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Short communication

Development and validation of sensitive liquid chromatography/tandem mass spectrometry method for quantification of bendamustine in mouse brain tissue

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ABSTRACT

A liquid chromatography–tandem mass spectrometry method for quantification of bendamustine in mouse brain tissue was developed and fully validated. Methanol was used to precipitate proteins in brain tissue. Bendamustine and internal standard (chlorambucil) were separated with reverse-phase chromatography on a C-18 column with a gradient of water and 95% methanol in 0.1% formic acid. Positive mode electrospray ionization was applied with selected reaction monitoring to achieve 5 ng/ml lower limits of quantitation in mouse brain tissue. The calibration curve for bendamustine in mouse brain was linear between 5 and 2000 ng/ml. The within- and between-batch accuracy and precision of the assay were within 15% at 10, 100 and 1000 ng/ml. The recovery and matrix effect of bendamustine in mouse brain tissue ranged from 41.1% to 51.6% and 107.4% to 110.3%, respectively. The validated method was then applied to quantitate bendamustine in an animal study. Results indicate the assay can be applied to evaluate bendamustine disposition in mouse brain tissue. This assay will be applied in the future to detect and quantify bendamustine in human brain tissue samples.

1. Introduction

Brain metastases, usually characterized by poor prognosis and short survival time, most commonly result from primary lung cancer (36–64%), breast cancer (15–25%), and melanoma (5–20%) [1,2]. A major long-standing challenge for treatment of brain tumors is the inability to achieve effective drug exposure of anticancer agents due to the tight junctions between endothelial cells, lack of fenestrations, and drug efflux transporter systems of the blood–brain barrier (BBB) [3–5]. Current treatment options for brain metastases include surgery, radiation therapy, stereotactic radiosurgery, and chemotherapy. Although stereotactic radiosurgery is an effective treatment option for patients with brain metastases, local recurrence can occur in approximately 30% of these patients [6–9]. Radiosensitizers can be used in radiation therapy and radiosurgery to enhance the sensitivities of tumor cells to radiation and consequently to improve the clinical outcome.

Bendamustine (BM) was first synthesized in 1963 in Germany. In 2008, BM was approved in the United States for treatment of chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma (NHL). Its anticancer mechanisms include inducing the formation of intra-strand and inter-strand crosslinks between DNA bases, causing significant DNA single/double-strand breaks, leading to concentration-dependent apoptosis and non-apoptotic cell death or mitotic catastrophe. Both preclinical and clinical studies indicate BM showed better anticancer activities compared with other alkylating agents, likely due to more durable DNA double-strand breaks induced by BM [9–13]. These properties indicate BM may be useful as a radiosensitizer. Currently, more than 100 clinical trials related to BM have been initiated in the United States to evaluate its activity in lymphoma, CLL, NHL, multiple myeloma, solid tumors, primary and metastatic brain tumors, and other diseases [14–19]. In particular, our group is conducting a phase I clinical trial of BM combined with fractionated stereotactic radiotherapy in patients with metastatic brain lesions from solid malignancies. One objective of

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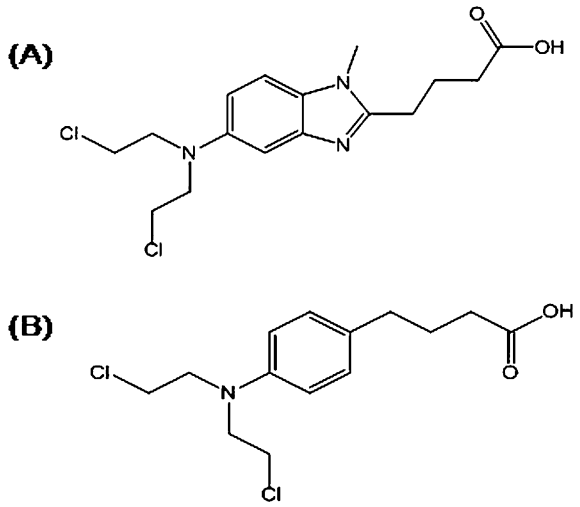

Fig. 1. Chemical structures for BM (A) and IS, chlorambucil (B).

this study will be to quantitatively assess BM plasma pharmacokinetics and concentrations in the metastatic tumor lesions surgically removed from patients following BM treatment. Therefore, development and validation of assays to achieve adequate sensitivity for accurately measuring potentially low levels in brain tissue will be critical to better understand the pharmacokinetics of BM, its penetration across the BBB, and the clinical outcomes from therapy.

Although high performance liquid chromatography/tandem mass spectrometry (LC–MS/MS) methods for plasma BM analysis have been presented in the published literature [20–23], no assays have been presented for quantification of BM in brain tissue. In this study, we developed and validated a highly sensitive LC–MS/MS assay for BM measurement in mouse brain tissue (as a surrogate for human brain tissue). The lower limit of quantification (LLOQ) of the method is 5 ng/ml based on Food and Drug Administration (FDA) guidelines criteria [24].

2. Experimental

2.1. Chemicals and reagents

BM (material description: CEP-18083; material control number: 06E087; Cephalon lot number: 059004A2), 4-{5-[bis(2-chloroethyl)-amino]-1-methyl-1H-benzoimidazol-2-yl}-butanoic acid, MW 358.26) was obtained from Cephalon Inc., Frazer, PA 19355. Methanol and HPLC grade water were purchased from ThermoFisher Scientific (Waltham, MA). The internal standard (IS), chlorambucil (MW 304.21) and all other chemicals were purchased from Sigma (St. Louis, MO). Chemical structures of BM and IS are shown in Fig. 1.

2.2. Preparation of stock solution and calibration samples

Stock solutions of BM and IS were prepared in methanol (MeOH) at the concentration of 1 mg/ml and stored in polypropylene centrifuge tubes (Life Science Products, Rochester, NY) at −20 °C for up to 1 month. BM standard solutions (10×) were prepared in MeOH from stock solution by serial dilution. IS solution with the concentration of 10 μg/ml was prepared in MeOH from IS stock solution.

Equal volumes of water were added to mouse brain tissue (e.g. 100 μl water was added to 100 mg tissue) and then sheared with a high-speed homogenizer (ProScientific, Oxford, CT). 10 μl standard solutions (10×) was spiked into each mouse brain homogenate to produce calibration samples at various final concentrations between 5 ng/ml and 2000 ng/ml. Quality control (QC) samples were produced at 10, 100 and 1000 ng/ml. Blank and zero samples were produced by adding 10 μl 20/80 water/MeOH and 10 μl 10 μg/ml IS to mouse brain homogenate, respectively.

2.3. Sample processing

10 μl of 10 μg/ml IS was spiked into mouse brain homogenate. 1 ml MeOH was added to each brain homogenate, then mixtures were vortex mixed for 30 s and centrifuged at 18,000 × *g* at 4 °C for 10 min. The supernatant was transferred to a clean centrifuge tube and dried with N_2 gas stream. Samples were reconstituted with 120 μl 20/80 H_2O/MeOH. After vortex (20 s) and centrifugation (18,000 × *g*, 4 °C, 10 min), 100 μl supernatant was loaded into autosampler vials for analysis.

2.4. LC–MS/MS instrumentation

A Shimadzu Prominence HPLC system connected to a TSQ Quantum Ultra EMR triple quadrupole mass spectrometer (Thermo Fisher Scientific Corporation, San Jose, CA) equipped with an electrospray ionization (ESI) source was used in this study. Xcalibur software was employed for system control and data processing. The HPLC system included a dual LC-20AD pump, SIL-20AC autosampler, CBM 20A communications bus module, and CTO-20A column oven. A reverse-phase Zorbax (Agilent) C-18 column (3.5 μm, 2.1 mm × 50 mm) with a Metaguard (Varian, Walnut Creek, CA) C-18 guard column (5 μm, 2 mm × 10 mm) was used to separate samples (20 μl injections). Mobile phases were 100% water with 0.1% formic acid (A) and 95/5 MeOH/water with 0.1% formic acid (B). Initial mobile phase composition was 100% A with a gradient to 100% B from 0.01 to 2.5 min. This was held for 2.5 min and followed by a 0.2-min linear gradient return to initial conditions for equilibration for the rest of the 8.2-min run. The flow rate was 0.3 ml/min and remained constant throughout the run.

BM and IS were ionized with ESI in positive mode and fragmented with collision gas for analysis using single reaction monitoring (SRM). Final parameter settings were as follows: collision energy, 25 V; scan time, 1.0 s; scan width, 0.5 *m/z*; Q1 and Q3 peak width, 0.7 full width at half-maximum *m/z*; collision gas pressure, 1.5 mTorr. Mass transitions monitored were 358.00 > 340.10 (BM) and 304.10 > 192.10 (IS), $[M+H]^+$. Peaks were integrated using the Interactive Chemical Integration System algorithm, and least squares regression was employed with equal weighting to fit a straight line for the peak area ratios (BM/IS) versus analyte concentration.

2.5. Method validation

The method validation for the BM assay was performed following the Food and Drug Administration guidelines [24] and included selectivity, linearity, sensitivity, precision, accuracy, recovery, matrix effect and stability of BM in mouse brain.

Calibration standards were produced at the concentrations of 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml in mouse brain. Quality control (QC) validation samples were prepared at 10, 100, and 1000 ng/ml. Blank (no BM and IS), zero (IS only) samples and calibration samples were included in validation runs, but blank and zero samples were not employed for calibration curve regression. Triplicate mouse brain QC samples at each QC levels were aliquoted (100 mg homogenized mouse brain) and stored in −70 °C for bench-top, freeze-thaw, short-term, long-term and post-preparative autosampler stability. Bench-top stability was evaluated by analyzing QC samples after 4-h storage at room temperature. Freeze-thaw QC samples were removed from the freezer and placed back into the freezer for at least 24 h after they were thoroughly thawed. The freeze-thaw cycles were repeated 3 times

and samples were analyzed within 2 weeks after initial freezing. Short-term and long-term stability samples were analyzed after 1 week and 1 month, separately. Post-preparative autosampler stability was determined by reinjection of samples 28 h later after the initial injection. Recovery was evaluated by comparing chromatographic peak areas and peak area ratios (BM/IS) in extracted pre-spiked mouse brain homogenate versus extracted post-spiked mouse brain homogenate. Matrix effect was calculated by comparing the peak areas of analytes between samples which were post-spiked to dry extracted blank mouse brain and neat solutions. A single source of 20 pooled mouse brain was used in matrix effect evaluation [25]. Triplicate samples at each QC levels were used to evaluate both recovery and matrix effect.

2.6. Pharmacokinetic studies in patients and mice

The assay was applied to mouse brain tissue samples collected after BM dosing. In the animal study, BM was injected through tail vein to ICR mice (4–6 weeks, male, $n = 3$) at a dose of 50 mg/kg. Both plasma and brain tissue were collected at 3, 5, 6, 9, and 15 min after injection and stored at −70 °C until analysis.

3. Results and discussion

3.1. Assay conditions

Chlorambucil was chosen as a suitable internal standard due to its similar structure to BM, The methanol precipitation method was initially evaluated and found to be acceptable for recovery from mouse brain tissue throughout the linear range.

The responses of BM and IS were evaluated with ESI and atmospheric pressure chemical ionization in both positive and negative ion modes. Positive mode ESI was selected as the preferred ionization source and polarity because it provided superior sensitivity of BM compared with other ionization modes evaluated. To minimize the possibility of carry-over from previous samples and to ensure accuracy of results, a 10-s pre-wash and post-wash with 50% acetonitrile was performed for each injection. Water and 20/80 water/MeOH both with 0.1% formic acid provided the best chromatographic and detector response results compared to various other mobile phases and modifiers evaluated. Gradient conditions were established to elute BM and IS at 3.85 and 4.59 min, separately.

3.2. Selectivity

The selectivity of the method was evaluated by comparing the chromatograms of blank sample without or with BM spiked at the LLOQ. BM and IS were eluted at 3.85 and 4.59 min, respectively, and no interfering peaks were observed at these retention times. Representative chromatograms of blank mouse brain and mouse brain spiked with 5 ng/ml BM are presented in Fig. 2.

3.3. Linearity and sensitivity

Calibration curves were generated by plotting the peak area ratios (BM/IS) of calibration standard versus nominalconcentration.

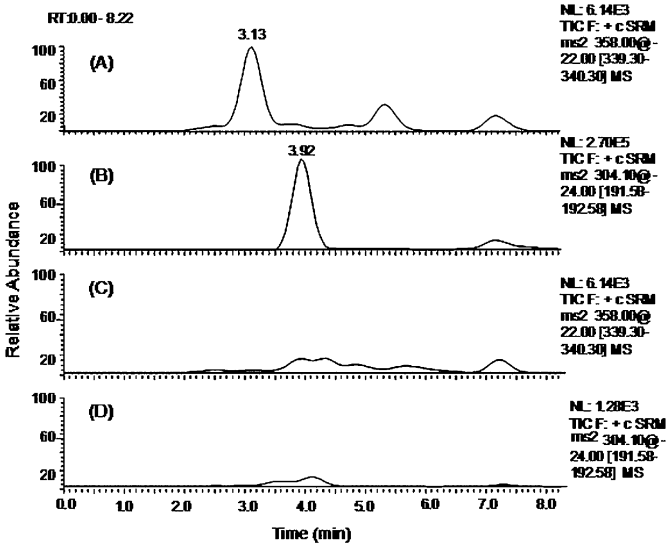

Fig. 2. Mass chromatograms of mouse brain spiked with 5 ng/ml BM (LLOQ) (A and B) and blank mouse brain (C and D). SRM channels include BM (358.00 > 340.10; A and C) and chlorambucil (304.10 > 192.10; B and D).

The limit of detection is 2 ng/ml for BM in mouse brain (data not shown). LLOQs, defined as the lowest concentration on the calibration curve with both accuracy and precision within ±20%, were 5 ng/ml for BM in mouse brain in this study. The calibration curves were linear in mouse brain tissue up to 2000 ng/ml.

3.4. Accuracy and precision

Three batches of mouse brain tissue QC samples at three concentration levels (10, 100, and 1000 ng/ml) were used for the calculation of accuracy and precision, which are shown in Table 1. Both within-batch and between-batch precisions are below 14.2% and accuracy values range from 99.4% to 114.1%, which are all within the FDA criteria.

3.5. Recovery and matrix effect

The pre-spiked QC samples were prepared by using methanol to precipitate protein after BM standard solutions and IS were added, while the post-spiked QC samples were prepared by precipitating protein before BM standard solutions and IS were added. Recovery was calculated by comparing the peak areas of pre-spiked QCs with those of post-spiked QCs. The mean recovery is 41.1 ± 4.8%, 51.6 ± 1.7% and 47.2 ± 1.1% for BM, and 60.3 ± 2.0%, 68.4 ± 3.6%, and 69.2 ± 3.9% for IS at concentrations of 10, 100, and 1000 ng/ml in mouse brain, respectively.

Matrix effect was evaluated by comparing the peak areas of post-spiked QCs with those of neat solutions, which were prepared by spiking BM and IS to 20/80 water/MeOH. The mean matrix effect (ME) in pooled mouse plasma at 10, 100, and 1000 ng/ml QC levels are 107.4 ± 5.2%, 108.1 ± 2.6%, 110.3 ± 7.3% for BM, and 45.7 ± 1.9%, 36.9 ± 1.3%, 36.4 ± 2.8% for IS, respectively.

Table 1
Within-batch and between-batch accuracy and precision for three validation runs.

Nominal conc. (ng/ml)	Within-batch			Between-batch		
	n	Precision (%CV)	Accuracy (%)	*n*	Precision (%CV)	Accuracy (%)
10	5	2.4	114.1	15	6.0	110.3
100	5	14.1	99.4	15	9.7	102.4
1000	5	14.2	102.2	15	10.8	98.0

Table 2
Stability of BM in mouse brain tissue ($n = 3$).

	Accuracy (mean ± SD, %)		
	10 ng/ml	100 ng/ml	1000 ng/ml
Bench-top stability	100.8 ± 8.0	103.6 ± 4.7	106.4 ± 8.3
Autosampler stability	92.7 ± 11.2	105.0 ± 4.5	104.7 ± 8.4
Short-term stability	106.8 ± 10.9	104.4 ± 4.9	92.7 ± 2.9
Long-term stability	112.0 ± 2.2	105.5 ± 2.2	96.5 ± 9.3
Freeze-thaw stability	91.5 ± 5.9	93.7 ± 7.9	87.4 ± 9.2

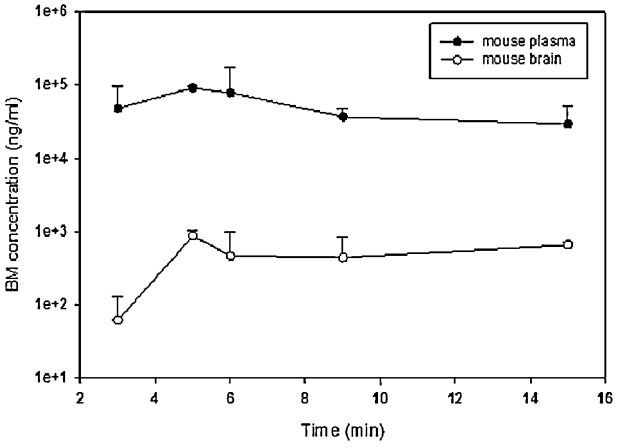

Fig. 3. Mouse BM plasma and brain concentration versus time plot with BM i.v. injection at 50 mg/kg. Data points are mean ± SD ($n = 3$).

3.6. Stability

The stability of BM in mouse brain is displayed in Table 2. Results indicated BM remained stable and no detectable loss or degradation under the conditions described above.

3.7. Quantification of BM levels in mouse brain

This method was successfully applied to detect BM levels in mouse brain tissue in an animal study. Both plasma and brain tissue were collected at various time points between 2 and 15 min after tail vein injection at the dose of 50 mg/kg. Mouse plasma samples were processed and analyzed with a sensitive LC/MS assay with similar methods and a LLOQ of 2 ng/ml (data for the plasma assay is not shown). The pharmacokinetic profile of the animal study is shown in Fig. 3 and the results indicate that BM levels can be quantified accurately in both mouse plasma and brain. Maximum measured BM levels in mouse brain were observed at the 5 min sampling time. Overall BM levels in mouse plasma were approximately 100-fold higher than in mouse brain.

4. Conclusion

An LC–MS/MS method for BM quantification in mouse brain was developed and validated with LLOQs of 5 ng/ml. The application of the method to quantify drug in mouse brain samples indicates the method will be useful for evaluating the ability of BM to penetrate metastatic brain tumors in human patients.

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