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ORIGINAL PAPER

# Analysis of methotrexate and its eight metabolites in cerebrospinal fluid by solid-phase extraction and triple-stacking capillary electrophoresis

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**Abstract** We establish a triple-stacking capillary electrophoresis (CE) separation method to monitor methotrexate (MTX) and its eight metabolites in cerebrospinal fluid (CSF). Three stacking methods with different mechanisms were combined and incorporated into CE separation. Complete stacking and sharp peaks were achieved. Firstly, the optimized buffer (60 mM phosphate containing 15% THF and 100 mM SDS) was filled into the capillary, which was followed by the higher conductivity buffer (100 mM phosphate, 2 psi for 45 s). The analytes extracted from CSF were injected at 2 psi for 99.9 s, which provided long sample zones and pH junction for focusing. Finally, the stacking step was performed by sweeping, and separation was achieved by micellar electrokinetic chromatography. The results of the linear regression equations indicated high linearity ($r \geq 0.9981$) over the range of 0.5–7 μM. In intra- and inter-batch results, all data of RSD and RE were below 11%, indicating good precision and accuracy of this method. The LODs (S/$N$=3) were 0.1 μM for MTX, 7-hydroxymethotrexate (7-OHMTX) and MTX-polyglutamates (MTX-$(Glu)_{n,\ n=2-5}$), 0.2 μM for MTX-$(Glu)_6$, and 0.3 μM for 2,4-diamino-$N^{10}$-methylpteroic acid (DAMPA) and MTX-$(Glu)_7$. Our method was implemented for analysis of MTX and its metabolites in the CSF, and could be used for evaluation of its curative effects of acute lymphoblastic leukemia patients. The data were also confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The results showed good coincidence.

**Keywords** Capillary electrophoresis · Cerebrospinal fluid · Metabolites · Methotrexate · Triple-stacking

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## Introduction

Methotrexate (MTX, p$K_a$ 4.3) is a cytotoxic drug containing antineoplastic and antifolate that help inhibit dihydrofolate reductase [1, 2]. MTX is widely used for treatment of various types of cancer diseases in humans, including acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma, osteosarcoma, choriocarcinoma, breast cancer, and other malignancies [3, 4]. MTX could be metabolized into 7-hydroxymethotrexate (7-OHMTX, pKa 5.5, one active form),

2, 4-diamino-$N^{10}$-methylpteroic acid (DAMPA, a minor metabolite) and MTX-polyglutamates (MTX-$(Glu)_{n,\ n=2-7}$) [5–7]. MTX-$(Glu)_n$ were metabolized by folylpolyglutamyl synthase, and could remain stacked for at least 24 h after removal of MTX [6, 8, 9]. The metabolites enhance the MTX effect, and also result in cytotoxicity, neurotoxicity and other side effects [6, 10]. MTX could penetrate the blood–brain barrier and get into the cerebrospinal fluid (CSF); at a sufficiently high level (1 μM), MTX and its metabolites could eliminate leukemia cells [11–13]. Therefore, it is important to develop an accurate and sensitive method to monitor MTX level during the elimination phase and the rescue therapy [14]. It would provide more information for determining clinical dosage and prevent meningeal spread of leukemia and central nervous system (CNS) relapse [14, 15].

Some techniques have been developed in the past for monitoring MTX concentration in biological fluids, such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [5, 16–22]. Due to its higher resolution, CE separation is now widely used and has become a powerful technology [23]. There are many advantages, including small sample consumption, rapid separation time, and high resolution and efficiency [24, 25]. Some on-line stacking techniques were used to enhance sensitivity [26–28]. The large-volume sample stacking (LVSS) method could help large volumes of analytes penetrate into the capillary [26, 29, 30]. Dynamic pH junction (DypH) could modify the mobility of analytes in different buffer pHs, and result in stacking [31, 32]. Sweeping and separation were performed by the interaction of micelles and analytes [31, 33, 34]. Some combinations of different stacking techniques [23, 35–38] were used for improving the sensitivity of the CE method, and for widening its applications. In this study, we investigate an efficient stacking technique in the CE method for analysis of MTX and its metabolites in CSF. Our goal is to meet the clinical requirements, and also monitor the correlation of diseases while using MTX.

## Materials and methods

### Chemicals and reagents

All chemicals and reagents were analytical grade. MTX, trifluoroacetic acid (TFA), cetyltrimethylammonium bromide (CTAB) and α-cyano-4-hydroxycinnamic acid (CHCA) were from Sigma (St. Louis, MO, USA) and 7-OHMTX, DAMPA, MTX-$(Glu)_{n,\ n=2-7}$ were from Schircks Laboratories (Jona, Switzerland). Naproxen (Biomol, PA, USA) was of internal standard (IS). Methanol (MeOH), tetrahydrofuran (THF), phosphate salt ($NaH_2PO_4$), sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), hydrochloride acid (HCl), acetonitrile (ACN), and formic acid (FA) were purchased as analytical grade. All reagents were used without any pretreatment. Milli-Q water (Millipore, Bedford, MA, USA) was used for preparing the buffer and the related aqueous solution. The OASIS® HLB SPE cartridges were purchased from Waters (Waters, Milford, MA, USA).

### Preparation of reference solutions and real samples

Stock solutions of analytes were prepared in 0.01 M NaOH at the concentration of 1 mM (MTX), and 200 μM (DAMPA, 7-OHMTX, MTX-$(Glu)_{n,\ n=2-7,}$ and IS solution), and were then suitably diluted to analytical concentration with the simulated CSF. Simulated CSF was used for optimized analytical and extraction method and calibration curve due to the limitation of blank CSF obtained from normal volunteers. Simulated CSF was prepared by mixing human serum with 20 mM ammonium phosphate buffer in a ratio of 1 part serum to 99 parts buffer, so the protein and salt content would be comparable to human CSF [39]. Some real patients' samples were obtained from a hospital. The stock solutions and samples were stored at temperature less than −20°C.

### Extraction of CSF

The extraction procedure integrated with deproteination and SPE processes, as in our previous research [38]. CSF samples (each 500 μL) with IS solution (45 μM) were added first with 15 μL of 70% perchloric acid, for deproteination, and were then vortex (30 s) centrifuged (7992 g, 10 min). Extraction was conducted by an auto-SPE instrument, Rapid Trace® (Zymark, Hopkinton, Germany). The SPE cartridge was preconditioned by MeOH and water (each 3 mL). The supernatant was transferred (1 mL/min) into SPE tube, held for 1 min under vacuum, and then washed with 5% MeOH (1 mL) and water (1 mL). The samples were eluted by 1 mL of MeOH, and evaporated by a centrifugal vaporizer (EYELA CVE-200D, Japan). The residue was reconstituted with 0.01 M NaOH (80 μL) before CE analysis.

### CE system

Beckman P/ACE MDQ system (Fullerton, CA, USA) equipped with UV detection and uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. and 40 cm effective length (50.2 cm, total length) were employed for separation. The UV absorbance wavelength was set at 300 nm. The capillaries were conditioned with MeOH, water, 1 M HCl, water, 1 M

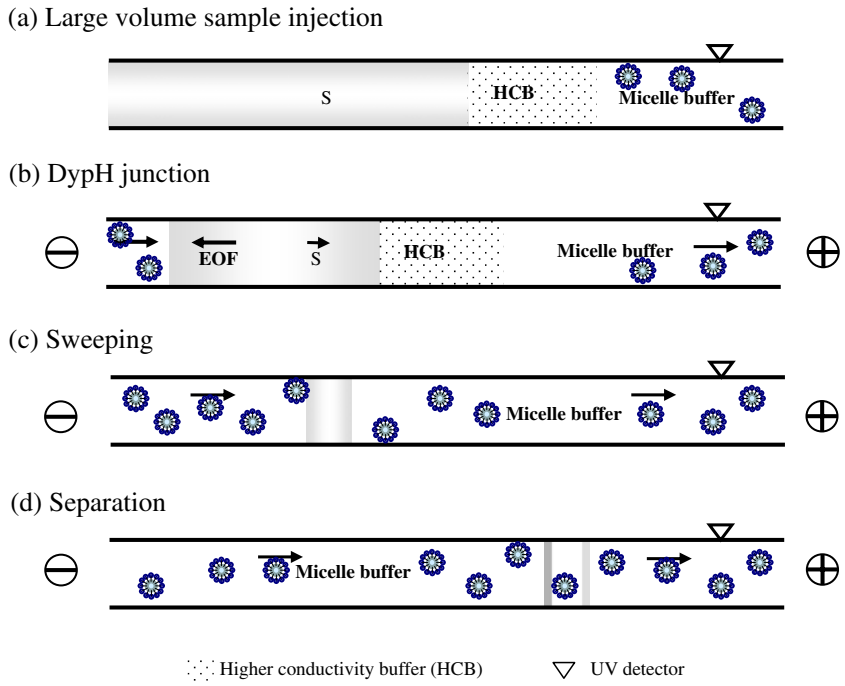


**Fig. 1** Mechanism of LVSS-DypH-sweeping method

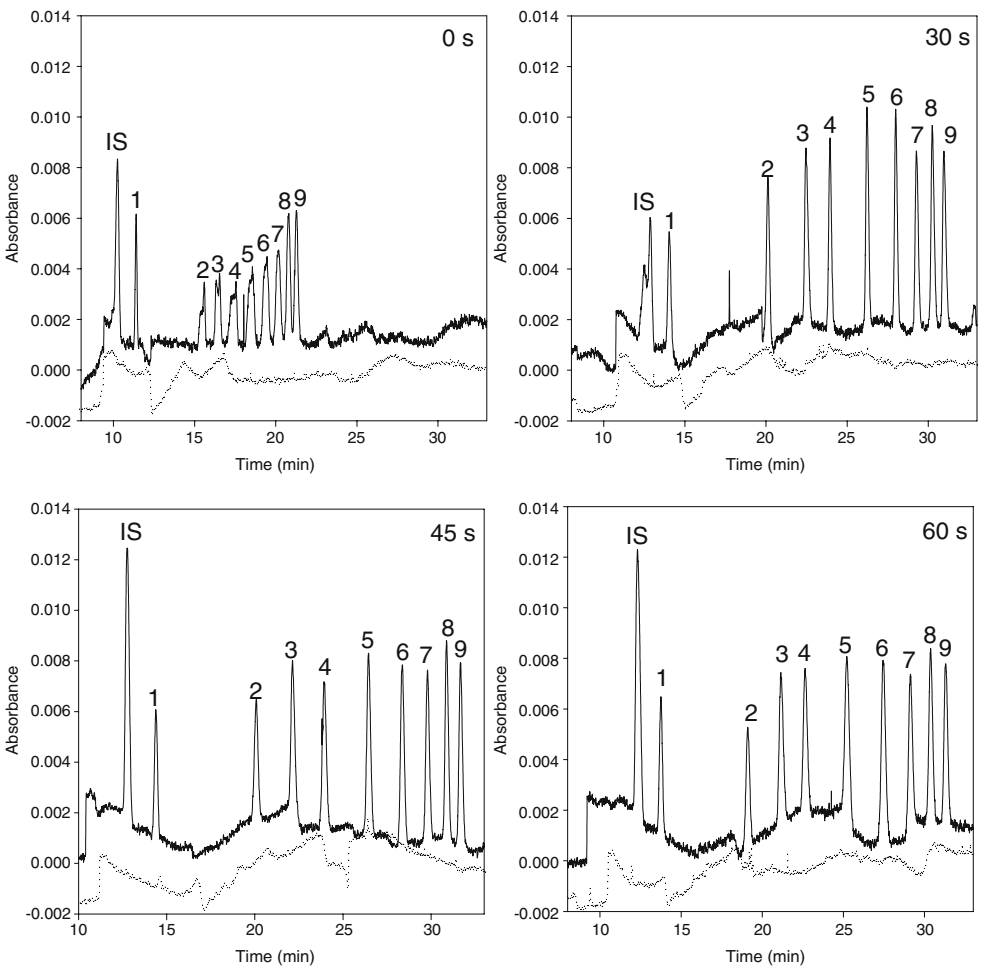


**Fig. 2** Effect of HCB zone by LVSS-DypH-sweeping. The HCB zone (100 mM phosphate) was introduced by 2 psi. The CE conditions: micelle buffer (60 mM phosphate, pH 3 containing 15% THF and 100 mM SDS), dynamic sample injection (2 psi, 99.9 s) and voltage (−20 kV). The concentrations of nine analytes are 1 μM. Electropherogram of blank CSF extract was shown as *dotted line*. Peak identified as *1* DAMPA; *2* MTX; *3* 7-OHMTX; *4* $MTX(Glu)_2$; *5* $MTX(Glu)_3$; *6* $MTX(Glu)_4$; *7* $MTX(Glu)_5$; *8* $MTX(Glu)_6$; *9* $MTX(Glu)_7$

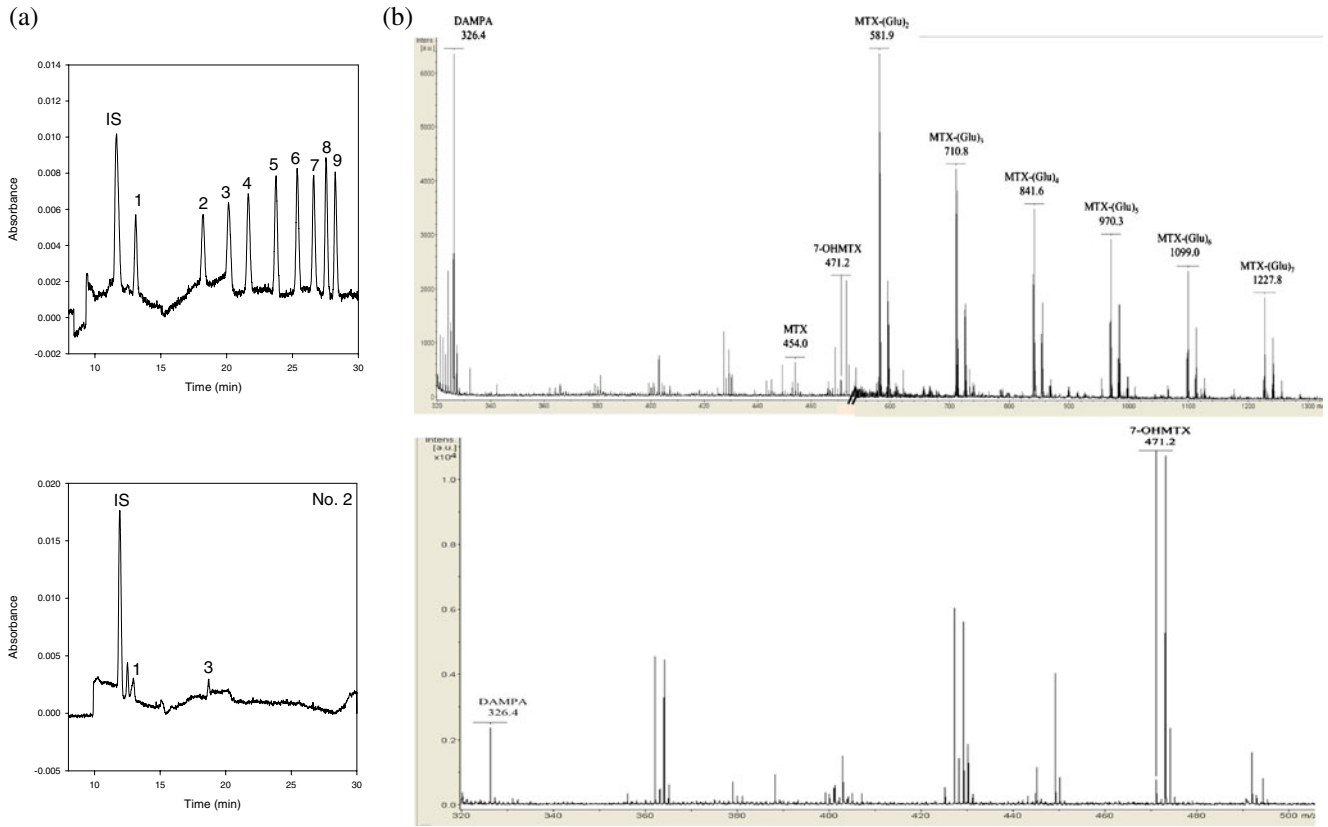


**Fig. 3** **a** Eletropherograms of standards mixture in simulated CSF and real sample obtained by LVSS-DypH-sweeping; **b** ion mass spectra of standards and real sample by MALDI-TOF-MS

**Table 1** Regression analysis for the determination of methotrexate and its eight metabolites in CSF

| 0.5–7μM | Regression equation | Coefficient of correlation (r) |
|---|---|---|
| Intra-batch[a] | | |
| DAMPA | $y = (0.3085 \pm 0.0106)x + (0.0274 \pm 0.0220)$ | 0.9984 |
| MTX | $y = (0.3310 \pm 0.0098)x + (0.0300 \pm 0.0169)$ | 0.9990 |
| 7-OHMTX | $y = (0.3087 \pm 0.0060)x + (0.0396 \pm 0.0132)$ | 0.9986 |
| MTX-$(Glu)_2$ | $y = (0.2840 \pm 0.0080)x + (0.0299 \pm 0.0147)$ | 0.9991 |
| MTX-$(Glu)_3$ | $y = (0.2853 \pm 0.0020)x + (0.0489 \pm 0.0084)$ | 0.9985 |
| MTX-$(Glu)_4$ | $y = (0.2783 \pm 0.0026)x + (0.0560 \pm 0.0110)$ | 0.9989 |
| MTX-$(Glu)_5$ | $y = (0.2535 \pm 0.0008)x + (0.0584 \pm 0.0148)$ | 0.9985 |
| MTX-$(Glu)_6$ | $y = (0.2698 \pm 0.0155)x + (0.0587 \pm 0.0209)$ | 0.9990 |
| MTX-$(Glu)_7$ | $y = (0.2500 \pm 0.0067)x + (0.0429 \pm 0.0326)$ | 0.9988 |
| Inter-batch[b] | | |
| DAMPA | $y = (0.2983 \pm 0.0033)x + (0.0256 \pm 0.0029)$ | 0.9991 |
| MTX | $y = (0.3156 \pm 0.0075)x + (0.0222 \pm 0.0087)$ | 0.9981 |
| 7-OHMTX | $y = (0.3116 \pm 0.0034)x + (0.0088 \pm 0.0078)$ | 0.9988 |
| MTX-$(Glu)_2$ | $y = (0.2936 \pm 0.0020)x + (0.0188 \pm 0.0013)$ | 0.9988 |
| MTX-$(Glu)_3$ | $y = (0.2919 \pm 0.0030)x + (0.0251 \pm 0.0015)$ | 0.9991 |
| MTX-$(Glu)_4$ | $y = (0.2856 \pm 0.0055)x + (0.0047 \pm 0.0126)$ | 0.9992 |
| MTX-$(Glu)_5$ | $y = (0.2602 \pm 0.0071)x + (0.0267 \pm 0.0075)$ | 0.9996 |
| MTX-$(Glu)_6$ | $y = (0.2632 \pm 0.0007)x + (0.0399 \pm 0.0104)$ | 0.9990 |
| MTX-$(Glu)_7$ | $y = (0.2385 \pm 0.0075)x + (0.0319 \pm 0.0067)$ | 0.9993 |

Concentration ranges for the intra- and inter-batch analysis: 0.5–7 μM

[a] Regression equations of intra-batch analysis were calculated from the assay values of prepared standards of one batch ($n$=3)

[b] Regression equations of inter-batch analysis were calculated from the assay values of prepared standards of five different batches ($n$=5)

NaOH, and water; each for 10 min before initial use. Before use, the capillary was rinsed with water, 1 M HCl, water, 1 M NaOH, and water; each for 5 min. Between runs, the capillary was washed with MeOH, 0.1 M NaOH and buffer, for 5 min. Data analysis and instruments were controlled by Beckman Coulter MDQ 32 Karat software.

### Triple stacking

Due to the low charge of analytes, hydrodynamic sampling was chosen. We also investigated different combinations of stacking methods for sensitivity improvement. First, the capillary was rinsed with 60 mM phosphate buffer (pH 3) containing 15% THF and 100 mM SDS, and this followed by injection of higher conductivity buffer (HCB) and 100 mM phosphate buffer, by applying hydrodynamic pressure (2 psi, 45 s). The sample was injected by pressure (2 psi 99.9 s). After sample injection, phosphate buffer (60 mM, pH 3) containing 15% THF and 100 mM SDS was used for separation. Applied voltage was −20 kV and the temperature was set at 25°C. The separation was performed under the condition of LVSS-DypH-sweeping mode.

### MALDI-TOF-MS analysis

A MALDI-TOF-MS system model Autoflex III Smartbeam equipped with a nitrogen laser radiating at 337 nm from Bruker Daltonics (Billerica, MA, USA) was used to obtain the data in positive ion reflector mode. One microliter of the sample was spotted on a stainless steel target (Bruker Daltonics), followed by addition of 1 μL of a 10 mg/mL matrix (50% ACN solution containing 0.1% TFA) and then 0.5 μL CTAB (1 mg/mL in 80:20 methanol–$H_2O$) was added. All mass spectra were recorded after summing up

**Table 2** Intra- and inter-batch analysis results for methotrexate and its eight metabolites in CSF

| | Concentration known (μM) | Intra-batch ($n$=3) | | | Inter-batch ($n$=5) | | |
|---|---|---|---|---|---|---|---|
| | | Concentration found (μM) | RSD (%) | RE[a] (%) | Concentration found (μM) | RSD (%) | RE[a] (%) |
| DAMPA | 0.8 | 0.75±0.02 | 2.7 | −6.3 | 0.86±0.02 | 2.3 | 7.5 |
| | 2.0 | 2.12±0.03 | 1.4 | 6.0 | 2.03±0.05 | 2.5 | 1.5 |
| | 6.0 | 5.98±0.18 | 3.0 | −0.3 | 6.09±0.13 | 2.1 | 1.5 |
| MTX | 0.8 | 0.78±0.04 | 5.1 | −2.5 | 0.85±0.02 | 2.4 | 6.3 |
| | 2.0 | 2.02±0.02 | 1.0 | 1.0 | 2.00±0.02 | 1.0 | 0.1 |
| | 6.0 | 6.04±0.08 | 1.3 | 0.7 | 6.03±0.05 | 0.9 | 0.5 |
| 7-OHMTX | 0.8 | 0.82±0.05 | 6.0 | 2.5 | 0.83±0.04 | 4.8 | 3.8 |
| | 2.0 | 2.05±0.05 | 2.7 | 2.5 | 1.98±0.03 | 1.5 | −1.0 |
| | 6.0 | 5.93±0.12 | 2.4 | −1.1 | 5.98±0.08 | 1.3 | −0.3 |
| $MTX-(Glu)_2$ | 0.8 | 0.85±0.04 | 4.7 | 6.3 | 0.82±0.02 | 2.4 | 2.5 |
| | 2.0 | 2.09±0.04 | 1.9 | 4.5 | 1.99±0.02 | 1.0 | −0.5 |
| | 6.0 | 6.13±0.12 | 2.0 | 2.2 | 5.99±0.06 | 1.0 | −0.2 |
| $MTX-(Glu)_3$ | 0.8 | 0.77±0.05 | 3.8 | −3.8 | 0.84±0.04 | 4.8 | 5.0 |
| | 2.0 | 2.15±0.03 | 1.4 | 7.5 | 2.01±0.04 | 2.0 | 0.5 |
| | 6.0 | 6.07±0.12 | 1.9 | 1.2 | 6.04±0.09 | 1.5 | 0.7 |
| $MTX-(Glu)_4$ | 0.8 | 0.74±0.03 | 4.1 | −7.5 | 0.82±0.02 | 2.4 | 2.2 |
| | 2.0 | 2.19±0.06 | 2.7 | 9.5 | 1.99±0.03 | 1.5 | −0.5 |
| | 6.0 | 5.81±0.29 | 4.5 | −3.1 | 6.03±0.10 | 1.7 | 0.5 |
| $MTX-(Glu)_5$ | 0.8 | 0.78±0.05 | 6.4 | −2.5 | 0.84±0.02 | 2.4 | 5.0 |
| | 2.0 | 2.12±0.16 | 7.5 | 6.0 | 1.99±0.03 | 1.5 | −0.5 |
| | 6.0 | 6.05±0.21 | 3.4 | 0.8 | 6.04±0.09 | 1.5 | 0.7 |
| $MTX-(Glu)_6$ | 0.8 | 0.76±0.07 | 9.2 | −5.0 | 0.84±0.01 | 1.2 | 5.0 |
| | 2.0 | 2.10±0.09 | 4.3 | 5.0 | 1.99±0.06 | 3.0 | −0.5 |
| | 6.0 | 6.01±0.10 | 1.7 | 0.2 | 6.09±0.08 | 1.3 | 1.5 |
| $MTX-(Glu)_7$ | 0.8 | 0.76±0.09 | 11.2 | −5.0 | 0.82±0.03 | 3.7 | 2.5 |
| | 2.0 | 2.04±0.11 | 5.4 | 2.0 | 1.99±0.06 | 3.0 | −0.5 |
| | 6.0 | 6.20±0.34 | 5.5 | 3.3 | 5.93±0.10 | 1.7 | −1.2 |

[a] RE(%)=(concentration found-concentration known)/(concentration known)×100

1000 laser shots. The laser power was adjusted between 20 and 30% of its maximal intensity; the Flex Analysis software was used for data processing (Bruker Daltonics).

## Results and discussion

Although many techniques have been developed and utilized for monitoring MTX concentration in biological fluids, including HPLC and CE, with different detectors or extraction processes [5, 16–22], most of these methods only detect MTX in samples. CSF is an important biofluid for monitoring neurotoxicity during chemotherapy and relapse [10]. MTX can penetrate the blood–brain barrier to get into CSF in suitable dosages for treatment [14, 15]. Therefore, it is necessary to establish an efficient CE method for clinical assay and evaluation. We develop this innovative triple-stacking CE method, which can monitor therapeutic levels of MTX and its metabolites for diagnostic information. This triple-stacking method includes the mechanism of LVSS-DypH-sweeping (Fig. 1). The capillary was rinsed with buffer containing micelles. After that, HCB with lower pH was loaded for collecting large volume samples (Fig. 1a). The analytes would have negative charges while in the sample matrix (higher pH), and migrate towards the detector. By applying the suitable voltage, the electro-osmotic flow turned toward the inlet direction (Fig. 1b). The pH junction was produced between sample zone and HCB zone, for stacking. The analytes were dissociated in the basic sample zone and protonated in acidic HCB zone. When analytes reached the pH junction boundary, their mobility was close to zero, causing the stacking effect. Then, the SDS buffer swept through and stacked into a compact zone (Fig. 1c). The separation was conducted in MEKC mode (Fig. 1d).

### Optimized conditions of triple-stacking CE mode

The factors influencing LVSS-DypH-sweeping in this system were investigated, including HCB zone, sample injection, micelle buffer, organic modifier, and SDS.

The advantage of HCB zone on peak shapes and peak height was introduced by Terabe and Quirino [40]. The HCB zone (100 mM phosphate, pH 2.5) has higher conductivity and provides the pH gap for focusing. When a large amount of analytes was injected into the capillary, HCB zone caused them to slow down and focus (Fig. 1b). The importance of HCB was evaluated by comparing no HCB with HCB (inject 2 psi, 30~60 s). Without HCB, the peaks would be destacked and become broad and crotched, as shown in Fig. 2. When increasing sample injection time, HCB zone should be increased sufficiently to facilitate accumulation. Samples were hydrodynamically injected (1~2.5 psi, 99.9 s). Considering the balance between HCB and sample volume, the optimized conditions were set as 100 mM phosphate, pH 2.5, 2 psi, 45 s for HCB and 2 psi, 99.9 s for sample injection. The other factors were micelle buffer, organic modifier, and SDS. We tried different levels of phosphate buffer (40~90 mM), organic modifier (THF, 0~20%), and SDS (50~125 mM). THF must be added in the buffer in order to modify analytes' ability to separate from micelles and provide a different velocity. Not adding THF makes analytes co-migrate; increasing

**Table 3** The recovery of MTX and its eight metabolites in CSF

| Analyte | Recovery (%) |
|---|---|
| DAMPA | 40.6 |
| MTX | 22.4 |
| 7OHMTX | 27.5 |
| MTX-$(Glu)_2$ | 22.8 |
| MTX-$(Glu)_3$ | 20.2 |
| MTX-$(Glu)_4$ | 25.0 |
| MTX-$(Glu)_5$ | 22.3 |
| MTX-$(Glu)_6$ | 18.8 |
| MTX-$(Glu)_7$ | 20.6 |

**Table 4** CE Data of real CSF samples from ALL patients

| No. | CE | |
|---|---|---|
| | Analyte found | Concentration (μM) |
| 1 | DAMPA | <0.5 |
| 2 | DAMPA | <0.5 |
| | 7-OHMTX | <0.5 |
| 3 | MTX | 7.0 |
| | 7-OHMTX | <0.5 |
| 4 | MTX | 1.1 |
| | 7-OHMTX | >7.0 |
| 5 | 7-OHMTX | >7.0 |
| 6 | 7-OHMTX | 6.2 |
| 7 | 7-OHMTX | >7.0 |
| 8 | 7-OHMTX | 7.0 |
| 9 | MTX | <0.5 |
| 10 | 7-OHMTX | 1.0 |
| 11 | 7-OHMTX | 0.5 |
| 12 | 7-OHMTX | 3.6 |
| 13 | 7-OHMTX | >7.0 |
| 14 | 7-OHMTX | <0.5 |
| 15 | 7-OHMTX | 1.2 |
| 16 | 7-OHMTX | 3.2 |
| 17 | 7-OHMTX | 4.2 |
| 18 | 7-OHMTX | 1.9 |
| 19 | 7-OHMTX | <0.5 |

the ratio of THF could provide better resolution but also prolonged analysis time. SDS micelles interacted with analytes and brought analytes towards detection (Fig. 1c). The best separation condition was defined as 60 mM phosphate buffer containing 15% THF and 100 mM SDS (Fig. 3a).

### Method validation results

The limits of detection (LOD, S/*N*=3) of analytes were between 0.1–0.3 μM. The limits of quantification (LOQ) were set at 0.5 μM. Once the concentrations were higher than 7 μM, the resolutions of analytes were become worse. Therefore, the upper limits of calibration curves were set at 7 μM. To evaluate the quantitative applicability of this method, five different levels of analytes in CSF were analyzed in the range of 0.5~7 μM. Linearity between the normalized peak area ratio (*Y*) of each analyte to IS, and the concentration of each analyte (*X* μM), is shown in Table 1. Regression equations of intra- and inter-day analysis were calculated from assay values of the three extracted CSF solutions, in a single batch ($n$=3) and in five consecutive batches ($n$=5). We calculated the data of the slope, intercepts and SD value of the regression line. The results of the linear regression equations indicated that high linearity ($r \geq 0.9981$) between *Y* and *X* was attained over the range studied. For evaluating precision and accuracy of the proposed method, the relative standard deviations (RSD) and relative error (RE) of all analytes spiked in CSF were tested at three different concentrations. As Table 2 shows, all data of RSD and RE were below 11%, indicating good precision and accuracy of this method. The detection limit (LOD, S/*N*=3, hydrodynamic injection 2 psi, 99.9 s) was observed to be 0.1 μM for MTX, 7-OHMTX, MTX-$(Glu)_{n,\ n=2-5}$, and 0.2 μM MTX-$(Glu)_6$, and 0.3 μM for DAMPA, and MTX-$(Glu)_7$. The pharmacokinetics and pharmacodynamics of MTX have been studied extensively, and a concentration of 1 μM MTX has been proposed as a minimum effective antileukemic concentration [11–13]. So the LODs provide sufficiently levels for detecting MTX and its metabolites in CSF samples. They were actually detected in CSF samples to obtain reliable data. The results indicate that this method could detect low concentration of MTX and its metabolites in CSF. The recovery was evaluated at 1 μM of all analytes in simulated CSF extracted by the relevant extraction procedures. The data was obtained by the peak area ratio of each analyte to IS, in extracted simulated CSF and in standard solutions. Recoveries of all analytes extracted by SPE procedures were 18.8% to 40.6%, as shown in Table 3. Triplicates of eight concentrations (five in quantitative range and three for precision and accuracy analysis) values were calculated for intra-batch analysis and demonstrated the system robustness. We examined the stability by analysis of one single sample over 48 h, and found the peak heights and shapes of this sample at different intervals did not show any significant differences. Mercaptopurine was commonly used with MTX, it would not be detected under 300 nm and make no interference in this method.

### Application of CSF samples

Consents were obtained from volunteers after providing them all relevant information, for the purpose of this study. The real CSF samples were from a hospital. The samples were acquired from 19 ALL patients after MTX treatment. All samples were collected and analyzed by the developed triple-stacking CE method. The results are shown in Table 4; their electropherograms are depicted in Fig. 3a. The peaks in real samples were confirmed by spiking standards. All CSF samples were further confirmed by MALDI-TOF-MS and matched analyzed data from CE (Fig. 3b). The results showed that only MTX, DAMPA and 7-OHMTX were found in CSF, none of polyglutamate metabolites were detected. This method could be used for clinical evaluation.

## Conclusions

Monitoring MTX in CSF is important for preventing CNS relapse and evaluation of therapeutic effects. In this study, we establish an effective CE method to analyze MTX and its metabolites in ALL patients' CSF. Furthermore, we investigate the correlation among derived diseases by MTX administration.

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## References

1. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chamner BA (1983) N Engl J Med 309:1094–1104
2. Wan H, Holmen AG, Wang Y, Lindberg W, Englund M, Nagard MB, Thompson RA (2003) Rapid Commun Mass Spectrom 17:2639–2648
3. Thompson PA, Murry DJ, Rosner GL, Lunagomez S, Blaney SM, Berg SL et al (2007) Cancer Chemother Pharmacol 59:847–853
4. Cole PD, Alcaraz MJ, Smith AK, Tan J, Kamen BA (2006) Cancer Chemother Pharmacol 57:826–834
5. Sczesny F, Hempel G, Boos J, Blaschke G (1998) J Chromatogr B 718:177–185
6. Genestier L, Paillot R, Quemeneur L, Izeradjene K, Revillard JP (2000) Immunopharmacology 47:247–257

7. Widemann BC, Sung E, Anderson L, Salzer WL, Balis FM, Monitjo KS et al (2000) J Pharmacol Exp Ther 294:894–901
8. Ranganathan P, McLeod HL (2006) Arthritis Rheum 54:1366–1377
9. Angelis-Stoforidis P, Vajda FJ, Christophidis N (1999) Clin Exp Rheumatol 17:313–320
10. Gool SWV, Meyer GD, Voorde AVd, Vanmechelen E, Vanderstichele H (2004) Neurotoxicology 25:471–480
11. Jonsson P, Hoglund P, Wiebe T, Schroder H, Seidel H, Skarby T (2007) Anticancer Drugs 18:941–948
12. Hiraga S, Arita N, Ohnishi T, Kohmura E, Yamamoto K, Oku Y et al (1999) J Neurosurg 91:221–230
13. Milano G, Thyss A, Serre Debeauvais F, Laureys G, Benoit Y, Deville A et al (1990) Eur J Cancer 26:492–495
14. Seidel H, Andersen A, Kvaloy JT, Nygaard R, Moe PJ, Jacobsen G et al (2000) Leuk Res 24:193–199
15. Balis FM, Blaney SM, McCully CL, Bacher JD, Murphy RF, Poplack DG (2000) Cancer Chemother Pharmacol 45:259–264
16. Li H, Luo W, Zeng Q, Lin Z, Luo H, Zhang Y (2007) J Chromatogr B 845:164–168
17. Anzai T, Jaffe N, Wang YM (1987) J Chromatogr 415:445–449
18. Albertioni F, Pettersson B, Beck O, Rask C, Seideman P, Peterson C (1995) J Chromatogr B 665:163–170
19. Dervieux T, Orentas Lein D, Marcelletti J, Pischel K, Smith K, Walsh M, Richerson R (2003) Clin Chem 49:1632–1641
20. Kuo CY, Wu HL, Kou HS, Chiou SS, Wu DC, Wu SM (2003) J Chromatogr A 1014:93–101
21. Kuo CY, Chiou SS, Wu SM (2006) Electrophoresis 27:2905–2909
22. Suzuki Y, Arakawa H, Maeda M (2003) Anal Sci 19:111–115
23. Horakova J, Petr J, Maier V, Znaleziona J, Stanova A, Marak J et al (2007) J Chromatogr A 1155:193–198
24. Britz-McKibbin P, Markuszewski MJ, Iyanagi T, Matsuda K, Nishioka T, Terabe S (2003) Anal Biochem 313:89–96
25. Horakova J, Petr J, Maier V, Tesarova E, Veis L, Armstrong DW et al (2007) Electrophoresis 28:1540–1547
26. Albert M, Debusschere L, Demesmay C, Rocca JL (1997) J Chromatogr A 757:291–296
27. Zhang H, Zhou L, Chen X (2008) Electrophoresis 29:1556–1564
28. Lin CH, Kaneta T (2004) Electrophoresis 25:4058–4073
29. Chien RL, Burgi DS (1992) Anal Chem 84:1046–1050
30. Burgi DS (1993) Anal Chem 65:3726–3729
31. Britz-McKibbin P, Terabe S (2003) J Chromatogr A 1000:917–934
32. Britz-McKibbin P, Chen DDY (2000) Anal Chem 72:1242–1252
33. Quirino JP, Terabe S (1999) Anal Chem 71:1638–1644
34. Quirino JP, Terabe S (1998) Science 282:465–468
35. Britz-McKibbin P, Otsuka K, Terabe S (2002) Anal Chem 74:3736–3743
36. Britz-McKibbin P, Terabe S (2002) Chem Rec 2:397–404
37. Shih CM, Lin CH (2005) Electrophoresis 26:3495–3499
38. Cheng HL, Liao YM, Chiou SS, Wu SM (2008) Electrophoresis 29:3665–3673
39. Howard GM, Schwende FJ (1997) J Chromatogr B 693:431–436
40. Quirino JP, Terabe S (2000) Anal Chem 72:1023–1030