

Simultaneous Determination of Methotrexate Concentrations in Human Plasma and Cerebrospinal Fluid Using Two-Dimensional Liquid Chromatography: Applications in Primary Central Nervous System Lymphoma

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Abstract

Background: In this study, we aimed to develop a method for the simultaneous quantification of methotrexate (MTX) samples extracted from human plasma and cerebrospinal fluid (CSF), using two-dimensional liquid chromatography (2D-LC). Furthermore, we intended to verify whether intravenous mannitol could increase MTX concentration in the CSF of patients.

Methods: The mobile phase of PUMP1 consisted of 10.0 mmol/L ammonium acetate and acetonitrile. PUMP2 solution consisted of an aqueous solution of 10.0 mmol/L ammonium acetate. The mobile phase of PUMP3 comprised 50.0 mmol/L ammonium acetate and acetonitrile, with a flow rate of 1.0 mL/min.

Results: The developed method was successfully employed to simultaneously determine drug levels in plasma and CSF from the patients treated with MTX. CSF samples were obtained by lumbar puncture 0.5 - 2 h after starting the high-dose methotrexate (HD-MTX) infusion (over 4 h) and immediately before the intrathecal (IT) administration of MTX. Venous blood samples were drawn 4 h after the start of infusion. The calibration curve was linear, with a range of 0.07

- 2.38 $\mu\text{mol/L}$ for CSF samples and a range of 0.11 - 5.51 $\mu\text{mol/L}$ for plasma samples. Precision ($> 95\%$) and accuracy ($> 97\%$) were within the acceptance criteria for each quality control (QC) level. Inter- and intra-day accuracy and precision values met the acceptance criteria for each QC level. The correlation between MTX concentrations in the plasma and CSF was moderate ($r = 0.502$). No significant difference was observed in MTX concentration in CSF between patients using intravenous mannitol and those not using intravenous mannitol ($P = 0.682$).

Conclusion: The developed method was useful for therapeutic drug monitoring of MTX and suitable for assessing the risks and benefits of chemotherapy in patients with primary central nervous system lymphoma. Intravenous mannitol did not increase MTX concentration in the CSF of patients.

Keywords: Primary central nervous system lymphoma; Methotrexate; Cerebrospinal fluid; Two-dimensional liquid chromatography

Introduction

Primary central nervous system lymphoma (PCNSL), a highly aggressive form of non-Hodgkin lymphoma (NHL), affects only the brain parenchyma, leptomeninges, cerebrospinal fluid (CSF), and vitreoretinal compartment, with no peripheral involvement [1, 2]. Over 95% of PCNSL cases are of diffuse large B-cell lymphoma (DLBCL), which constitutes about 3-4% of all intracranial malignancies [3]. PCNSL is known for its marked aggressiveness and poor prognosis, both of which contribute to dismal clinical outcomes [4]. Untreated PCNSL patients have an overall survival time of just 1.5 months. Despite the effectiveness of chemotherapy, 35-60% of PCNSL patients experience a relapse within 2 years of initial treatment [3, 5]. High-dose methotrexate (HD-MTX) administered intravenously is the standard induction therapy for PCNSL, as it can cross the blood-brain barrier (BBB). Methotrexate (MTX) doses in most regimens typically range from 1 to 8 g/m^2 [6]. Recent years have seen significant advances in PCNSL prognosis, largely due to the use of HD-MTX [7]. However, MTX's

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poor selectivity harms not only tumor cells but also normal cells, resulting in severe side effects. The kidneys eliminate over 90% of MTX, accounting for most of its total clearance rate. Delayed elimination of MTX can expose patients to serious toxicity risks such as nephrotoxicity, hepatotoxicity, myelosuppression, severe mucositis, pancytopenia, and other adverse effects [8, 9]. These adverse events can lead to prolonged hospital stays, increased economic burdens, delayed chemotherapy, reduced doses, treatment failures, or even death. Safe HD-MTX treatment involves maintaining blood MTX levels within an effective range and rapidly reducing them to a safe range. Implementing prophylactic measures, including fluid hydration, urine alkalization, and leucovorin rescue, is essential [10, 11]. Additionally, therapeutic drug monitoring (TDM) measures plasma MTX concentrations to ensure HD-MTX's safety and effectiveness [12].

TDM is a clinical discipline focused on developing mechanisms, technologies, methods, and standards for personalized drug therapy. It leverages research outcomes to optimize the rational use of medications in clinical settings. TDM plays a critical role in creating personalized drug delivery plans that consider factors like the drug treatment window [13]. Currently, TDM is commonly used for drugs that show significant variability between and within individuals, and those with narrow, highly toxic therapeutic windows [14]. HD-MTX therapy is highly toxic and shows significant variability in MTX clearance among individuals during each treatment cycle. For safety, MTX concentrations must be monitored at 24, 48, and 72 h after administration to meet specific detection criteria. Specifically, plasma MTX concentrations should not exceed 10, 1, and 0.1 $\mu\text{mol/L}$ at 24, 48, and 72 h, respectively [15]. Although HD-MTX-based therapies are standard for PCNSL induction, their effectiveness is still debated. The blood-brain barrier (BBB) significantly limits drug delivery to brain tissues, restricting chemotherapy's reach within the central nervous system. However, evidence indicates that the BBB is compromised at PCNSL sites, potentially enhancing drug penetration [16, 17]. Intravenous mannitol, a safe method for disrupting the BBB to increase intracerebral MTX levels, has not yet seen widespread clinical use.

In this study, we aimed to establish a simple and rapid method for simultaneously measuring MTX concentrations in CSF and plasma. We also attempted to prove the correlation between the concentration of MTX in plasma and that in CSF, so as to infer the concentration of MTX in the CSF of patients based on the concentration of MTX in the plasma of patients, avoiding the traumatic damage and economic loss caused by collecting CSF from patients. Additionally, this study also sought to explore the correlation of MTX concentration in the CSF between patients who received intravenous mannitol treatment and those who did not, in order to determine whether intravenous mannitol can enhance the permeability of the BBB to MTX.

Materials and Methods

This study protocol was reviewed and approved by Ethics Committee of Shandong Cancer Institute (approval number

2024003025). All the relevant ethical safeguards have been met with the patient.

Reagents and chemicals

The MTX reference material (free base, purity > 99%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and methanol were high-performance liquid chromatography (HPLC) grade, and ammonium phosphate, ethylene glycol, and ammonium acetate were analytical grade. All reagents and chemicals were procured from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Distilled water was used throughout the experiments. Blank human plasma, used for setting up the experiments, was obtained from the hospital medical examination center, and CSF was acquired from untreated patients in the Department of Lymphology and Hematology (Shandong Cancer Hospital and Institute, Jinan, China). All patients provided duly signed informed consent forms.

Chromatographic system

The 2D-LC system included a first-dimensional liquid chromatography system (LC1), an interface, and a second-dimensional liquid chromatography system (LC2). LC1 included an LC1 column (first separation column), an autosampler device (SIL-20A, 500- μl quantitative loop, Shimadzu; SIL), and a chromatography pump (LC-20ATvp, Shimadzu, Kyoto, Japan; PUMP1). The interface housed two capture columns (A and B), 6- and 10-port switching valves (Rheodyne, Oak Harbor, WA), and an auxiliary high-pressure pump (LC-20ATvp; PUMP2). LC2 consisted of an LC2 column (second separation column), a UV detector (SPD-20A, Shimadzu), a workstation (LC solution ver. 1.26, Shimadzu), and a low-pressure gradient chromatography pump (LC-20ATvp; PUMP3). The system included three columns: an LC1 column (Aston FS SC2, 3.5 \times 50 mm, 5 μm , ANAX, Changsha, China), a capture column (Aston SN, 4.6 \times 10 mm, 5 μm , ANAX), and an LC2 column (Aston SCX4, 4.6 \times 125 mm, 5 μm , ANAX). Other instruments included a Mini-15K micro-high-speed centrifuge (Allsheng Instruments, Hangzhou, China), a GH-202 electronic analytical balance (A&D, Tokyo, Japan), an XW-80 vortex mixer (QITE, Shanghai, China), a medical refrigerator (MEILING, Hefei, China), and a TDZ4-WS low-speed centrifuge (XIANGYI, Xiangtan, China).

PUMP1's mobile phase consisted of 10.0 mmol/L ammonium acetate and acetonitrile in a 90:10 v/v ratio, with the pH adjusted to 3.8 using acetic acid, and a flow rate of 1.2 mL/min. The PUMP2 solution was an aqueous mix of 10.0 mmol/L ammonium acetate, with the pH adjusted to 3.0 using acetic acid. PUMP3's mobile phase included 50.0 mmol/L ammonium acetate and acetonitrile in an 82.5:17.5 v/v ratio, pH adjusted to 5.2 with acetic acid, and a flow rate of 1.0 mL/min. The detection wavelength was set at 302 nm. The detector cell temperature was maintained at 40 $^{\circ}\text{C}$, and the autosampler's injection volume was 200 μL .

Stock and standard solutions

A mixed solution of methanol and ethylene glycol (3:1, v/v) was used to prepare the standard stock solution of MTX at 23.80 and 55.10 $\mu\text{mol/L}$. Two groups of working solutions were prepared by serial dilution from the stock solution with 25% ethylene glycol. The first group comprised concentrations of 0.24, 2.38, and 11.90 $\mu\text{mol/L}$, while the second group comprised concentrations of 1.10, 5.51, and 27.55 $\mu\text{mol/L}$. All these working solutions were freshly prepared and used for calibration purposes. Calibration concentrations were set at 0.07, 0.15, 0.30, 0.59, 1.19, and 2.38 $\mu\text{mol/L}$ for CSF and 0.11, 0.28, 1.10, 2.76, 3.86, and 5.51 $\mu\text{mol/L}$ for human plasma. They were prepared by spiking blank CSF and plasma with the corresponding working solutions. Internal quality control (QC) samples were similarly prepared at three different concentrations, comprising low-, mid-, and high-level QC samples of 0.24, 0.71, and 1.49 $\mu\text{mol/L}$ for CSF and 0.55, 2.20, and 4.13 $\mu\text{mol/L}$ for plasma, respectively. All stock and working solutions, calibration solutions, and QC samples were stored at -80°C and brought to ambient temperature before use.

Sample preparation

In the initial step, 200 μL plasma or CSF sample was pipetted into a 1.5 mL polypropylene microtube. Subsequently, 600 μL of an 85:15 (v/v) solution of 6% perchloric acid-methanol was added. The tube was vigorously vortexed for 30 s and centrifuged at $14,500 \times g$ for 5 min at ambient temperature. Finally, the clear supernatant (600 μL) was transferred into a glass injection vial for analysis. Both the blank and patient samples were treated identically.

Clinical samples

All patients underwent an HD-MTX-based chemotherapy regimen, with each HD-MTX treatment administered as a 4-h infusion. CSF and corresponding serum samples were not collected simultaneously. CSF samples were obtained by lumbar puncture 0.5 - 2 h after starting the HD-MTX infusion (over 4 h) and immediately before the intrathecal (IT) administration of MTX. Venous blood samples were drawn 4 h after the start of infusion. Additionally, as part of routine clinical treatment drug monitoring, plasma MTX concentrations were measured at 24, 48, and 72 h after infusion completion. To ensure a steady-state concentration of MTX in the plasma, CSF samples and their corresponding plasma MTX concentrations were included in the data analysis only if blood sampling had been performed less than 5 h after the start of infusion. The availability of the data is determined based on the administration time of MTX and the blood collection time as recorded in the case system.

Validation methodology

Validation procedures employed a method for establishing and

validating bioanalysis according to FDA guidelines [18].

Results

Method design and development

During optimization, various concentrations of mobile phase were tried in an isocratic and gradient mode. The final selected concentrations were as follows. The mobile phase of LC1 consisted of ammonium acetate and acetonitrile (90: 10, v/v, pH 3.8), with a flow rate of 1.2 mL/min. The mobile phase of LC2 consisted of 50.0 mmol/L ammonium acetate and acetonitrile (82.5:17.5, v/v, pH 5.2), with a flow rate of 1.0 mL/min. In PUMP1, the flow rate had a significant effect on the retention time and peak area. In PUMP3, an increase in the proportion of ammonium acetate increased the retention time but did not significantly increase the peak area. Chromatographic separation in LC1 and LC2 was distinct, with no observed interference (Fig. 1). For the accurate detection of MTX, achieving maximum absorption, and minimizing interference, the wavelength of 302 nm was selected as the absorption spectrum. When samples were treated with perchloric acid and acetonitrile, it was found that the samples treated with perchloric acid were no interference of endogenous components, and the recovery rate of the samples was higher. To enhance the recovery rate of the samples, plasma and CSF samples were treated with perchloric acid. The detector cell operated at a temperature of 40°C , and the autosampler injection volume was 200 μL . Under these conditions, the chromatographic separation was clear, the MTX peak exhibited a satisfactory shape, and no interference was observed. The analysis time was approximately 7.55 min (Fig. 2).

Method validation

Selectivity

Selectivity was ascertained using six drug-free CSF and plasma samples each, along with spiked CSF samples (at 2.38 $\mu\text{mol/L}$) and spiked plasma samples (at 5.51 $\mu\text{mol/L}$). These samples were processed without the inclusion of target compounds. Representative chromatograms are displayed in Figure 3, indicating a satisfactory MTX peak shape, a reasonably steady baseline, and the absence of other peaks.

Accuracy and precision

The inter- and intra-day precision and accuracy were evaluated across three QC levels (low, mid, and high). In the intra-day accuracy experiments, five replicates of each QC level were assayed in a single run. In the inter-day experiments, three replicates of each QC level were assayed for five consecutive days. The precision and accuracy of the method were expressed as relative standard deviation (RSD, %) and as the

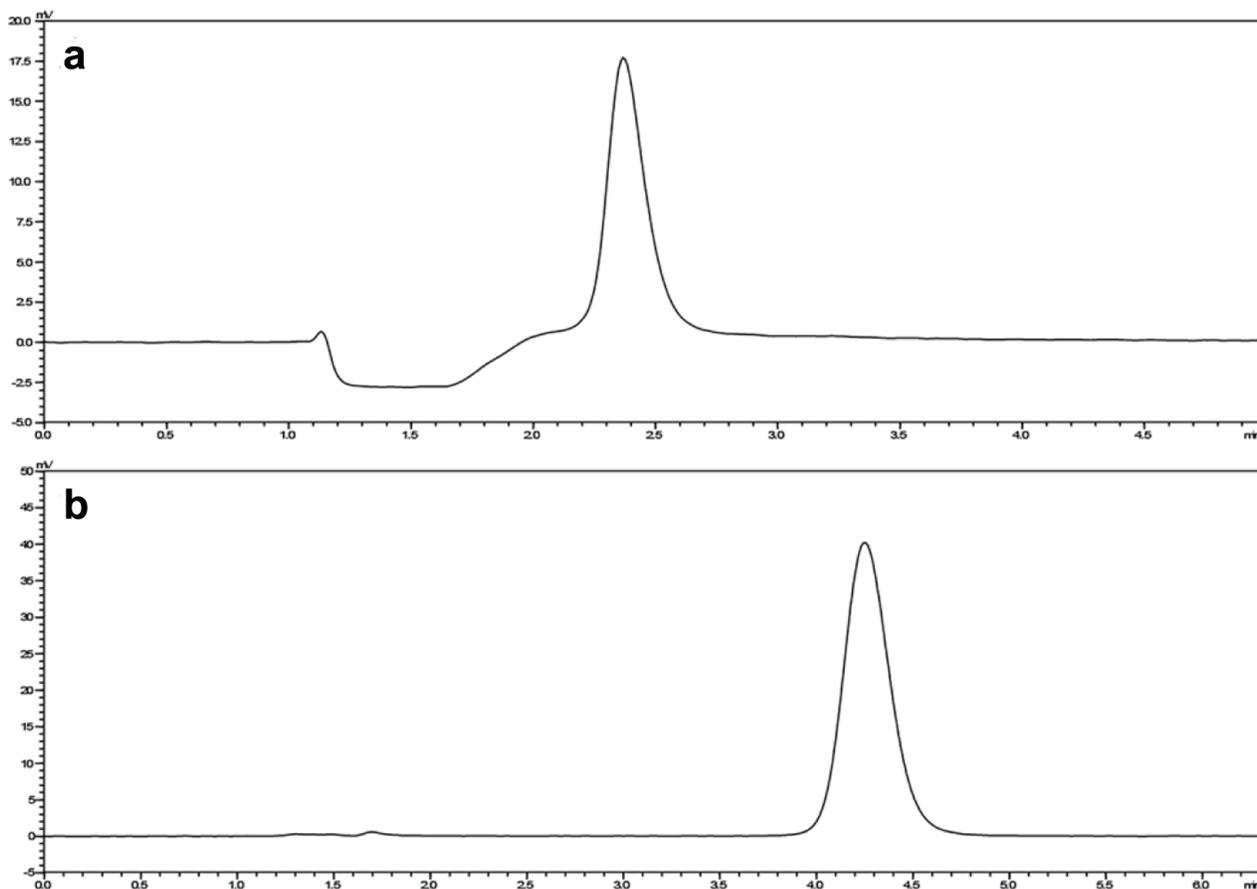


Figure 1. The peak area of MTX LC1 (a) and LC2 (b). LC1: first-dimensional liquid chromatography system; LC2: second-dimensional liquid chromatography system; MTX: methotrexate.

percent deviation of measured concentration from the nominal value, respectively. Table 1 summarizes the validation sample data for assay performance. Precision (< 5%) and accuracy (< 3%) were within the acceptance criteria for each QC level. Both inter- and intra-day accuracy and precision values met the acceptance criteria for each QC level.

Sensitivity and linearity

Six concentration levels of MTX and a blank CSF sample were used to construct a standard curve 1. The calibration curve (n = 6) exhibited linearity within the concentration range of 0.07 - 2.38 μmol/L for MTX (Fig. 4a). The calibration curve equation is presented in Equation (1). Using six concentration levels of MTX and a blank sample, a standard curve 2 was generated. The calibration curve (n = 6) displayed linearity within the concentration range of 0.05 - 5.51 μmol/L for MTX (Fig. 4b). The calibration curve equation is presented in Equation (2). These two curves were observed to coincide, indicating that the method is suitable for the simultaneous determination of MTX concentration in both CSF and plasma. Curve 3 was generated through curve fitting for all samples using Equation (3) (Fig. 4c).

$$y = 118709.23 (\pm 0.2048)x - 4275.04 (\pm 0.1827) (R^2 = 0.999) \tag{1}$$

$$y = 118680.99 (\pm 0.1192)x - 4409.68 (\pm 0.3583) (R^2 = 0.999) \tag{2}$$

$$y = 118663.53 (\pm 0.1419)x - 4304.72 (\pm 0.0623) (R^2 = 0.999) \tag{3}$$

In both cases, the lowest limit of quantitation (LLOQ) was 0.05 and 0.07 μmol/L, respectively, with a signal-to-noise ratio of ≥ 10.

Recovery

Extraction recovery was determined by comparing the peak areas of processed low-, mid-, and high-level QC samples (n = 5 for each level) with reference methanol solutions containing the target compound at equivalent concentrations. The extraction recoveries for CSF samples were 100.69±4.04%, 100.85±1.61%, and 99.84±1.46% for the low-, mid-, and high-level QC samples, respectively (Table 2). The extraction recoveries for plasma samples were 99.33±2.12%, 100.55±2.70%, and 99.99±2.90% for the low-, mid-, and high-level QC samples, respectively.

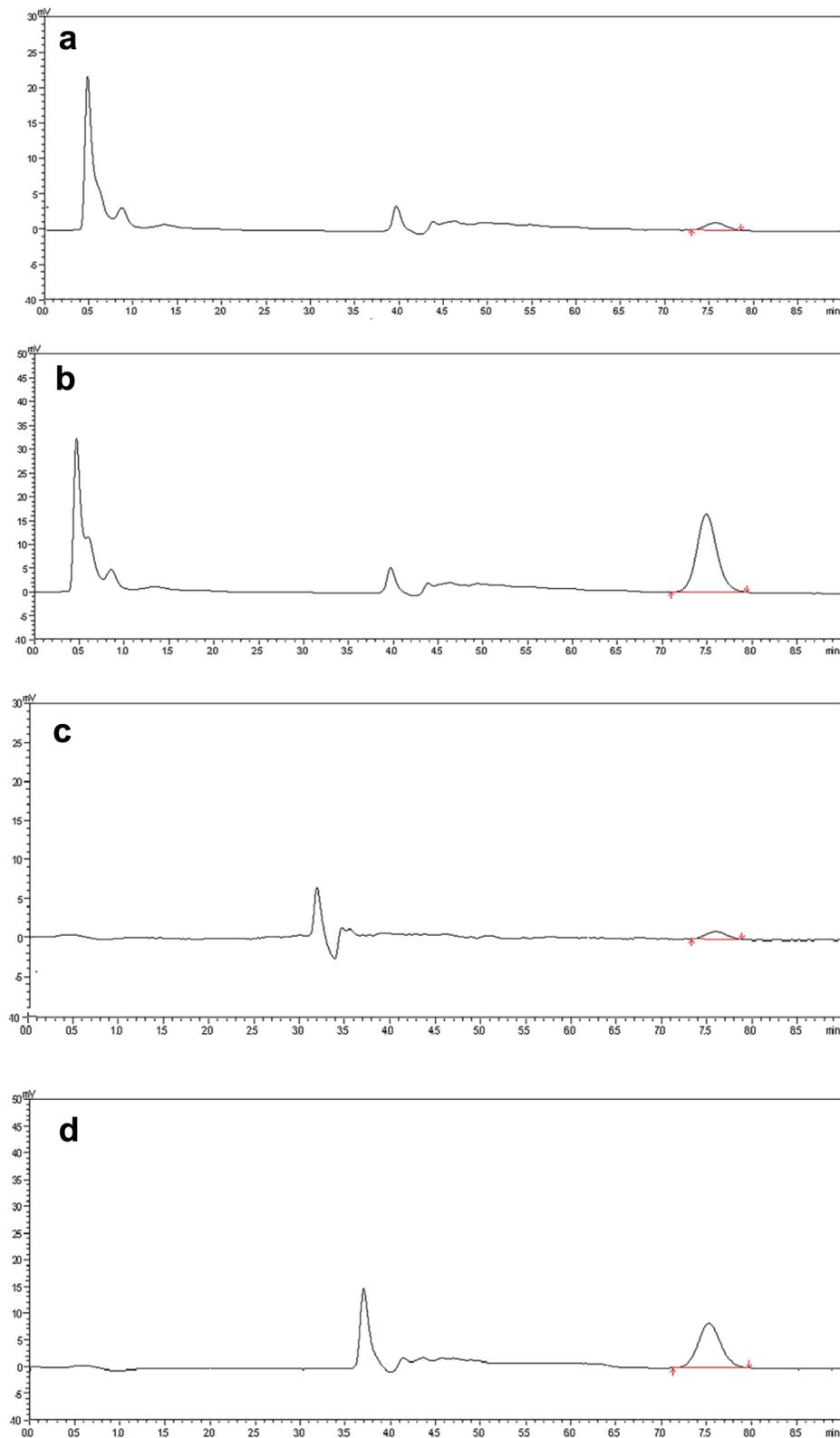


Figure 2. The chromatogram of (a) blank human plasma with MTX 0.05 $\mu\text{mol/L}$ (LLOD) and (b) clinical patient plasma with MTX (5.51 $\mu\text{mol/L}$). The chromatogram of (c) blank cerebrospinal fluid with MTX 0.05 $\mu\text{mol/L}$ (LLOD) and (d) clinical patient cerebrospinal fluid with MTX (2.38 $\mu\text{mol/L}$). MTX: methotrexate.

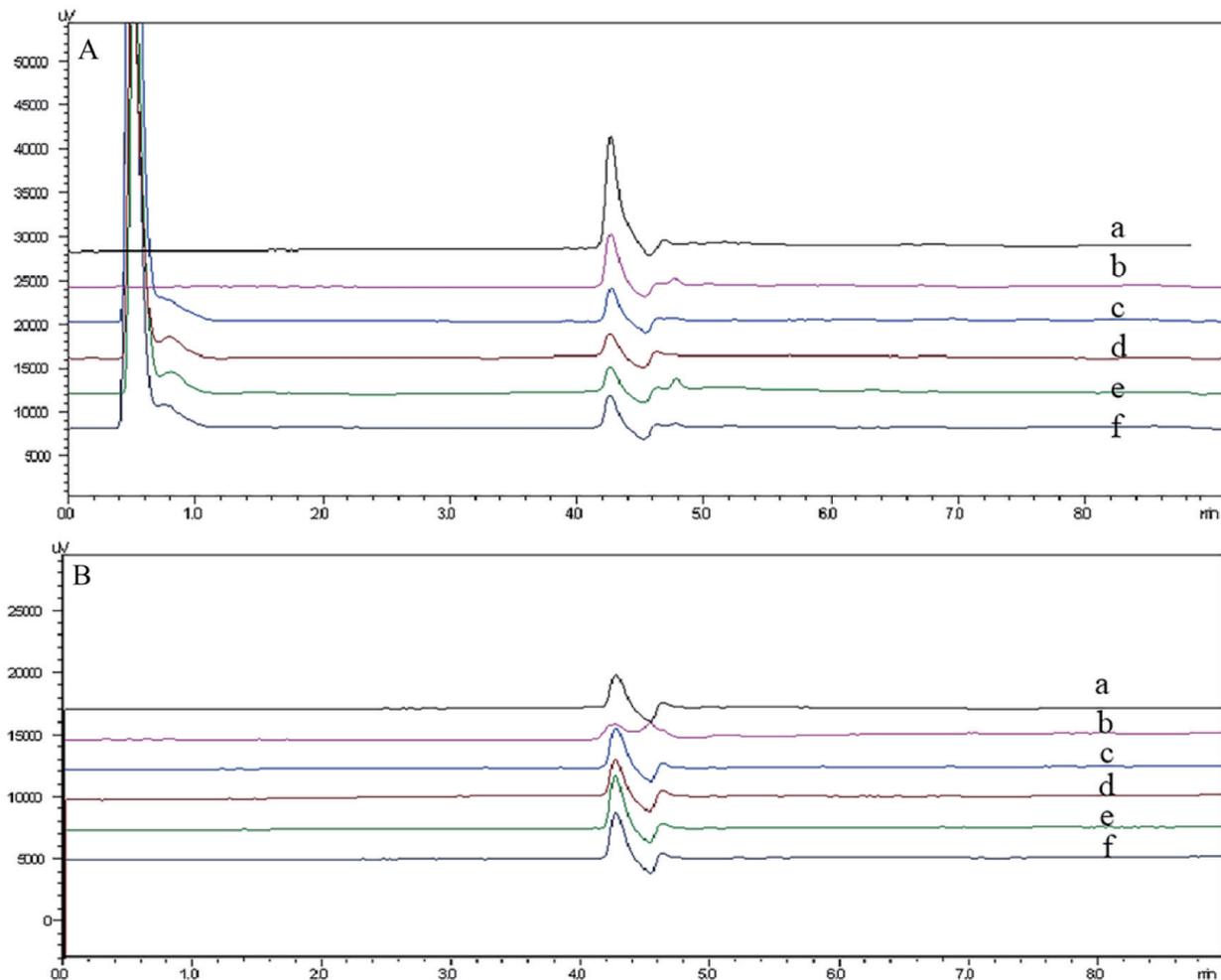


Figure 3. (A) (a) The chromatogram of blank plasma. (b-f) Clinical patient plasma without MTX. (b) Patient 1 drugs: cyclophosphamide, cytarabine, omeprazole, dexamethasone, ondansetron, levocarnitine, methotrexate, and magnesium isoglycyrrhizinate. (c) Patient 2 drugs: vancomycin, caspofungin, spironolactone, lidocaine, reduced glutathione, methylprednisolone, imipenem and cilastatin, voriconazole, and linezolid. (d) Patient 3 drugs: vitamin B6, furosemide, pantoprazole, methotrexate, magnesium isoglycyrrhizinate, vitamin C, tropisetron, rituximab, indomethacin, and promethazine. (e) Patient 4 drugs: cefoperazone and sulbactam, torasemide, pantoprazole, palonosetron, rituximab, promethazine, methotrexate, levoleucovorin calcium, and recombinant human interleukin. (f) Patient 5 drugs: idarubicin hydrochloride, vindesine, sulfate mesna, cyclophosphamide, omeprazole, prednisone acetate, and meropenem. (B) (a) The chromatogram of blank cerebrospinal fluid. (b-f) Clinical patient cerebrospinal fluid without MTX. (b) Patient 1 drugs: cyclophosphamide, cytarabine, omeprazole, dexamethasone, ondansetron, levocarnitine, methotrexate, and magnesium isoglycyrrhizinate. (c) Patient 2 drugs: vancomycin, caspofungin, spironolactone, lidocaine, reduced glutathione, methylprednisolone, imipenem and cilastatin, voriconazole, and linezolid. (d) Patient 3 drugs: vitamin B6, furosemide, pantoprazole, methotrexate, magnesium isoglycyrrhizinate, vitamin C, tropisetron, rituximab, indomethacin, and promethazine. (e) Patient 4 drugs: cefoperazone and sulbactam, torasemide, pantoprazole, palonosetron, rituximab, promethazine, methotrexate, levoleucovorin calcium, and recombinant human interleukin. (f) Patient 5 drugs: idarubicin hydrochloride, vindesine, sulfate mesna, cyclophosphamide, omeprazole, prednisone acetate, and meropenem. MTX: methotrexate.

This method exhibited a high extraction recovery rate, thereby meeting the requirements of clinical testing.

Stability

Stability studies were performed on the low-, mid-, and high-level QC samples. These samples were frozen in a refrigera-

tor (-80 °C) for 12 h and subsequently thawed at room temperature for three freeze-thaw cycles to analyze freeze-thaw stability. For short-term stability testing, samples were placed at room temperature for 6 h, whereas for long-term stability testing, samples were stored at -80 °C for 30 days. The stability results indicated that the level of stability, as denoted by the RSD, was less than 4% (Table 3). MTX remained stable in plasma and CSF samples after three freeze-thaw cycles (-80 °C

Table 1. Intra- and Inter-Assay Precision and Accuracy Results of MTX (n = 5)

	QC levels (μmol/L)	Intra-day		Inter-day	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Human plasma	0.07	-0.46	4.93	0.26	0.60
	0.55	-0.33	1.34	0.33	3.79
	1.38	1.10	3.25	-1.33	3.78
	4.13	0.60	1.93	0.66	2.31
Cerebrospinal fluid	0.05	1.32	2.72	0.47	0.67
	0.24	0.36	0.49	1.33	2.82
	0.71	2.17	4.05	2.30	3.56
	1.49	1.60	0.36	1.48	2.42

MTX: methotrexate; QC: quality control.

to room temperature) as well as when stored at room temperature for 6 h and at -80 °C for 30 days. No detrimental effect was observed on the plasma and CSF concentrations of MTX under the studied conditions (Table 3).

Clinical application

We measured the concentrations of MTX in CSF and plasma

from 35 patients and analyzed these two concentrations. The test results are presented in Table 4 and Figure 5. MTX concentrations of 1 μmol/L or greater were detected in 76.47% of CSF samples. In our study, the correlation between MTX concentrations in the plasma and CSF was moderate (r = 0.502). The concentrations of MTX in CSF and plasma exhibited a significant correlation with the dosage of MTX administered (P = 0.0002 and P = 0.0372, respectively). Additionally, we analyzed the difference in MTX concentration in CSF between

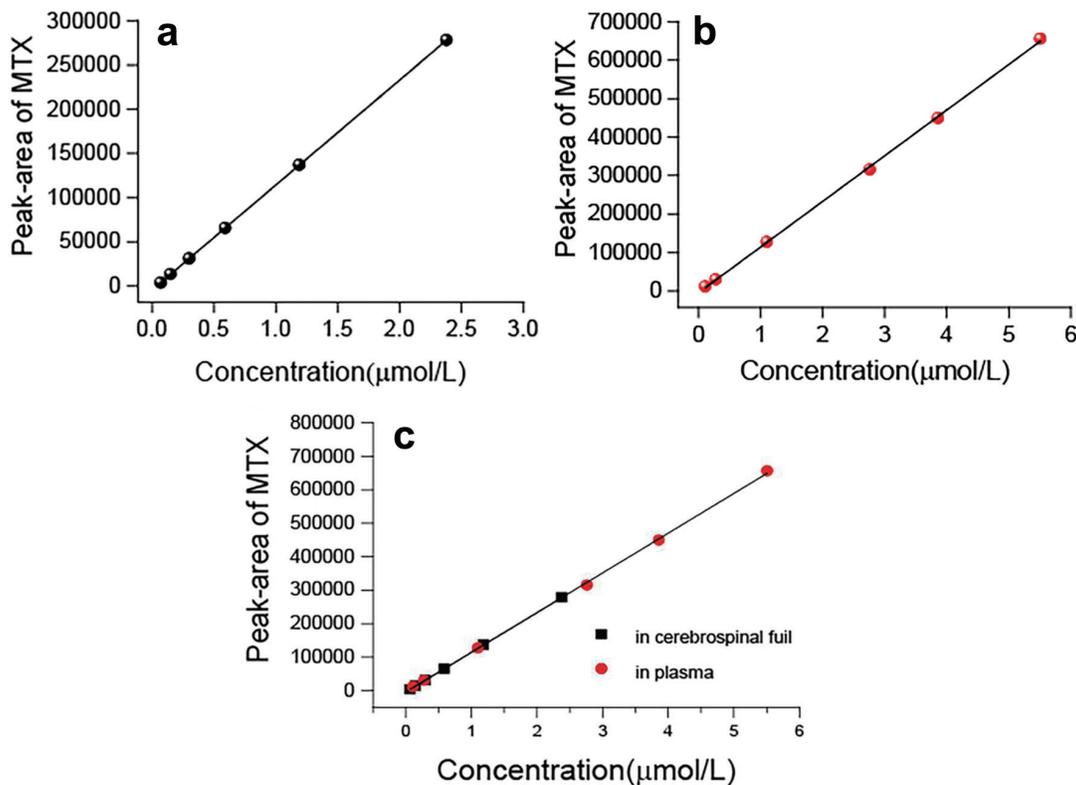


Figure 4. (a) The linearity of the standard curve was observed at MTX concentrations that ranged 0.07 to 2.38 μmol/L (cerebrospinal fluid). (b) The linearity of the standard curve was observed at MTX concentrations that ranged 1.10 to 5.51 μmol/L (human plasma). (c) The linearity of the standard curve was observed at MTX concentrations that ranged 0.07 to 5.51 μmol/L (cerebrospinal fluid and human plasma). MTX: methotrexate.

Table 2. Recovery Results of MTX

	QC levels ($\mu\text{mol/L}$)	Recovery	
		Mean \pm SD (%)	RSD (%)
Human plasma	0.55	99.33 \pm 2.12	3.88
	1.38	100.55 \pm 2.70	1.95
	4.13	99.99 \pm 2.90	0.7
Cerebrospinal fluid	0.24	100.69 \pm 4.04	1.67
	0.71	100.85 \pm 1.61	2.25
	1.49	99.84 \pm 1.46	3.61

MTX: methotrexate; QC: quality control; RSD: relative standard deviation; SD: standard deviation.

15 patients treated with intravenous mannitol and 20 patients not treated with intravenous mannitol, and the results were not significantly different. These results indicated that intravenous mannitol may not increase the concentration of MTX in the CSF of patients.

Discussion

2D-LC is renowned for its superior robustness and real-time performance [19, 20]. In China, more than 200 units use 2D-LC, and its accuracy is widely recognized. A core patent of this new two-dimensional system addresses the challenge of controlled transfer of objects between one and two dimensions

(US10859476 B2; JP6639527). The LC1 and LC2 columns of the fully automated 2D-LC used the unique 2D column system (RP1-IEX-RP2), wherein the retention mechanisms of RP1 and IEX differed substantially, leading to strong orthogonality of RP1-IEX. The LC1 column first separated MTX and impurities via an RP separation mechanism, followed by an IEX column segregating MTX and co-eluted components via an ion-exchange separation mechanism. Compounds with a strong ion-exchange capacity, such as MTX, were retained by the IEX column, whereas the co-eluted nonionic and anionic components were removed. This demonstrated the enhanced resolving power and high sensitivity of 2D-LC, making it suitable for determining extremely complex, multi-component mixtures. The new 2D-LC featured dual-level control and hierarchical diagnosis, enabling both positioning research and independent analysis of one-dimensional coarse separated substances. Its capture column not only captured and transferred the analyte but also performed separation, thereby further enhancing the separation ability of the fully automated 2D-LC. Moreover, factors such as laboratory environments (e.g., ambient temperature and uncontrolled humidity state) and sample status (e.g., hemolyzed and high-fat samples) did not significantly influence testing. Changes in ion concentration and pH in the mobile phase, influenced by the proficiency of the operator and column type, did not significantly affect accuracy. All processes were controlled by the time program of the chromatographic workstation, eliminating the need for manual intervention and ensuring high automation and rapid analysis.

Currently, liquid chromatography mass spectrometry (LC-

Table 3. Results of Stability (n = 5)

	Condition	QC levels ($\mu\text{mol/L}$)	Mean \pm SD (%)	RSD (%)
Human plasma	Freeze-thaw stability (three cycles)	0.55	98.12 \pm 1.70	3.16
		1.38	99.47 \pm 1.74	1.27
		4.13	100.89 \pm 4.16	1
	Short-term stability (6 h at room temperature)	0.55	99.76 \pm 0.74	1.34
		1.38	99.04 \pm 4.73	3.46
		4.13	99.86 \pm 1.55	0.37
	Long-term stability 30 days at -80 °C)	0.55	100.54 \pm 1.57	2.84
		1.38	100.74 \pm 2.95	2.12
		4.13	99.80 \pm 0.35	0.85
Cerebrospinal fluid	Freeze-thaw stability (three cycles)	0.24	99.31 \pm 0.42	1.75
		0.71	100.47 \pm 1.42	1.99
		1.49	100.33 \pm 0.87	0.66
	Short-term stability (6 h at room temperature)	0.24	98.75 \pm 0.85	3.61
		0.71	99.15 \pm 1.85	2.63
		1.49	100.56 \pm 2.00	2.46
	Long-term stability (30 days at -80 °C)	0.24	99.17 \pm 0.30	1.26
		0.71	100.09 \pm 1.17	1.64
		1.49	100.83 \pm 0.47	0.31

QC: quality control; RSD: relative standard deviation; SD: standard deviation.

Table 4. Test Results of Plasma and Cerebrospinal Fluid Concentration of Subjects

No.	Mode of administration	Sex	Age (years)	Weight (kg)	Fluid concentration ($\mu\text{mol/L}$)	Plasma ($\mu\text{mol/L}$)	Combined with intravenous mannitol
1	6.5 g	Male	59	67.5	1.275	299.644	No
2	5 g	Male	62	69	4.306	155.573	No
3	6.5 g	Male	59	69	3.310	171.422	No
4	3.5 g	Female	45	70	0.848	77.380	Yes
5	3.5 g	Male	72	65	2.101	87.899	No
6	6 g	Female	46	66	1.403	88.177	Yes
7	6 g	Male	50	80	1.162	152.761	Yes
8	5.16 g	Male	66	60	3.961	223.446	Yes
9	6 g	Male	72	66	2.394	145.875	No
10	6 g	Male	50	85	1.420	120.460	Yes
11	6 g	Female	46	66	1.793	161.457	No
12	6.5 g	Male	59	69	3.983	12.554	Yes
13	3.5 g	Male	62	69.5	2.168	129.279	No
14	6 g	Male	72	66	2.109	158.315	No
15	6 g	Male	50	88	1.943	108.490	Yes
16	4.5 g	Female	46	65	1.543	151.782	No
17	6 g	Male	50	88	3.089	103.449	Yes
18	4.7 g	Female	70	60	1.703	122.792	Yes
19	5 g	Male	68	59	2.668	209.483	No
20	4 g	Male	52	58	1.307	42.774	Yes
21	6 g	Male	55	90	2.580	89.987	No
22	1.7 g	Female	68	67.5	0.946	35.847	No
23	6 g	Male	72	66	2.617	148.489	Yes
24	3 g	Male	52	57	0.728	43.607	No
25	6 g	Male	55	90	3.743	77.833	Yes
26	5 g	Male	61	68	3.057	4.967 ^a	No
27	3 g	Female	48	47	0.512	0.436 ^a	Yes
28	5.7 g	Female	35	56	0.097 ^a	3.295 ^a	No
29	3.5 g	Male	61	65	0.611	2.169 ^a	No
30	5.7 g	Female	35	54	1.309	0.267 ^b	Yes
31	3.5 g	Male	61	65	0.037	1.863 ^a	No
32	6.4 g	Male	52	64	1.491	0.991 ^a	Yes
33	1.8 g	Male	32	63	0.358	1.461 ^c	No
34	3.5 g	Female	42	57	0.526	0.099 ^c	Yes
35	1.5 g	Female	57	60	1.975	0.042 ^c	No

^a24 h. ^b48 h. ^c72 h.

MS)/MS is primarily used for the quantitative analysis of MTX [12, 21]. Despite being highly sensitive and unaffected by interference from MTX metabolites, this method entails expensive instrumentation and complicated operations. In contrast, the 2D-LC method requires only regular maintenance of chromatographic columns, detectors, and chromatographic pumps, without requiring advanced professional knowledge or train-

ing. It is simpler for technicians to learn and operate, offering a significant separation, automation, robustness, and real-time performance. In our study, the linear range of MTX was 0.07 - 2.38 $\mu\text{mol/L}$ for CSF samples and 0.11 - 5.51 $\mu\text{mol/L}$ for plasma samples. LLOQ was 0.05 and 0.07 $\mu\text{mol/L}$, respectively, and the retention time was 7.55 min. Although still inferior to MS in terms of anti-interference capability, sensitivity, and

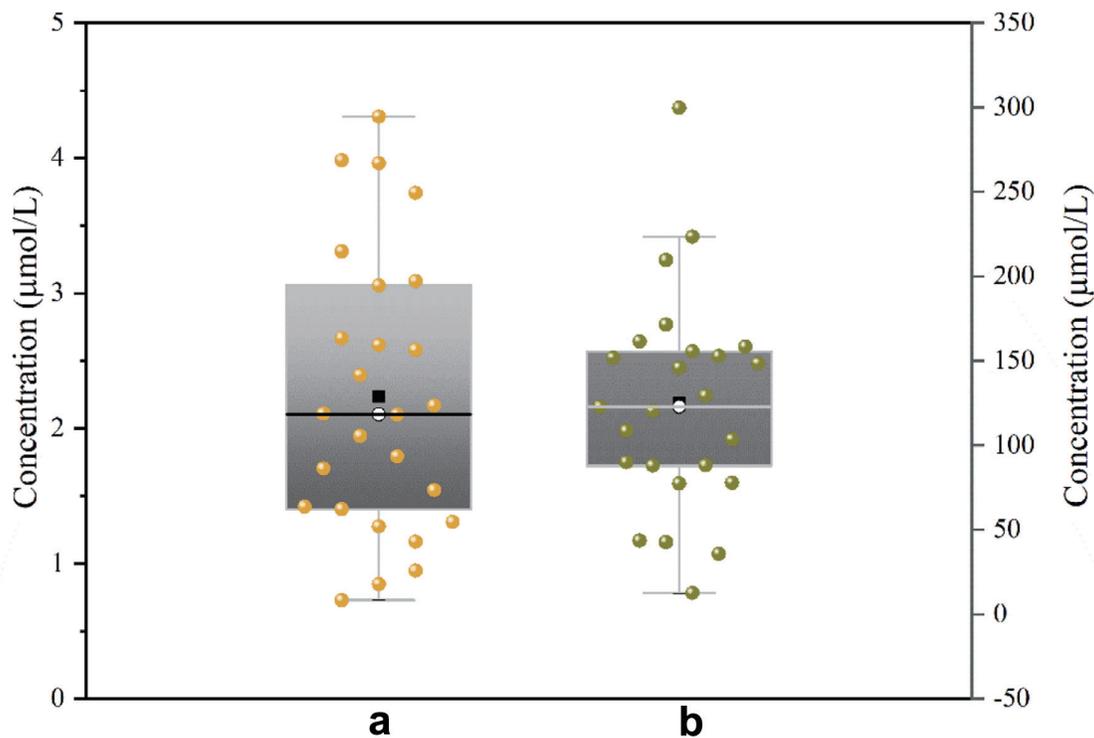


Figure 5. Figure 5. Test results from samples of (a) CSF and (b) plasma. CSF: cerebrospinal fluid.

simultaneous determination of various components, the detection sensitivity of this assay was adequate for performing TDM in PCNSL patients.

Limitations

Our study presents several limitations. First, the analysis relied on routine monitoring data of MTX. Additionally, we did not collect numerous covariates, including genetic polymorphisms, hydration volume, alkalization volume, and the calcium folinate rescue regimen, precluding multivariate analyses that could have identified predictors of outcomes and confounding variables. Second, this is a single-center retrospective study, which may introduce multiple biases. Third, obtaining an adequate number of CSF samples is necessary, and the procedure itself is challenging. Among the CSF samples collected, only those obtained 0.5 - 2 h after the initiation of the HD-MTX infusion were selected for analysis. Therefore, whether all samples reached steady-state concentration in the CSF remains uncertain.

Conclusion

In this study, we developed a robust 2D-LC method with enhanced resolving power and high sensitivity, suitable for determining extremely complex, multi-component mixtures. This method can simultaneously and accurately determine the total

and free concentrations of MTX in both CSF and plasma. It was successfully applied to analyze plasma and CSF levels in patients with PCNSL. There are no significant differences between patients treated with intravenous mannitol and patients not treated with intravenous mannitol. However, this conclusion may be limited by the small number of samples we collected. Collecting CSF samples poses potential hazards and presents additional challenges for clinicians. We will continue our study and collect a sufficient number of samples to further explore the effect of intravenous mannitol on MTX across the BBB.

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Conflict of Interest

The authors have no conflict of interest to declare.

Informed Consent

All patients signed informed consent.

Author Contributions

Yan Hong Wang tested the samples and is the main contributor to the manuscript. Qiang He mainly operated the experiment. Wang Feng provided the technical support. Jing Shi completed part of the experiment. Hao Jiang analyzed and screened the patient's data. Ji Ma and Yu Guo Liu designed the experiment. All the authors have read and approved the final manuscript.

Data Availability

All data generated or analyzed during this study are included in this published article.

Abbreviations

MTX: methotrexate; CSF: cerebrospinal fluid; 2D-LC: two-dimensional liquid chromatography; QC: quality control; PC-NSL: primary central nervous system lymphoma; DLBCL: diffuse large B-cell lymphoma; HD-MTX: high-dose methotrexate; BBB: blood-brain barrier; TDM: therapeutic drug monitoring; LC1: first-dimensional liquid chromatography system; LC2: second-dimensional liquid chromatography system; IT: immediately before the intrathecal; HPLC: high-performance liquid chromatography; LC-MS: liquid chromatography mass spectrometry

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