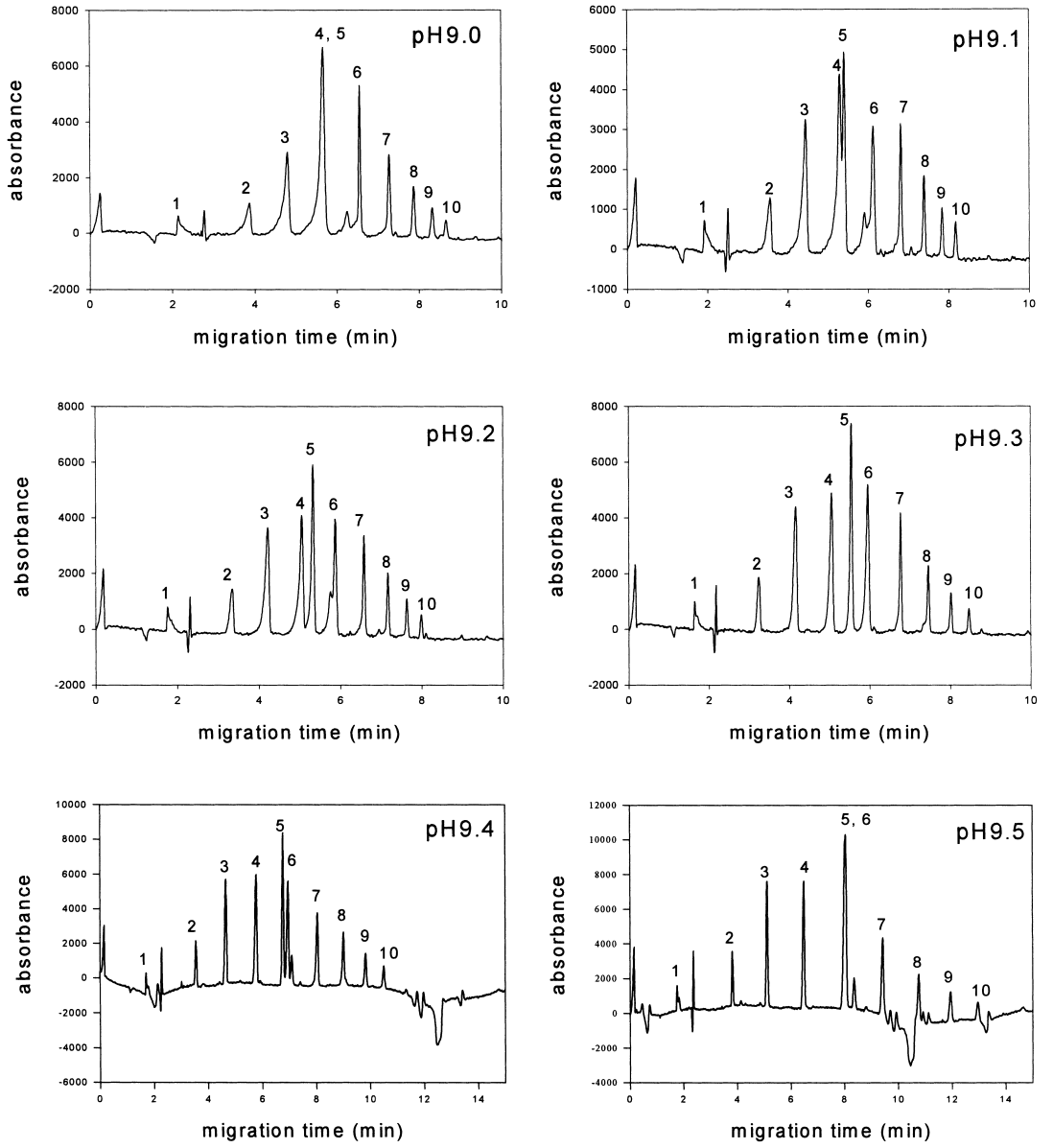

Fig. 3. Effects of pH of glycine buffer on analyte separation in whole blood. Peaks: 1 = I.S.; 2 = DAMPA; 3 = MTX; 4 = MTX–$(Glu)_2$; 5 = 7-OHMTX; 6 = MTX–$(Glu)_3$; 7 = MTX–$(Glu)_4$; 8 = MTX–$(Glu)_5$; 9 = MTX–$(Glu)_6$; 10 = MTX–$(Glu)_7$.

2.5. Method validation parameters

The proposed method for the quantitative determination of MTX and its metabolites in whole blood was validated by a set of parameters. Calibration curves were established with the corrected peak-area ratio of each analyte to I.S. as ordinate (y) versus the concentration of each analyte as abscissa (x) following the procedure in Section 2.4. Intra-day precision was tested by analyzing three identically spiked whole blood samples for three concentrations: 25, 35, 45 μM for each analyte. Inter-day precision was calculated from repeated analysis of identically spiked samples on 5 successive days for these three concentrations of those analytes. Their relative recoveries from the matrix were determined by calculating concentration divided with spiked concentration. The limits of detection were determined by spiking samples with decreasing concentrations of each analyte until the signal-to-noise ratio equalled 3 ($S/N=3$).

3. Results and discussion

Optimized methods allow simultaneous measurement of the MTX and its metabolites within a few minutes as shown in Fig. 2. Effects of concentration and pH of glycine buffer were studied to determine the optimal separation conditions.

3.1. Concentration and pH of glycine buffer

Capillary zone electrophoresis (CZE) of the analytes using glycine buffer (pH 9.3) at different concentration was studied. Glycine is a zwitterionic buffer. Its advantage is the low current draw and thus reduced Joule heating. The results indicate that baseline resolution is obtainable when glycine buffers were greater than 1 M. The optimal concentration is set at 1.2 M, even in this high level the current is 156 μA.

Different pH values of the glycine buffer (1.2 M) were studied. The peaks are broad and migrate

Table 1
Regression analysis for the determinations of MTX and its metabolites in whole blood

Concentration range	Regression equation	r^2
Intra-day[a] (10.0–50.0 μM)		
DAMPA	$y=(0.0232\pm0.0005)x-(0.1751\pm0.0108)$	0.9930
MTX	$y=(0.0478\pm0.0013)x-(0.2371\pm0.0231)$	0.9931
MTX–$(Glu)_2$	$y=(0.0423\pm0.0002)x-(0.1493\pm0.0152)$	0.9967
7-OHMTX	$y=(0.0379\pm0.0006)x-(0.0858\pm0.0251)$	0.9985
MTX–$(Glu)_3$	$y=(0.0364\pm0.0090)x-(0.01333\pm0.0588)$	0.9969
MTX–$(Glu)_4$	$y=(0.0183\pm0.0009)x-(0.0485\pm0.0107)$	0.9974
MTX–$(Glu)_5$	$y=(0.0111\pm0.0004)x-(0.0269\pm0.0165)$	0.9968
MTX–$(Glu)_6$	$y=(0.0054\pm0.0001)x-(0.0049\pm0.0048)$	0.9979
MTX–$(Glu)_7$	$y=(0.0032\pm0.0002)x-(0.0016\pm0.0058)$	0.9973
Inter-day[b] (10.0–50.0 μM)		
DAMPA	$y=(0.0222\pm0.0009)x-(0.1513\pm0.0194)$	0.9947
MTX	$y=(0.0445\pm0.0023)x-(0.1815\pm0.0372)$	0.9953
MTX–$(Glu)_2$	$y=(0.0385\pm0.0029)x-(0.0950\pm0.0402)$	0.9968
7-OHMTX	$y=(0.0347\pm0.0033)x-(0.0171\pm0.0576)$	0.9968
MTX–$(Glu)_3$	$y=(0.0345\pm0.0036)x-(.01133\pm0.0467)$	0.9963
MTX–$(Glu)_4$	$y=(0.0160\pm0.0016)x-(0.0143\pm0.0232)$	0.9969
MTX–$(Glu)_5$	$y=(0.0101\pm0.0009)x-(0.0178\pm0.0072)$	0.9978
MTX–$(Glu)_6$	$y=(0.0047\pm0.0005)x-(0.0028\pm0.0052)$	0.9966
MTX–$(Glu)_7$	$y=(0.0032\pm0.0015)x-(0.0032\pm0.0080)$	0.9971

[a] The regression equations of intra-day analysis were calculated from the assay values of prepared standards on a single day ($n=3$).
[b] The regression equations of inter-day analysis were calculated from the assay values of prepared standards on 5 different days ($n=5$).

slowly when pH<9. Fig. 3 shows that a baseline resolution was obtained at pH 9.3.

3.2. Method validation

For evaluating the quantitative applicability of the method, five different concentration of whole blood sample solution were analyzed using PTP as I.S. The regression lines of all analytes were quantitative over the range of 10.0–50.0 μ*M*. The r^2 values were all >0.9930 in intra- and inter-day analysis (Table 1). The detection limit ($S/N=3$, injection 5 s) were found to be 1 μ*M* for MTX, 7-OHMTX and MTX–$(Glu)_{n=2-4}$, 3 μ*M* for MTX–$(Glu)_{n=5-6}$; 5 μ*M* for MTX–$(Glu)_{n=7}$, and 8 μ*M* for DAMPA. The reproducibility and reliability of the proposed method for spiked samples were assessed at three different concentrations and evaluated as RSD and relative error (RE). The precision and accuracy of the method for each analyte spiked in human whole blood were less than 5.92% for RSD and −10.80% for RE for intra- and inter-day assays (Tables 2 and 3). The relative recoveries of all analytes were more than 94.3% (Table 4). MTX and its metabolites spiked in human whole blood were stable for 30 days under −70 °C. Comparing with all published papers,

Table 2
Precision and accuracy for the determination of MTX and its metabolites in whole blood in an intra-day analysis

	Concentration known (μ*M*)	Concentration found (μ*M*)	RSD (%)	R.E. (%)
Intra-day analysis ($n=3$)				
DAMPA	25	22.30±0.06	0.27	−10.80
	35	32.72±0.05	0.15	−6.51
	45	45.59±1.48	3.25	1.31
MTX	25	23.80±0.10	0.42	−4.80
	35	34.70±0.23	0.66	−0.86
	45	45.48±0.60	1.32	1.07
MTX–$(Glu)_2$	25	24.08±0.14	0.58	−3.68
	35	33.07±0.47	1.42	−5.51
	45	46.62±0.75	1.61	3.60
7-OHMTX	25	23.46±0.14	0.60	−6.16
	35	36.58±0.45	1.23	4.51
	45	46.88±0.04	0.09	4.18
MTX-$(Glu)_3$	25	23.40±0.19	0.81	−6.40
	35	34.21±0.43	1.26	−2.26
	45	46.60±0.07	0.15	3.56
MTX–$(Glu)_4$	25	26.12±0.31	1.19	4.48
	35	35.87±0.08	0.22	2.49
	45	45.75±0.10	0.22	1.67
MTX–$(Glu)_5$	25	26.20±0.45	1.72	4.80
	35	37.47±0.65	1.73	7.06
	45	47.52±0.21	0.44	5.60
MTX–$(Glu)_6$	25	26.33±0.44	1.67	5.32
	35	36.67±0.16	0.44	4.77
	45	45.15±0.76	1.68	0.33
MTX–$(Glu)_7$	25	26.04±0.30	1.15	4.16
	35	34.94±0.98	2.80	−0.17
	45	45.52±0.74	1.63	1.16

Table 3
Precision and accuracy for the determination of MTX and its metabolites in whole blood in an inter-day analysis

	Concentration known (μ*M*)	Concentration found (μ*M*)	RSD (%)	RE (%)
Inter-day analysis ($n=5$)				
DAMPA	25	22.33±0.11	0.49	−10.68
	35	32.65±0.63	1.92	−6.71
	45	43.88±2.02	4.60	−2.49
MTX	25	23.43±0.53	2.26	−6.28
	35	33.94±0.89	3.72	3.03
	45	45.42±0.66	1.45	0.93
MTX–$(Glu)_2$	25	24.47±1.40	5.66	−1.04
	35	34.32±1.40	4.08	−1.94
	45	46.52±0.68	1.46	3.38
7-OHMTX	25	23.80±1.41	5.92	−4.80
	35	35.61±1.03	2.89	1.74
	45	45.76±1.12	2.45	1.69
MTX–$(Glu)_3$	25	23.97±0.79	3.30	−4.12
	35	34.42±1.61	4.68	−1.66
	45	46.49±0.17	0.37	3.31
MTX–$(Glu)_4$	25	26.10±0.29	1.11	4.40
	35	36.97±1.11	3.00	5.63
	45	46.82±1.21	2.58	4.04
MTX–$(Glu)_5$	25	26.40±0.38	1.44	5.60
	35	36.50±1.51	4.14	4.29
	45	47.32±0.59	1.25	5.16
MTX–$(Glu)_6$	25	26.59±0.41	1.54	6.36
	35	36.16±0.65	1.80	3.31
	45	44.65±0.73	1.63	−0.78
MTX–$(Glu)_7$	25	25.99±0.26	1.00	3.96
	35	35.10±2.04	5.81	0.29
	45	46.40±1.03	2.22	3.11

this method provides the best selectivity and reliable accuracy and precision for a rapid analysis of MTX and its eight metabolites.

3.3. Application in a patient dosing with MTX

An 8-year-old boy with acute lymphoblastic leukemia was treated with MTX (5.0 g/m^2 per 24 h infusion). The blood samples were collected in EDTA-treated tubes at 7 h after stopping infusion and stored at −70 °C until analysis. The electropherogram resulting from the analysis of the blood sample is shown in Fig. 4. With the method presented here both MTX and 7-OHMTX can be measured. Those peaks were confirmed by adding the standards. The blood levels are calculated to be 26.2 μ*M* for MTX and 6.4 μ*M* for 7-OHMTX. MTX blood concentrations of 10–1000 μ*M* have been reported following high-dose MTX therapy (1–15 g/m^2) [17]. Our results are in agreement with this information. High dose MTX is an important element of treatment protocols in childhood acute lymphoblastic leukemia. The CE method presented here allows fast testing for MTX and its metabolites in future prospective studies.

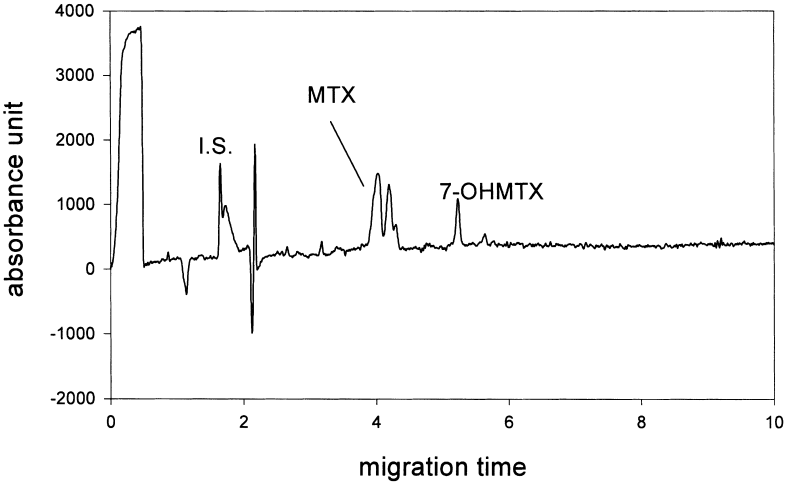

Fig. 4. Electropherogram of blood from one 8-year-old boy with acute lymphoblastic leukemia treated with 5.0 g MTX/m^2 per 24 h.

Table 4
Relative recoveries of MTX and its metabolites in whole blood

Analyte	Concentration spiked (μM)	Concentration found (μM)	Relative recovery (%)
DAMPA	10	11.11±0.36	111.1±3.6
	30	29.63±0.65	98.8±2.2
	50	50.86±0.81	101.7±1.6
MTX	10	11.00±0.48	110.0±4.8
	30	29.40±0.57	98.0±1.9
	50	50.76±0.79	101.5±1.6
MTX–$(Glu)_2$	10	10.51±0.73	105.1±7.3
	30	29.91±0.91	99.7±3.0
	50	50.22±0.48	100.4±1.0
7-OHMTX	10	9.85±0.74	98.5±7.4
	30	30.38±0.89	101.3±3.0
	50	49.69±0.79	99.4±1.6
MTX–$(Glu)_3$	10	10.62±0.51	106.2±5.1
	30	30.10±0.68	100.3±2.3
	50	50.50±0.71	101.0±1.4
MTX–$(Glu)_4$	10	9.71±0.82	97.1±8.2
	30	30.70±0.85	102.3±2.8
	50	49.49±0.41	99.0±0.8
MTX–$(Glu)_5$	10	10.20±0.77	102.0±7.7
	30	30.25±0.42	100.8±1.4
	50	50.03±0.97	100.1±1.9
MTX–$(Glu)_6$	10	9.43±0.47	94.3±4.7
	30	30.85±0.87	102.8±2.9
	50	49.40±0.54	98.8±1.1
MTX–$(Glu)_7$	10	9.71±0.28	97.1±2.8
	30	30.75±0.62	102.5±2.1
	50	49.41±0.91	98.8±1.8

4. Nomenclature

MTX	methotrexate
7-OHMTX	7-hydroxymethotrexate
DAMPA	2,4-diamino-N^{10}-methylpteroic acid
MTX–$(Glu)_n$	methotrexate polyglutamates
PTP	protriptyline
BGE	background electrolyte

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