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Development and Validation of a Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination of Temozolomide in Mouse Brain Tissue

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RESEARCH ARTICLE

Development and validation of a liquid chromatography-tandem mass spectrometry method for the determination of temozolomide in mouse brain tissue

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Temozolomide is a Food and Drug Administration-approved anticancer drug that has poor drug delivery via oral or intravenous routes. A potential strategy to combat this problem is investigating alternative routes of administration, requiring quantitation of the drug in the brain tissues by liquid chromatography-mass spectrometry. However, current methods used to extract the drug from brain tissues resulted in poor recovery and substantial matrix effects. Herein, we reported a new two-step extraction method that involves the use of Proteinase K to lyse tumor tissues to efficiently release the drug, followed by ethanol protein precipitation. The extracts were then separated on a C18 column and analyzed in positive electrospray ionization, a multiple reaction monitoring mode of the triple quadrupole. We found this new method led to a recovery of 82% with negligible matrix effects. The method has been validated in accordance with Food and Drug Administration guidance for linearity, specificity, selectivity, accuracy, precision, carry-over, stability, and lower limit of quantitation. In conclusion, we have developed and validated a liquid chromatography-mass spectrometry method with a novel sample preparation method that was able to efficiently extract temozolomide from mouse brain tissue with high recovery.

KEYWORDS
mass spectrometry, mouse brain, quantitation, temozolomide, validation

1 | INTRODUCTION

It has been challenging to treat brain tumors due to systemic toxicity that limits the amount of the drug that can be administered orally or intravenously. To illustrate, Temozolomide (TMZ) is an anticancer drug approved by the US Food and Drug Administration (US FDA) for the treatment of newly diagnosed glioblastoma multiforme (GBM) in adults. GBM is a brain tumor affecting 10 per 100,000 people across the world and patients usually die within a few months after diagnosis [1]. Administration of TMZ with concurrent radiation therapy followed by a maintenance dose has been found to increase the life span of

Article Related Abbreviations: %RE, percent relative error; GBM, glioblastoma multiforme; IS, internal standard; LLOQ, lower limit of quantitation; *m/z*, mass-to-charge ratio; QC, quality control; TMZ, temozolomide; US FDA, US Food and Drug Administration.

GBM patients to 14.6 months [2]. However, GBM remains incurable because the administration of TMZ via intravenous and oral routes delivers only 35%–39% of the drug to the tumor site owing to poor permeability and abnormal vasculature of brain tumors [3]. Furthermore, if a higher dose of TMZ is administered to increase drug delivery, it leads to systemic toxicity worsening the already compromised lifestyle of the patient. The same problem was also encountered when other drugs were used to treat brain tumors.

Potential alternatives for effective treatment with minimal side effects are intra-thecal, intracerebroventricular drug administration, and other targeted drug delivery systems [4–7]. To better assess the feasibility of these novel routes of administration, a suitable analytical method is required to quantify TMZ in brain tissues. Goldwirt et al. and Liu et al. have reported the use of LC-MS methods for determining TMZ in mouse brain after intraperitoneal administration and focused ultrasound treatment [8, 9]. However, it was found that the extracted methods used led to poor recovery of TMZ from the mouse brain and a significant matrix effect. For example, Goldwirt et al. reported a mean recovery of 63% and mean matrix effects of 220%.

We hypothesized that poor extraction of TMZ from brain tissue might be due to the lack of a lysis buffer to effectively release drugs from brain tissues and the matrix effect resulting from incomplete protein precipitation. Therefore, we studied different methods to extract drug molecules from brain tissues. We found that a two-step extraction procedure; the use of Proteinase K to lyse brain tissues and to effectively release drug molecules, followed by ethanol protein precipitation, performed better than the reported extraction method [8]. Specifically, brain tissue homogenization augmented with a lysis buffer containing Proteinase K could more effectively release TMZ from mouse brain tissue, achieving a mean recovery of 82% as compared to 63% achieved with the current method. We also found that we could reduce the matrix effect to within 15% by precipitating proteins in the above lysis system with ethanol. Because this new method worked better than the reported method, we have developed and validated an LC-MS/MS method, in which it was used to extract TMZ from mouse brain tissues, for quantitation of TMZ in mouse brain tissues.

2 | MATERIALS AND METHODS

2.1 | Chemicals

TMZ ($C_6H_6N_6O_2$, > 98%), Theophylline (as an internal standard, IS, $C_7H_8N_4O_2$, 99%), HPLC grade formic acid (CO_2H_2, 98%), LCMS grade ammonium acetate ($C_2H_7NO_2$, 99.99%) and HPLC grade ethanol were purchased from Sigma Aldrich (St. Louis, MO, USA). LCMS grade methanol was obtained from Alfa Aesar (Ward Hill, MA, USA). Proteinase K was purchased from Qiagen (Hilden, Germany). Deionized water was obtained from Barnstead D3750 nano pure water purification system by Thermo Scientific (Waltham, MA, USA).

2.2 | Preparation of stock solutions and mobile phase

The standard stock solution of TMZ (1 mg/ml) was prepared by dissolving an appropriate amount of TMZ in a known volume of acidic methanol consisting of ammonium acetate (10 mM):methanol (1:4 v/v) (pH 3.5). The standard stock IS solution (1 mg/ml) was prepared by dissolving an appropriate amount of IS in a known volume of methanol. Both the standard stock solutions were stored at −20°C in separate glass vials. The working standard solutions for calibrators (2.04, 6.80, 34.0, 68.0, 170, and 340 μg/ml) and quality controls (QCs, 6.12, 40.8, and 272 μg/ml) were prepared by serial dilution of the stock solution with mobile phase A. These working standards were used to prepare mouse brain calibrators and QCs. The working standard solution of IS (34 μg/ml) was prepared by diluting the standard stock solution of IS with methanol. Mobile phase A (pH 3.5) was prepared by dissolving 500 μl of formic acid and ammonium acetate (equivalent to 10 mM) in 100 ml of water and bringing the total volume to 500 ml. Mobile phase B consisted of 100% methanol.

2.3 | Preparation of calibrators and QCs

TMZ mouse brain calibrators (1.02, 3.40, 17.0, 34.0, 85.0, and 170 μg/g) and QCs (3.06, 20.4, and 136 μg/g) were prepared by spiking 10 μl of working standard solutions of TMZ to 20 mg aliquots of the blank mouse brain and treated the same way as study samples.

2.4 | Mouse brain sample preparation

Male C57BL/6 J mice were purchased from Jackson Laboratory. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Cleveland State University and Baylor Scott & White Healthcare Institutional Animal Care and Use Committee.

To 20 mg slices of the homogenized frozen mouse brain, 10 µl of IS solution (34 µg/ml), 10 µl of formic acid, and 40 µl of Proteinase K solution (20 mg/ml) were added, vortexed for 1 min and incubated at 37°C for 1 h. 270 µl of Ethanol was added to the lysed samples and incubated at -20°C for 1 h to allow protein precipitation. The samples were then centrifuged at 14,000 rpm for 10 min. 50 µl of the supernatant was transferred into an autosampler vial and the volume was made up to 1 ml with mobile phase A for subsequent LC-MS/MS analysis.

2.5 | Instrumentation

UHPLC was performed on the Shimadzu UHPLC system (Columbia, MD, USA) which consisted of binary pumps (Nexera LC-30 AD), degasser (a DUG20A3R), autosampler (SIL-30 AC), column oven (CTO-10AVP) and system controller (CBM 20A). Mass spectrometric detection was carried out on an AB SCIEX 5500 QTRAP mass spectrometer (Toronto, Canada) equipped with an ESI probe and a syringe pump. The instrument operation, acquisition, and processing of data were performed using AB SCIEX Analyst software (version 1.6.1).

2.5.1 | Liquid chromatography

Chromatographic separation of TMZ and IS was achieved at 30°C on a Waters symmetry C18 column (2.1 mm x 150 mm, 3.5 µm). The gradient elution program was as follows: 5% B (initial), 5%–30% B (2 min), 30% B (2 min), 30%–90% B (2 min), 90%–5% B (2 min) and 5% B (1 min). The flow rate was set at 0.4 ml/min with a run time of 8 min.

2.5.2 | Mass spectrometry

MS detection was carried out in positive electrospray ionization mode by utilizing the multiple reaction monitoring feature of the quadrupole instrument. The source and compound-dependent parameters were optimized by manual infusion of TMZ and IS solutions separately into the ionization source. The source-dependent parameters were as follows: curtain gas at 30 psi; collision-assisted dissociation gas at high; ion spray voltage at 5500 V; source temperature at 100°C; sheath gas at 10 psi and desolvation gas at 10 psi. The compound dependent parameters were as follows: declustering potential at 150 V; entrance potential at 10 V; collision energy at 15 eV (TMZ), 25 eV (IS), and collision cell exit potential at 20 V (TMZ), 10 V (IS). The mass transitions for TMZ were *m/* 195.1 → 138.1 (quantifier) 195.1 → 110.0 (qualifier) and for IS was mass-to-charge ratio (*m/z*) 181.1 → 124.1.

3 | RESULTS AND DISCUSSION

3.1 | Sample preparation

The first step in sample preparation was the addition of 10 µl of formic acid to prevent hydrolysis of TMZ at pH < 5 by maintaining a pH of ~3.5. Next, in order to achieve a high percent recovery of TMZ, we tested homogenization with various lysis buffers such as SDS, RIPA, guanidine hydrochloride and Proteinase K. Proteinase K produced the most transpicuous solution thereby effectively homogenizing the brain tissue and hence was chosen for lysis. We further optimized homogenization volume by treating equal-weight frozen brain aliquots with 0.4, 4, and 40 µl of Proteinase K and incubated them at 37°C for 1 h. Samples treated with 40 µl of Proteinase K produced the most transpicuous solution, and thus this volume was selected for homogenization.

In addition to water, mouse brain consists of around 25% proteins, 11% phospholipids, and 4% cholesterol [10]. Protein precipitation is one of the most common sample preparation methods to remove proteins from the sample matrix. However, Goldwirt et al.'s method suffered from severe matrix effects of about 220% possibly due to the incomplete removal of proteins from the matrix. The amount of zinc sulfate used in Goldwirt et al.'s method was lower than the typically recommended ratio of 2:1 (zinc sulfate to matrix), which might have resulted in incomplete protein precipitation. Additionally, zinc sulfate was also believed to precipitate drug molecules along with proteins present in tissues [11]. To address this issue, we evaluated alternative protein precipitating agents, TFA and ethanol.

Equal-weight frozen brain aliquots were first acidified with formic acid, spiked with TMZ and IS, and lysed with Proteinase K. For TFA protein precipitation, one set of the above samples was treated with 40 µl of TFA, diluted with 130 µl of water and incubated at 4°C for 1 h. The second set of samples was treated with 270 µl of ethanol and incubated at -20°C for 1 h. All samples were then centrifuged and processed in the same way as described in Section 2.4. The peak area ratios of both sample sets were compared against those of the neat solution (without matrix). The peak area ratios of ethanol-treated samples were consistent with negligible matrix effects and suggested higher recovery compared to those of TFA-treated samples. Hence, ethanol was chosen for protein precipitation.

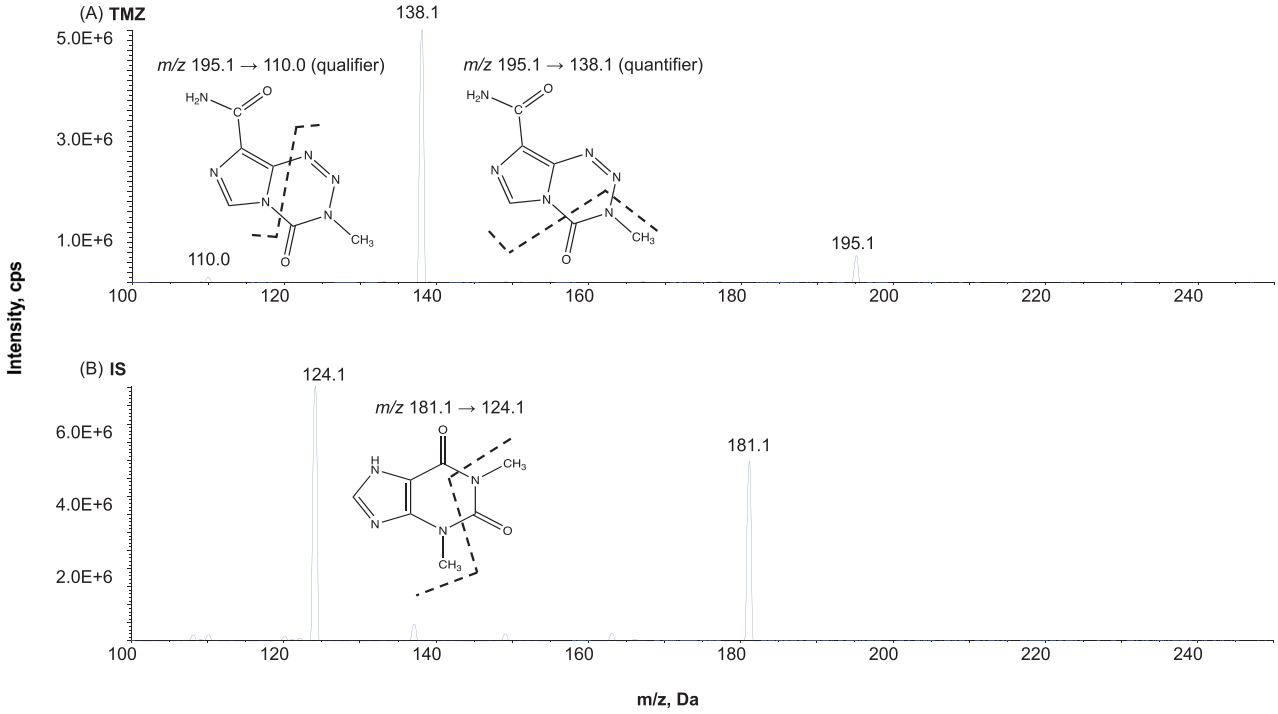

FIGURE 1 The mass spectra of Temozolomide (TMZ) and internal standard (IS) along with their probable fragmentation pattern in ESI+ mode. (A) Precursor ion of TMZ—*m/z* 195.1, product ions of TMZ—*m/z* 110.0 (qualifier), and 138.1 (quantifier). (B) Precursor ion of IS—*m/z* 181.1, product ion of IS—*m/z* 124.1

3.2 | Method validation

The LC-MS method was validated in accordance with the guidelines established by the US FDA for industry on bioanalytical method validation [12].

3.2.1 | Specificity and selectivity

To ensure specificity, in addition to *m/z* 195.1 → 138.1 (quantifier), another mass transition *m/z* 195.1 → 110.0 (qualifier) was monitored throughout the analysis. (Figure 1)

To evaluate selectivity, six double blanks and single blanks from six different mouse brains were analyzed for interferences at retention times of TMZ and IS. As shown in Figure 2, minuscule peaks were detected at the retention time of TMZ and IS in double and single blanks, but they were insignificant as their peak area was < 10% of that of lower LOQ (LLOQ).

3.2.2 | Linearity and LLOQ

The relationship between the concentration of TMZ in the calibrators and detector response was studied in the range of 1.02–170 μg/g. A standard calibration curve for TMZ was constructed using a double blank, a single blank, and six non-zero calibrators in the mouse brain matrix (1.02, 3.40, 17.0, 34.0, 85.0, and 170 μg g^{-1}). The peak area ratios of TMZ to IS were plotted against the concentrations of TMZ in mouse brain calibrators with 1/x as the weighing factor. The equation derived from the calibration curve was $y = 0.0356x + 0.2771$ with a correlation coefficient of 0.9975. The accuracy and precision of individual mouse brain calibrators were ≤14% for four non-zero calibrators and ≤17% for upper LOQ and LLOQ.

The absolute peak area of TMZ at 1.02 μg/g, that is, LLOQ was more than five times the absolute peak area of TMZ in double and single blanks as required by the US FDA. LLOQ was further evaluated in five different mouse brain samples on the same day and five different days for intra- and inter-day accuracy and precision respectively. The accuracy and precision of LLOQ were 6% and 15%, respectively, which were below the 20% limit set by the US FDA.

3.2.3 | Accuracy and precision

For this study, we estimated intra-day and inter-day accuracy and precision in three QCs at three concentrations (3.06, 20.4, and 136 μg/g) prepared from five individ-

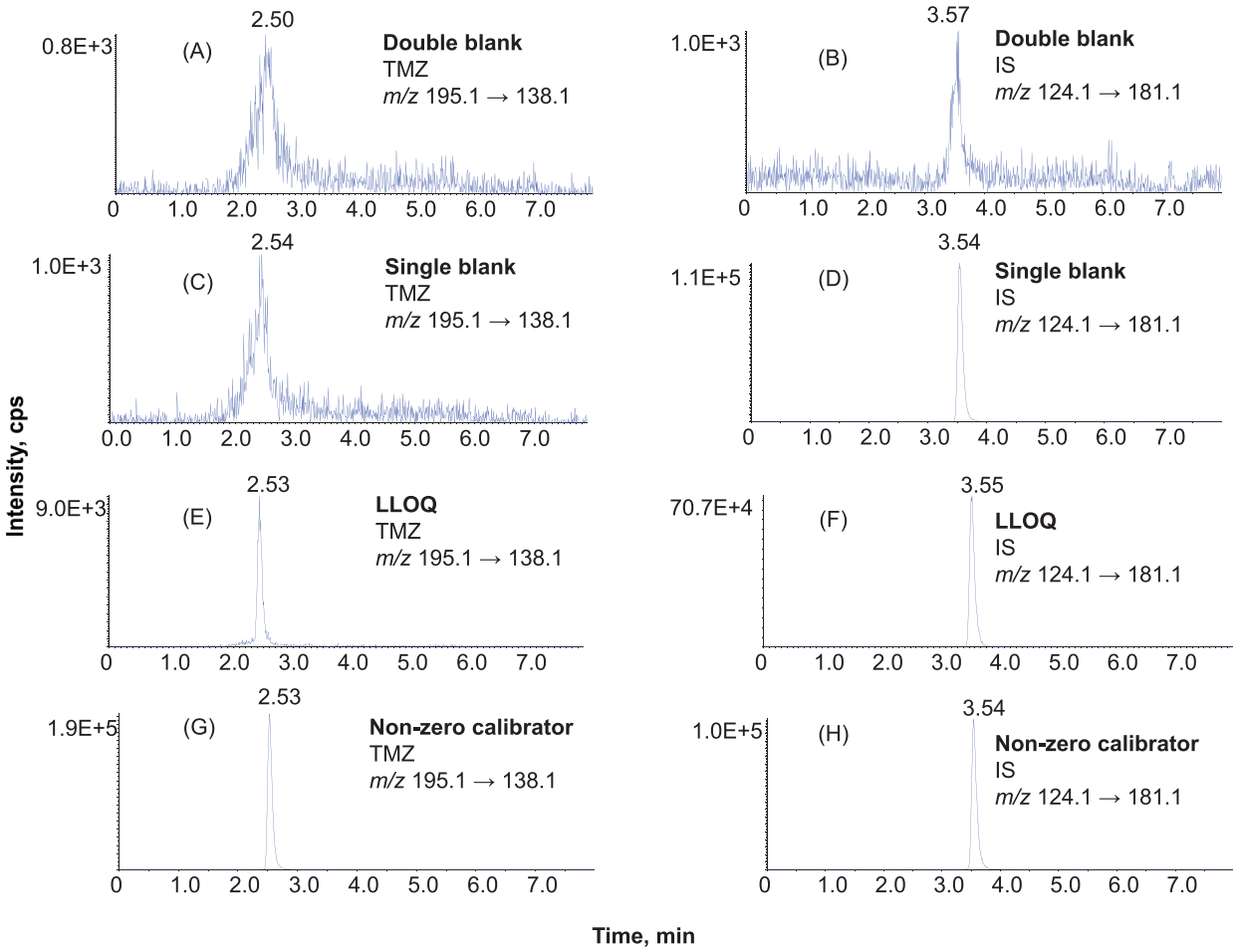

FIGURE 2 The LC-MS/MS chromatograms of Temozolomide (TMZ) and internal standard (IS) in mouse brain. Double blank (TMZ and IS absent) chromatograms of (A) TMZ quantifier (B) IS. Single blank (TMZ absent and IS at 17 μg/g) chromatograms of (C) TMZ quantifier (D) IS. Lower limit of quantitation (LLOQ) (TMZ at 1.02 μg/g and IS at 17 μg/g) chromatograms of (E) TMZ quantifier (F) IS. Calibrator (TMZ at 17 μg/g and IS at 17 μg/g) chromatograms of (G) TMZ quantifier (H) IS

TABLE 1 Accuracy and precision of Temozolomide (TMZ) in five individual blank mouse brain samples measured on the same day and five different days ($n = 5$)

		Intra-day			Inter-day		
QC level	Spiked concentration (μg/g)	Determined concentration ± SD (μg/g)	%RE[a]	%CV[b]	Determined concentration ± SD (μg/g)	%RE[a]	%CV[b]
LQC	3.06	3.13 ± 0.05	2	2	3.27 ± 0.13	4	4
MQC	20.4	19.0 ± 0.63	-7	3	19.2 ± 0.66	–6	4
HQC	136	137 ± 4.64	1	3	141 ± 4.87	2	4

Abbreviations: HQC, high quality control; LQC, low quality control; MQC, medium quality control.

%RE[a] (percent relative error) = [(determined concentration − spiked concentration)/(spiked concentration)] x 100.

%CV[b] (percent coefficient of variation) = (standard deviation/mean) x 100.

ual mouse brains. Intra-day accuracy and precision were determined by five sets of three QCs prepared from five individual mouse brains on the same day. Inter-day accuracy and precision were determined by five sets of three QCs prepared from five individual mouse brain samples on five different days.

As shown in Table 1, intra-day accuracy expressed as percent relative error (%RE) was ≤7%, and precision expressed as percent coefficient of variation (%CV) was ≤3%. The inter-day accuracy was ≤6% and precision was ≤10%. It should be noted that even though analyses were done using QCs from five individual mouse brain samples, all

TABLE 2 Absolute, relative and internal standard (IS) matrix effect in five individual mouse brain samples ($n = 5$)

Mouse brain sample	QC level	Spiked concentration (μg g^{-1})	Absolute matrix effect[a] (%)	ISmatrix effect[b] (%)	Relative matrix effect[c] (%)
1	LQC	3.06	97 ± 4.67	98 ± 1.57	98 ± 4.23
	MQC	20.4	98 ± 2.29	101 ± 1.85	98 ± 0.76
	HQC	136	98 ± 1.30	102 ± 0.99	96 ± 1.37
2	LQC	3.06	96 ± 1.16	97 ± 2.06	99 ± 3.25
	MQC	20.4	94 ± 1.20	95 ± 2.51	98 ± 3.14
	HQC	136	90 ± 0.84	89 ± 1.33	101 ± 0.84
3	LQC	3.06	106 ± 1.77	104 ± 3.76	102 ± 5.35
	MQC	20.4	94 ± 0.55	94 ± 0.32	100 ± 0.31
	HQC	136	85 ± 1.57	82 ± 0.45	103 ± 2.13
4	LQC	3.06	108 ± 2.71	111 ± 2.35	98 ± 1.53
	MQC	20.4	95 ± 2.40	98 ± 0.41	97 ± 2.72
	HQC	136	91 ± 0.13	94 ± 1.18	97 ± 1.19
5	LQC	3.06	103 ± 2.69	101 ± 0.87	102 ± 2.97
	MQC	20.4	99 ± 1.50	101 ± 1.96	99 ± 3.34
	HQC	136	92 ± 0.55	91 ± 1.27	100 ± 1.17

Abbreviations: HQC, high quality control; LQC, low quality control; MQC, medium quality control.
Absolute matrix effect[a] = [(mean peak area of TMZ in extracted mouse brain/mean peak area of TMZ in neat solution)] x 100.
IS matrix effect[b] = [(mean peak area of IS extracted mouse brain /mean peak area of IS in extracted neat solution)] x 100.
Relative matrix effect[c] = [(mean peak area ratio of TMZ/IS in extracted mouse brain/mean peak area ratio of TMZ/IS in neat solution)] x 100.

values were well below the 15% limit set by the US FDA demonstrating very good accuracy and precision of the method.

3.2.4 | Matrix effect and recovery

For this study, we evaluated the absolute and relative matrix effects in five individual mouse brain samples in three QCs at three concentrations (3.06, 20.4, and 136 μg/g). The absolute matrix effect of TMZ was determined by calculating the percentage of the mean peak area of TMZ in extracted mouse brain matrix over that of TMZ in neat solution. The relative matrix effect was determined by calculating the percentage of the mean peak area ratio of TMZ and IS at three concentrations in the extracted mouse brain matrix over that of TMZ and IS in neat solution. As shown in Table 2, the absolute matrix effects were within 85%–108% and relative matrix effects were within 96%–102%. All the values were below the 15% limit set by the US FDA suggesting that we have successfully addressed the matrix effect issue of Goldwirt et al.'s method by using ethanol for more complete removal of proteins that are present in the brain matrix.

Recovery was studied by assessing the absolute and relative recoveries in two individual mouse brain samples in three QCs at three concentrations (3.06, 20.4, and 136 μg/g). Absolute recovery was estimated by calculating the percentage mean peak area of TMZ spiked in the mouse brain matrix before extraction over that of TMZ spiked in extracted mouse brain matrix. Relative recovery was evaluated by calculating the percentage of the mean peak area ratio of TMZ and IS spiked in the mouse brain matrix before extraction over that of TMZ and IS spiked in the extracted mouse brain matrix.

As shown in Table 3, the mean absolute recovery was 82%, and the mean relative recovery was 111%. These values were higher than those reported by Goldwirt et al. who reported a mean recovery of 63% suggesting that our method has improved the recovery of TMZ from mouse brain by 19% which could be attributed to the use of a lysis buffer to aid in homogenization.

3.2.5 | Stability studies

Studies were conducted to assess stock solution, autosampler, benchtop, and freeze-thaw cycle stabilities. The stability of TMZ in the autosampler, benchtop, and freeze-thaw samples was expressed as a percentage of the measured mean peak area ratio of TMZ to IS against those of TMZ to IS in freshly prepared samples. Stock solution stability was assessed by keeping the stock solution (1 mg/ml) of TMZ at room temperature (25°C) on the bench top for 12 h before diluting it to 10 and 100 ng/ml. Bench top and autosampler stability studies were performed by leaving QCs on the bench top at 25°C for 12 h and autosampler at 15°C respectively. Three freeze-thaw cycles were conducted by freezing

TABLE 3 Absolute, relative and internal standard (IS) recovery in two individual mouse brains ($n = 2$)

Mouse brain sample	QC level	Spiked concentration (μg/g)	Absolute Recovery[a] (%)	IS Recovery[b] (%)	Relative Recovery[c] (%)
1	LQC	3.06	80 ± 0.03	71 ± 0.01	113 ± 0.05
	MQC	20.4	85 ± 0.02	78 ± 0.02	109 ± 0.02
	HQC	136	79 ± 0.01	72 ± 0.00	109 ± 0.02
2	LQC	3.06	81 ± 0.00	67 ± 0.02	121 ± 0.03
	MQC	20.4	84 ± 0.02	78 ± 0.01	108 ± 0.02
	HQC	136	84 ± 0.02	77 ± 0.02	108 ± 0.02

Abbreviations: HQC, high quality control; LQC, low quality control; MQC, medium quality control.
Absolute recovery[a] = [(mean peak area of TMZ in mouse brain/mean peak area of TMZ in extracted mouse brain matrix)] x 100.
IS recovery[b] = [(mean peak area of IS in mouse brain/mean peak area of IS in extracted mouse brain matrix)] x 100.
Relative recovery[c] = [(mean peak area ratio of TMZ/IS in mouse brain/mean peak area ratio of TMZ/IS in extracted mouse brain matrix)] x 100.

TABLE 4 Stability studies of Temozolomide (TMZ) under various test conditions (n = 3)

Test conditions	Sample	Nominal concentration	Temperature	Duration	%Recovery[a] ± SD
Bench top	Stock solution	10 ng/ml	25°C	12 h	116 ± 0.02[a]
	Stock solution	100 ng/ml			125 ± 0.04[a]
Bench top	LQC	3.06 μg/g	25°C	12 h	100 ± 0.04[b]
	HQC	136 μg/g			102 ± 0.05[b]
Autosampler	LQC	3.06 μg/g	15°C	18 h	109 ± 0.06[b]
	HQC	136 μg/g			90 ± 0.04[b]
Freeze-thaw cycles	LQC	3.06 μg/g	−20–25°C	Frozen for 12 h	123 ± 0.08[b]
	HQC	136 μg/g			116 ± 0.04[b]

Abbreviations: HQC, high quality control; LQC, low quality control; MQC, medium quality control.
%Recovery[a] = [mean peak area ratio of TMZ/IS in stability sample/ mean peak area ratio of TMZ/IS in fresh sample] x 100.

QCs at -20°C for 12 h followed by thawing them unassisted at 25°C.

The stability of samples under various test conditions described above was expressed as %RE and is presented in Table 4. The stock solution showed a recovery of 116% when diluted to 10 ng/ml and 125% when diluted to 100 ng/ml indicating that it should not be stored at room temperature for more than 12 h. The %RE of QCs was ≤10% suggesting that QCs were stable for 12 h at the bench top and 18 h in the autosampler. The %RE for low QC was 2% higher than the 20% limit set by FDA signifying that freezing and thawing QCs should be kept under three cycles.

3.3 | Method application

Finally, we conducted a study by injecting 40 μg of TMZ into mouse brain tissues using a 1 cc syringe fitted with a 31 gauge (5/16 inch) needle. The mouse brain tissues that were injected with 40 μg of TMZ were then immediately subjected to sample preparation and analyzed with the new method developed in this study. It was found that the amount of TMZ determined in the tissues was 39.55 μg, suggesting a percent recovery of 99%. This simple application study showed that the method developed in this study can be used to accurately quantify TMZ present in brain tissues.

4 | CONCLUDING REMARKS

In conclusion, an accurate LC-MS/MS method for the quantitative determination of TMZ in mouse brain tissues has been developed and validated for the first time. The method consisted of a two-step extraction process involving a lysis buffer for homogenization and protein precipitation with ethanol, both crucial for achieving 82% recovery and matrix effects within 15% CV. The method developed was applied to study samples and might be useful in studying the pharmacokinetics of TMZ in the mouse brain.

AUTHOR CONTRIBUTIONS

Raghavi Kakarla: Conceptualization, investigation, validation, and writing; Kim Yacoub: Conceptualization, investigation, and writing; Rebecca Bearden: Validation

and writing; Aimin Zhou, Sanjib Mukherjee, and Frank Y. Shan: Conceptualization and resources; Baochuan Guo: Conceptualization, investigation, validation, and writing

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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