

**Development and validation of simple step protein precipitation
UHPLC-MS/MS methods for quantitation of temozolomide in patient
plasma samples**

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Abstract

Temozolomide (TEMODALTM) (TMZ) is an antineoplastic agent that is primarily used
for the treatment of glioblastoma and anaplastic gliomas, two aggressive forms of brain
cancer. Due to the poor prognosis of brain tumour patients, there is an increasing body
of research into improving the stability and delivery of TMZ past the blood brain barrier
using carrier molecules. These require accurate determination of TMZ levels for

biodistribution and pharmacokinetic evaluation. Unfortunately, current methodologies for the determination of TMZ in human plasma suffer from low reproducibility, recovery, sensitivity or cost ineffective procedures associated with extensive sample cleaning. To surpass these disadvantages, we developed two bioanalytical methods with high sensitivity and excellent recovery for the determination of TMZ in human plasma at minimum cost. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used and both methods were validated under US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines. The two methods had minor differences in the sample pre-treatment and each method was developed and applied in separate laboratories. Theophylline was selected as internal standard (IS). Calibration curves were linear over the range of 10-500 ng/mL with extraction recovery ranging from 77.3-97.3% while all validation parameters met the acceptance criteria and proved the methods' reliability. The validated methods were successfully applied to plasma samples donated from cancer patient following treatment with temozolomide.

Abbreviations

LC-MS/MS, liquid chromatography-tandem mass spectroscopy; IS, internal standard; TMZ, temozolomide; UHPLC, ultra-high performance liquid chromatography; RT, retention time; LOQ, limit of quantification; LOD, limit of detection;

Keywords

temozolomide; LC-MS/MS; human plasma; method validation; theophylline; MTIC;

1. Introduction

Temozolomide (3-methyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide) (TMZ) is a cytotoxic alkylating agent that is rapidly hydrolyzed to its active metabolite 3-methyl-(triazene-1-yl)imidazole-4-carboxamide (MTIC), upon exposure to pH above 7 [1, 2]. MTIC is instantaneously degraded to the extremely reactive methyl diazonium cation that methylates guanines in DNA at the O⁶ or N⁷ position [3, 4]. The cytotoxic effect of TMZ is primarily based on the formation of O⁶ methylguanine which results in mispairing during replication and subsequent cell death [5].

TMZ is the first-line chemotherapeutic option for the treatment of glioblastoma, anaplastic astrocytoma and certain brain metastases [6, 7]. Along with radiotherapy, it

can increase the mean survival rate of glioblastoma patients and the two-year survival rate [8, 9]. In combination with other therapies, it has shown encouraging results in the treatment of metastatic melanoma [10-15].

TMZ is generally used as a chemotherapeutic against brain cancers primarily due to its biodistribution and its pharmacokinetic properties. It is characterized by 100 % oral bioavailability and due to its small size is able to penetrate the blood-brain barrier (BBB) [16-19]. Nonetheless, its efficacy is limited due to its poor stability in the slightly alkaline conditions found in human plasma, [6]. TMZ is rapidly converted to MTIC and as a result has a short half-life of 1.8 h, so the concentration of TMZ both in serum and tumor sites drops to suboptimal levels very rapidly [20]. Moreover, the active metabolite MTIC, has a very poor BBB penetration and limited cellular absorption [21]. Therefore, high doses of TMZ need to be administrated to achieve a sufficient concentration of MTIC at the site of the tumor [22]. Due to the severe side effects brought about by the need to treat patients with high doses of TMZ to achieve the desired therapeutic effect there is increasing interest in trying to stabilize TMZ to delay its conversion to MTIC before passing the BBB. This conversion is pH- and temperature-dependent since TMZ is unstable at 37 °C in human plasma but stable in acidified plasma at the same temperature [6, 23]. The control of the pH in plasma using various acids (phosphoric, hydrochloric etc.) during the collection, processing and analysis of the biological samples is of great significance for the stabilization and preservation of TMZ [17, 24-26].

The most common techniques for the determination and quantification of TMZ in human plasma are liquid chromatography coupled with mass spectrometry or UV detection [19, 24, 27, 28]. The efficient extraction of TMZ from the plasma samples and the set-up of the optimal chromatographic conditions are the crucial steps in these techniques that greatly affect the detection accuracy. Several extraction methods, like solid phase extraction (SPE), liquid-liquid extraction (LLE) and protein precipitation (PP) have been described for the recovery of TMZ [1, 24, 29]. However, depending on the method, many issues can be encountered like low recovery, uncleaned samples, poor chromatographic resolution, complexed and time-consuming sample processing or even costly consumables.

Herein, we aimed to use PP, as a simple step protein precipitation, in order to extract the drug from plasma samples avoiding the high-cost SPE and the low recoveries offered by LLE [29]. Following the PP, we conducted two alternative routes for the

sample reconstitution and the chromatographic parameters in our effort to decrease even more the time of sample preparation. Thus, two different methods were developed and established in different LC-MS/MS instrumentations and separate laboratories, which succeeded high recovery and sensitivity while at the same time describe fast and simple procedures for the sample preparation. Since TMZ is currently the standard of care for the treatment of aggressive brain tumors, a lot of research is being done into improving it as a drug or combining it with other drugs for an enhanced efficacy. Thus, speed, simplicity and accuracy in the quantification of TMZ in human plasma will assist such studies in enabling biodistribution or pharmacokinetics charting and will also be useful for clinical studies/trials where TMZ is used in combination with other treatments. The two assays for the detection and quantitation of TMZ in human plasma were validated according to FDA and EMEA guidelines and applied to plasma samples extracted from cancer patient treated with TMZ.

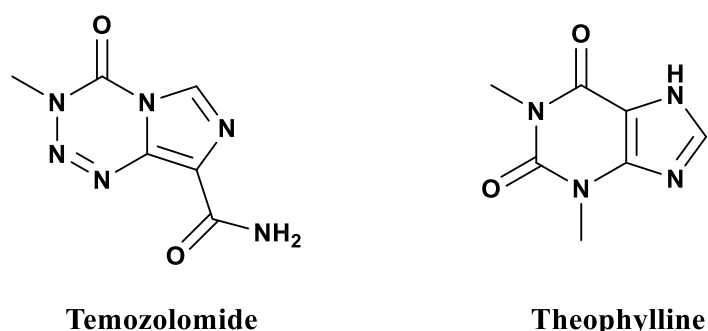


Fig. 1. Chemical structures of TMZ and theophylline.

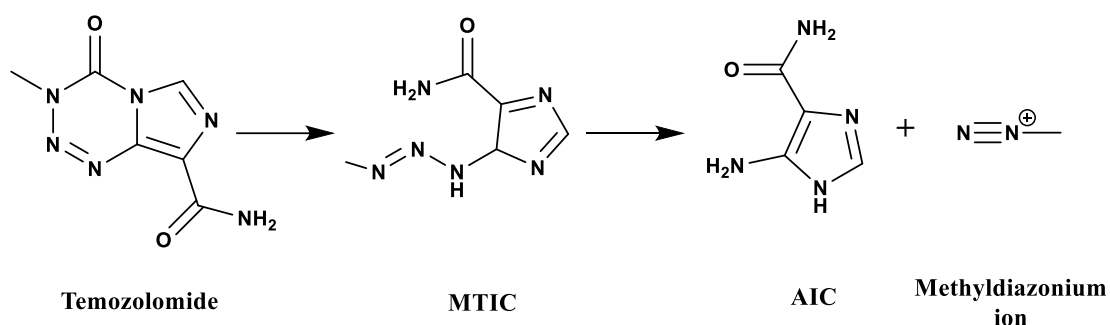


Fig. 2. The two-step degradation route of TMZ, first to its active metabolite MTIC and then to 5-amino-imidazole-4-carboxamide (AIC) and methyldiazonium cation.

2. Experimental

2.1. Reagents and chemicals

TMZ was purchased from Sigma and theophylline monohydrate from Alfa Aesar. Methanol, acetonitrile, *N, N*-dimethylsulfoxide (DMSO) and ammonium formate (LC-MS grade) were purchased from Sigma-Aldrich. Formic, phosphoric and hydrochloric acid (HCl) were purchased from Merck (Germany). Purified water (18.2 MΩ) was prepared using a Milli Q-Integral system (Merck-Millipore, Watford, UK). All solvents were filtered through 0.22 μm filters (Titan Membrane, Merck Millipore). Samples were filtered with 0.2 μm Minisart RC 4 (Sartorius) syringe filters. Human plasma from healthy donors was a kind offer of Blood Donation Centre of University Hospital of Ioannina.

2.2. Instrumentation

2.2.1. Chromatographic conditions

Method A: Chromatographic analyses were performed on a Waters HPLC system (Alliance HT 2795) equipped with a temperature-controlled autosampler and a degasser. Chromatographic analysis was achieved using a Synergi Hydro-RP column 100 × 2 mm, 4 μm, Proguard 2 to 8 mm (Phenomenex). The mobile phase consisted of ammonium formate (pH 3.0; 10 mM) (solvent A) and acetonitrile (solvent B). A gradient elution was started with 5% of solvent B, then increased with a linear increment to 95% until 0.5 min and maintained for 1.0 min. The elution was then decreased to 5% of solvent B in 0.5 min, and this composition was maintained until the end of the run (6 min). The column temperature was maintained at 40 °C throughout all measurements, whereas the sample temperature was kept at 15 °C. A volume of 50 μL of each sample was injected with a flow rate 0.3 mL min⁻¹.

Method B: Reversed phase liquid chromatography assay was performed using Advance Ultra High Performance Liquid Chromatography (UHPLC) system (Bruker, Germany). Chromatographic separation of TMZ and IS was performed on a Kinetex C18 column 100 mm × 2.1 mm, 2.1 μm with Ultra SecurityGuard pro-column (Phenomenex). The mobile phases were composed of LC-MS grade water with formic acid 0.1% (A) and acetonitrile with formic acid 0.1% (B). Applying gradient elution, the following profile was used: initial phase (B) concentration 5%, increased to 90% within 3.0 min, then kept constant for 0.5 min, reduced to 5% at 3.6 min and kept constant till the end of the run at 4 min. Column and autosampler temperature were set

at 40 °C and 15 °C, respectively. The injection volume was set at 5 μL with a flow rate 0.25 mL min^{-1} .

2.2.2. Mass spectrometric conditions

Method A: Micromass Quattro Micro tandem MS system (Waters, Milford, MA, USA), was used for monitoring the analytes. The mass spectrometer was operated in positive ionization mode, using the electrospray ionization source (ESI). The tuning parameters of TMZ and IS were optimized by infusing a solution containing 500 ng mL^{-1} of the analytes with a flow rate of 30 $\mu\text{L min}^{-1}$ to the mass spectrometer. The analytes were detected by monitoring the precursor $\rightarrow$ product ion transition using Multiple Reaction Monitoring (MRM) scan mode with 500 ms dwell time for each transition.

The selected transitions were m/z 195>138.1 and 181.3>124 for TMZ and IS, respectively. The source temperature was 100 °C, desolvation temperature was 400 °C, desolvation gas flow was 500 L h^{-1} and cone gas flow was 50 L h^{-1} . The capillary voltage was set at 3.5 kV, while the cone voltage values for TMZ and the IS was optimized at 18 and 35 V, respectively. The multiplier was set at 650 V and argon was used as collision gas. Lastly, the optimized collision energies for TMZ and IS were found to be 15 eV for both compounds. All data were acquired using the MassLynxv. 4.0 software.

Method B: For the ionization and detection of TMZ and IS, EVOQ Elite ER TQ mass spectrometer (Bruker) was operated in positive ESI. The optimal MRM transitions were acquired by direct infusion to the mass spectrometer of a solution containing 500 ng mL^{-1} of the analytes at a flow rate of 15 $\mu\text{L min}^{-1}$.

Utilizing MRM builder, a feature of the Bruker MS workstation software, that estimates the fragmentation energies and the most abundant product ions, the optimal transitions were found to be m/z 195.2>138.2 and 195.2>55.2 for TMZ and 181.2>124.1 and 181.2>96.3 for IS. ESI parameters including nebulizer, spray voltage and heated probe temperature were optimized in order to obtain a consistent response for all analytes and were found to be as follows: cone temperature, 300 °C; heated probe temperature 150 °C; nebulizer gas flow, 50 units; heated probe gas flow, 50 units; cone gas flow, 20 units; spray voltage 4.5 kV; and exhaust gas on. Total control of LC and MS as also data acquisition was performed with MSWS software, version 8.2.1 (Bruker, Germany).

2.3. Preparation of calibrator and spiked control samples

Method A: Standard stock solutions of TMZ and IS at a concentration of 5 mg mL⁻¹ were prepared by dissolving in DMSO and methanol, respectively. Therefore, these solutions were subsequently used to prepare working solutions (WS) of TMZ (1-30 µg mL⁻¹) and IS (12.5 µg mL⁻¹). Calibration standards (CS) and quality control (QC) samples were prepared by spiking appropriate amounts of WS into the drug-free plasma. Final concentrations of the CS were 10, 50, 100, 150, 200 and 300 ng mL⁻¹ for TMZ and 125 ng mL⁻¹ for IS, while the QCs were 10, 30, 125 and 250 ng mL⁻¹. CS and QC samples were freshly prepared on the day of extraction. All stock solutions (WS, CS and QC) were immediately stored at -20 °C.

Method B: A stock solution of TMZ at concentration 1 mg mL⁻¹ was prepared by dissolving the appropriate amount in an acidic solution containing methanol-water (80:20, v/v) and formic acid 0.1%. Further WS of TMZ (2-100 µg mL⁻¹) were prepared by appropriate dilutions of standard stock solution with the above acidic solution. The final concentrations of CS at 10, 50, 100, 150, 200, 300 and 500 ng mL⁻¹ were prepared by spiking the appropriate amounts of WS in drug-free plasma. QC samples were prepared from WS at concentrations of 10, 30, 250 and 450 ng mL⁻¹. Stock solution of IS at concentration 1 mg mL⁻¹ was prepared by dissolving the appropriate amount in methanol. WS at concentration 30 µg mL⁻¹ was prepared by further dilution of stock solution. All CS and QC samples were freshly prepared on every experimental day and all stock solutions were kept at -20 °C during validation period. Plasma from healthy donors and patient was acidified (pH<4) by adding 50 µL HCl (1N) per 1 mL plasma.

2.4. Sample preparation

Method A: All plasma samples were thawed at room temperature, vortexed and centrifuged at 2500 g for 10 minutes at approximately 4 °C. 300 µL of acetonitrile (1% H₃PO₄) was immediately added to an aliquot of 100 µL of TMZ spiked plasma samples (10 µL aliquot of each working solution of analytes, 10 µL of IS working solution, 80 µL drug-free plasma) in order to facilitate the precipitation of plasma proteins. The samples were subsequently vortexed for 1 min and centrifuged at 10.000 g for 10 min (4 °C). The supernatants were transferred into another tube and samples were dried on a Centrивap Cold Trap concentrator (Labconco, USA) for ~ 3 h. The dried extracts were

reconstituted in 1 mL of ammonium formate (pH 3.0; 10 mM) filtered through RC 0.22 μm syringe filters (Phenomenex) and finally 50 μL of the solution were injected into the LC-MS.

Method B: Drug-free plasma acidified with HCl (1N) was left to thaw at room temperature prior to analysis. In 80 μL drug-free acidified plasma, 10 μL of the respective WS of TMZ was added along with 10 μL of IS working solution. 400 μL of ice-cold acetonitrile was added in order to precipitate the plasma proteins. Samples were vortex-mixed and centrifuged at 10.000 g for 10 min. 100 μL of the supernatant were collected and added to a new vial containing 300 μL water (LC-MS grade) with 0.1% formic acid. The samples were vortex-mixed, filtered with 0.2 μm Minisart RC 4 syringe filters (Sartorius) and transferred to LC-MS vials for analysis. For human patient plasma samples, the same procedure was followed but without the addition of 10 μL TMZ working solution.

2.5. Stability assay

Stability studies of the spiked human plasma (n=5) with TMZ were performed: (a) after remaining for a week in the refrigerator (4–8 $^{\circ}\text{C}$) and (b) after 3 freeze-thaw cycles (freeze at -20°C and thaw at room temperature) at 30, 125 and 250 ng mL^{-1} for method A and 30, 250 and 450 ng mL^{-1} for method B.

2.6. Method validation

Both methods were validated according to the bioanalytical method validation guidelines of the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [30, 31] under ISO17025 criteria for (1) linearity, (2) precision (repeatability and intermediate precision), (3) accuracy and (4) stability.

2.7. Patient samples

Plasma from one cancer patient following treatment with TMZ was provided from the Department of Radiation Oncology, University Hospital of Ioannina, Ioannina, Greece, after informed consent.

2.8. Statistical analysis

Bland-Altman (B&A) plot (Supplementary Data, Fig. S3) has been conducted to describe the agreement between the two quantitative methods (A and B) [32].

3. Results and discussion

3.1. Method development

3.1.1 Optimization of the extraction procedure

As mentioned above a crucial point in method development is the efficient extraction recovery of the drug from the plasma samples. Generally, there are three ways to extract a drug from a biological fluid: protein precipitation, liquid-liquid extraction and solid phase extraction. In the literature, several extraction methods for TMZ are presented following these three basic methods, with each exhibiting specific advantages and disadvantages [1, 2, 6, 24, 29, 33].

Shen *et al.*, developed a HPLC-based method for the quantification of TMZ in human plasma, where they recruited SPE and succeeded a % recovery of 86-90% [24]. However, SPE is a time-consuming and high-cost procedure, especially when it comes to handling a large number of samples.

The most commonly used extraction method for TMZ is LLE, where several solvents have been used like methanol, acetonitrile, ethyl acetate, diethyl ether and dichloromethane [2, 6, 29, 33]. Despite the wide use of this method, results concerning the recovery rate of TMZ are quite disappointing and this is due to the hydrophilic behavior of the drug [33]. Jain *et al.*, managed to drastically increase the % recovery of TMZ through the application of the doublesalting-out-assisted liquid-liquid extraction (SALLE) [33]. By adding sodium chloride and potassium sulfate in a two-step extraction procedure, they took advantage of the enhanced immiscibility -provoked by the salts- between the aqueous and the organic phase.

In this work, our effort was concentrated on the development of a simple and rapid extraction method that would follow the principles of PP and at the same time maintain high rates of TMZ recovery. Typically, the most common solvents for PP are methanol and acetonitrile. When we added methanol in the spiked plasma samples, the % recovery was very low as TMZ is nearly insoluble in that solvent [33]. We then tried to reproduce the extraction procedure presented by Goldwirt *et al.*, where methanol (200 μ L) and ZnSO₄ (200 μ L) were added to precipitate the plasma proteins- to mice plasma containing TMZ and IS diluted in ammonium acetate buffer (200 μ L). However, this procedure led to high turbidity in the samples that eventually caused clogging of the

ESI probe. This was probably due to insufficient precipitation of the plasma components being prompted by the addition of the aqueous solution of ZnSO₄ and ammonium acetate. In addition, methanol is less effective for protein precipitation than acetonitrile [34]. Even when we tried to increase the volume of methanol, maintaining the ZnSO₄ and ammonium acetate volumes the turbidity remained. While an aqueous solvent hampers the PP, in the case of TMZ it is necessary as it aids proper retention at the chromatographic column. Therefore, we combined different volumes of acetonitrile, ZnSO₄ and/or ammonium formate and checked the PP by measuring the protein content of the supernatant with BCA protein assay kit. Among all cases, the more effective way to precipitate the plasma proteins was to add only acetonitrile, whose presence ensures the collection of clean samples and at the same time the adequate recovery of TMZ which has a solubility of up to 10 mg mL⁻¹ [33]. The necessity of an aqueous solvent in the final analysis samples was circumvented by two alternative methods presented here. The first is the evaporation of the supernatants after the PP using speedvac and their reconstitution in 1 mL of 10 mM ammonium formate pH 3.0. The second is the further dilution of the supernatant in water (LC-MS grade). Both methods were validated and their parameters are presented below.

3.1.2 Optimization of chromatographic conditions

Method A: Chromatographic conditions, especially the composition of the mobile phase, the column type, the flow rate and the oven temperature were optimized through several trials. It was found that a gradient elution of a mixture of ammonium formate (pH 3.0; 10 mM) (solvent A) and acetonitrile (solvent B) could achieve this purpose, and therefore was finally adopted as the mobile phase. The Synergi Hydro-RP column 100 × 2 mm, 4 µm, Proguard 2 to 8 mm (Phenomenex) column operated at 40 °C produced a good peak shape and response, even at the lowest concentration level of the analytes. PP was employed for sample preparation. PP is helpful in producing a clean sample and also minimizing the experimental costs. Among the different solvents investigated for their suitability both alone and in combination, acetonitrile (containing 1% H₃PO₄) was found to be optimal as it produced a clean chromatogram for the drug-free plasma sample and yielded the highest recovery for the analyte from plasma. A good internal standard must mimic the analyte during extraction and compensate for any analyte loss. Theophylline monohydrate was found to be the optimal choice for the

quantification of TMZ, due to its similar extraction characteristics, retention time, stability and detector response.

Method B: Several chromatographic tests were conducted in order to maximize resolution for sharper peak shapes and shorter run times. Different ratios of mobile phases and added salts (ammonium acetate, ammonium formate) were tested. The presence of 0.1% formic acid in mobile phase A and B improved peak shapes and increased the signal for all compounds. Also best resolution for TMZ and IS was achieved on a Kinetex C18 RP column under gradient elution mode. PP was also used in this method with acetonitrile serving a dual purpose since not only did it produce clean chromatograms on drug-free plasma samples but it also improved the recovery efficacy of the method.

3.2. Method Validation

3.2.1. Linearity and Sensitivity

The linearity of the methods was calculated using the IS calibration method and evaluated over a concentration range of 10-300 ng mL⁻¹ and 10-500 ng mL⁻¹ for method A and B, respectively, in three validation runs. The calibration curves were constructed by plotting TMZ versus IS area against the nominal concentrations of the calibration standards and were found to be linear with a mean regression coefficient $r^2 \geq 0.999$ in both cases (**Table 1**). The standard errors of intercept and slope were 0.034 and 0.078 for method A and 0.013 and 0.074 for method B. In both cases, the intercept did not deviate statistically significantly ($p < 0.05$) from zero.

LLOQ for both methods was determined at 10 ng mL⁻¹, with signal to noise ratio >10 estimated from MS software based on the respective LLOQ chromatograms (**Fig. 3B and Supplementary Data, Fig. S1**). Precision (expressed as % relative standard deviation, %RSD) and accuracy (expressed as % relative error, %RE) at LLOQ (n=5) were found to be $\leq 6.51\%$ and $\leq 3.23\%$ for method A and $\leq 9.3\%$ and $\leq 10.4\%$ for method B and within the acceptable limits of the guidelines (<20%). These data further confirmed that the analytical method was able to quantify the LLOQ with high accuracy and sensitivity.

Table 1. Linearity data of TMZ spiked in human plasma (n =5).

Method	Concentration (ng mL ⁻¹)	Equation	r ²	LLOD (ng mL ⁻¹)	LLOQ (ng mL ⁻¹)	Relative error
A	10-300	0.0446x + 0.1898	0.999	3	10	2.99
B	10-500	0.6947x + 0.0215	0.999	3	10	3.28

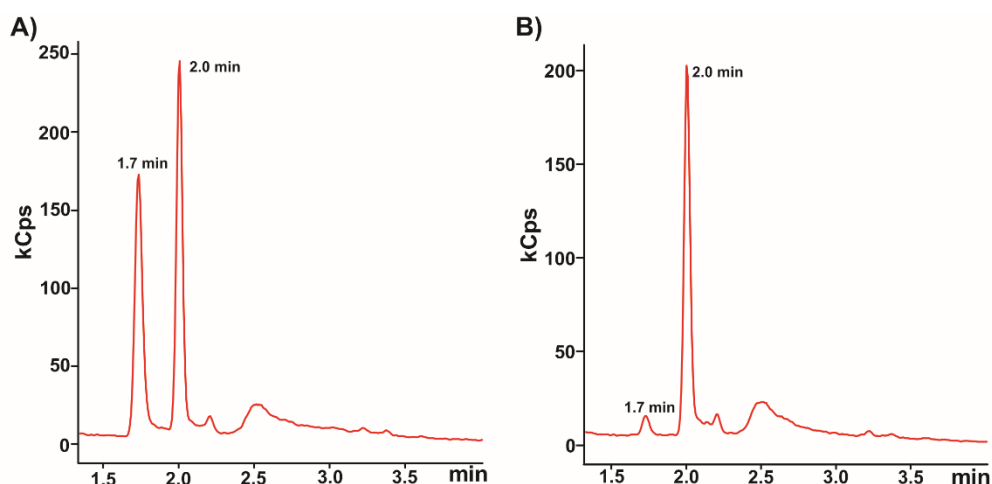


Fig. 3. Representative chromatograms of TMZ and IS in human plasma at **A)** 250 ng mL⁻¹ concentration and **B)** at 10 ng mL⁻¹ (LLOQ).

3.2.2. Precision and accuracy

The intra- and inter- day accuracy and precision were determined using spiked samples at three different concentrations: low (30 ng mL⁻¹), medium (125 ng mL⁻¹) and high (250 ng mL⁻¹) for method A and 30 ng mL⁻¹, 250 ng mL⁻¹ and 450 ng mL⁻¹ for method B with five replicates for each concentration. Inter-day accuracy and precision were determined on three consecutive intra-day runs (n=15). The intra- and inter- day accuracy and precision were found to be ≤ 2.55% and ≤ 6.78%, respectively for method A and ≤ 10.32% and ≤ 11.22% for method B and within the acceptable limit (<15%) (Table 2).

Table 2. Intra- and inter- day (%) recovery, precision (%RSD), and relative error (%RE) of TMZ spiked in human plasma (n =5).

Method	Level	Nominal concentrations (ng mL ⁻¹)	Intra-day			Inter-day		
			Recovery (%)	RE (%)	RSD (%)	Recovery (%)	RE (%)	RSD (%)
A	Low	30	79.80	2.47	4.99	81.47	2.55	2.3

	Mid	125	77.31	1.72	6.78	84.90	0.70	1.49
	High	250	82.24	0.75	1.89	78.56	1.22	4.14
	Low	30	90.92	-9.01	10.32	91.65	-8.41	11.22
B	Mid	250	92.03	-7.92	8.71	89.82	-10.22	7.41
	High	450	97.38	3.46	4.32	97.32	2.71	7.92

3.2.3. Selectivity and carry over

Selectivity was evaluated by analyzing human drug-free plasma samples from six different sources in order to test the interference in the retention time of TMZ during the analysis. The chromatograms did not reveal any interfering peaks in the retention time of TMZ (**Fig. 4A and Supplementary Data, Fig S2A**) in both methods. Normally, the absence of interfering components is accepted where the response is less than 20% of the lower limit of quantification for the analyte and 5% for the internal standard. Carry-over was assessed by injecting blank samples (containing only acetonitrile) after injection of a high concentration sample, at the upper limit of quantification of the two methods. The response of the blank samples at the retention time of the analyte was <20% of the corresponding peak area of the LLOQ sample (**Fig. 4B and Supplementary Data, Fig S2B**) and therefore, the carry-over was considered acceptable.

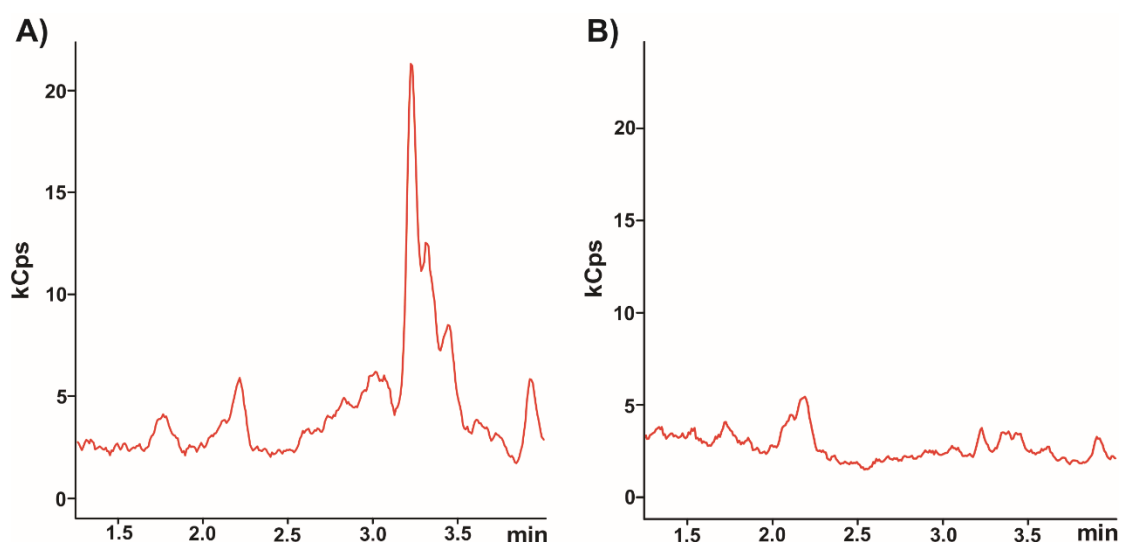


Fig. 4. Representative chromatograms of **A)** drug-free human plasma where no interfering peaks are observed in the retention time of TMZ and **B)** blank sample analyzed immediately after the injection of a high concentration TMZ sample (450 ng mL⁻¹).

3.2.4. Recovery and matrix effect

Recovery for TMZ was assessed at three QC concentration levels (30, 125, 250 ng mL⁻¹) for method A and 30, 250, 450 ng mL⁻¹ for method B (n=5). Recovery was calculated 79.8±1.9%, 77.3±0.9% and 82.2±2.3% for low, mid and high concentration levels with method A and 90.9±2.6%, 92.3±1.1% and 97.3±3.8% with method B (**Table 2**). IS recovery (n=5) was calculated 101.4±4.1% and 98.6±2.7% for method A and B, respectively. After the extraction procedures, the recoveries were consistent and precise between analytes and the IS. The percentage matrix effect ME% was assessed by comparing the signals of analytes spiked in plasma (n=5) after extraction with those of analytes dissolved in mobile phase at the same concentration. The matrix effects were 92.4±1.8%, 97.6±0.7% and 95.6±3.6% for TMZ at the three QC concentration levels (low, mid and high) in method A and 95±2.4%, 102.3±4.8% and 111.5±7.8% in method B. Matrix effects for IS in method A was 93.5±2.7% at the working concentration.

3.2.5. Stability assay

TMZ plasma samples that were analyzed after incubation for a week at 4 °C and after three freeze/thaw cycles at -20 °C are presented in **Table 3**. Intra- and inter- day precision and accuracy values for both methods were <7.26% and <9.05%, respectively revealing an excellent stability of TMZ under the tested conditions and within the acceptable limits (<15%).

Table 3. Stability of TMZ spiked in human plasma (n=5) after three freeze/thaw cycles at -20 °C and after a week in the refrigerator at 4 °C.

Method	Nominal concentrations (ng mL ⁻¹)	After 3 cycles freeze/thaw (-20°C)			After a week in the refrigerator (4°C)		
		Recovery (%)	RSD (%)	RE (%)	Recovery (%)	RSD (%)	RE (%)
A	30	77.32	2.30	1.54	78.52	4.99	4.78
	125	78.65	1.49	3.65	79.03	5.09	1.98
	250	83.51	4.14	2.98	75.00	1.88	2.32
B	30	87.62	7.22	7.66	86.34	6.37	6.99
	250	88.95	6.47	-9.05	85.22	7.26	8.65
	450	91.25	4.27	5.36	87.36	5.55	7.81

3.3. Method application to cancer patient

The validated method was applied to human plasma sample from cancer patient following oral treatment with 75 mg/m² TMZ in combination with radiotherapy. The blood sample was collected 1 h after oral administration of TMZ and it was analyzed with the two validated methods. TMZ levels were estimated at 450 ng mL⁻¹ and 550 ng mL⁻¹ for method A and B, respectively. Since the concentration levels surpassed the upper calibration point for both methods, we processed plasma sample of the same patient stored at -80 °C and we proceeded in dilution (1:1 v/v) of the analysis samples just before the LC-MS injection with reconstitution buffer solution. TMZ levels were estimated at 207 ng mL⁻¹ and 249 ng mL⁻¹ for method A and B, respectively.

4. Conclusions

Two rapid cost effective and sensitive methods were developed and validated for the determination of TMZ in human plasma without sacrificing sensitivity and accuracy. To allow simplicity and speed in sample processing both methods adapted PP treatment but differentiated in the reconstitution sample process. The methods were characterized by high sensitivity with LLOQ at 10 ng mL⁻¹ and high reliability over the concentration range of 10-500 ng mL⁻¹. To the best of our knowledge, this is the first time that a validated method for TMZ in human plasma is reported and characterized by LOQ at very low levels, accompanied by high extraction recovery. The two methods were successfully applied to plasma samples from patient following treatment with TMZ. The simple protein precipitation step, as it is described herein, could shape the basis for further modifications on sample pre-treatment procedures and is applicable also for the detection of other drugs. The accurate and sensitive methods reported, could be integrated in research platforms aiming to enhance the stability of TMZ in human plasma and in the numerous ongoing clinical studies/trials where TMZ is used in combination with other treatments. We envisage that these methods will be of utmost importance for assessing the novel TMZ nano-formulates that aim to maximize the stability, efficacy and biodistribution profile of the parent drug, leading to an improved treatment of complex and aggressive forms of brain cancers such as glioblastoma and anaplastic astrocytomas.

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Conflicts of interest

The authors declare no conflicts of interest.

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