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VALIDATED HPLC METHOD FOR DETERMINATION OF TEMOZOLOMIDE IN HUMAN PLASMA

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Abstract: The aim of the study was to develop a bioanalytical method for the determination of temozolomide (TMZ) in human plasma. Plasma concentration of TMZ was determined on a C18 column after liquid-liquid extraction. Isocratic elution was applied with the mixture of aqueous acetic acid and methanol. Theophylline was used as the internal standard. To prevent chemical degradation of TMZ at physiological pH, plasma samples were acidified to pH < 3. All validation parameters met the acceptance criteria. Calibration curve, prepared using freshly spiked plasma samples, was linear within the range of 0.10–20.00 µg/mL. The method was found to be sufficiently accurate and precise over the studied range of concentrations. TMZ was stable in the acidified plasma samples for at least 50 days at ≤ –14°C and ≤ –65°C. The method recovery of TMZ from human plasma was consistent and ranged 37.1–41.1%. The developed method is suitable for pharmacokinetic studies in humans after oral administration of TMZ.

Keywords: temozolomide, HPLC, validation, pharmacokinetics, human plasma

Temozolomide (TMZ) is an imidazotetrazinone derivative with methylating properties, which is used in the treatment of malignant primary brain tumors, e.g., glioblastoma (1) and malignant glioma (2, 3), because it has the ability to penetrate the blood–brain barrier and cerebrospinal fluid (1). TMZ undergoes chemical degradation at physiological pH to the active metabolite, i.e., methyl-triazeno-imidazolecarboxamide (MTIC) (Fig. 1). Thereafter, MTIC rapidly degrades to the inactive derivative – 5-aminoimidazole-4-carboxamide (AIC) and methyldiazonium cation. This process is irreversible and depends on the pH (4). Kim et al. demonstrated that TMZ was unstable at 37°C in human plasma, however, it was stable in human plasma acidified to pH < 4 (5). The stability of TMZ in human plasma can be achieved using an appropriate portion of 8,5% phosphoric acid (1, 5–10) or 1 M hydrochloric acid (2, 11–16).

The most commonly used techniques for the analysis of TMZ are high-performance liquid chromatography with UV detection (HPLC-UV) (1, 2, 5–7, 11, 14–16) and liquid chromatography coupled to mass spectroscopy (LC/MS/MS) (8–10, 12). TMZ was extracted from plasma samples using liquid-liquid extraction (5, 6, 9, 12, 14) or solid-phase extraction (2, 13, 15, 16).

The aim of the presented study was to validate the HPLC-UV method for the determination of temozolomide in human plasma to allow pharmacokinetic studies in humans after the oral administration of TMZ.

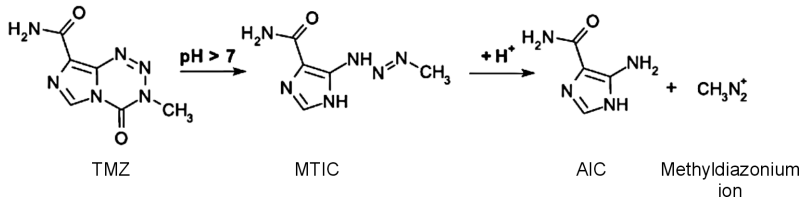

Figure 1. Scheme of TMZ and pH-dependent conversion

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EXPERIMENTAL

Materials and chemicals

Temozolomide (reference standard) was prepared at Pharmaceutical Research Institute in Warszawa, Poland. Teophylline anhydrous (the internal standard, I.S., Fig. 2) was supplied by Sigma-Aldrich, Saint Louis, USA. Acetonitrile (ACN) and methanol (MeOH) (both of HPLC grade) and ethyl acetate 99,8% were purchased from POCH, Gliwice, Poland. Hydrochloric acid 35–38% (HCl), acetic acid 100% (HAc) were supplied by CHEMPUR (Piekary Śląskie, Poland) and Merck (Darmstad, Germany), respectively. Purified water from Milli-Q system, Millipore, Molsheim, France, was used throughout the study.

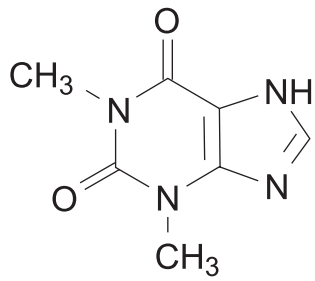

Figure 2. Chemical structure of teophylline (I.S.)

Instrument

The high-performance liquid chromatography system consisted of a system controller SCL-10A VP, a pump LC-10AD VP connected to an autosampler SIL-10A, an UV-Vis detector SPD-10A VP and a column oven of detector Decade II. The data processing software was Class-VP v. 6.13 Shimadzu (Shimadzu, Duisburg, Germany). Separation was performed on Agilent dC-18 analytical column (150 × 4.6 mm, 3.0 µm) from Waters (Milford, MA, USA) which was preceded by a guard column SecurityGuard C-18 (4 × 3 mm) from Phenomenex (Torrance, CA, USA).

Chromatographic conditions

The mobile phase consisted of MeOH / 0.5% HAc (20:80, v/v). The flow rate of the mobile phase was 1.1 mL/min in the isocratic elution mode. The

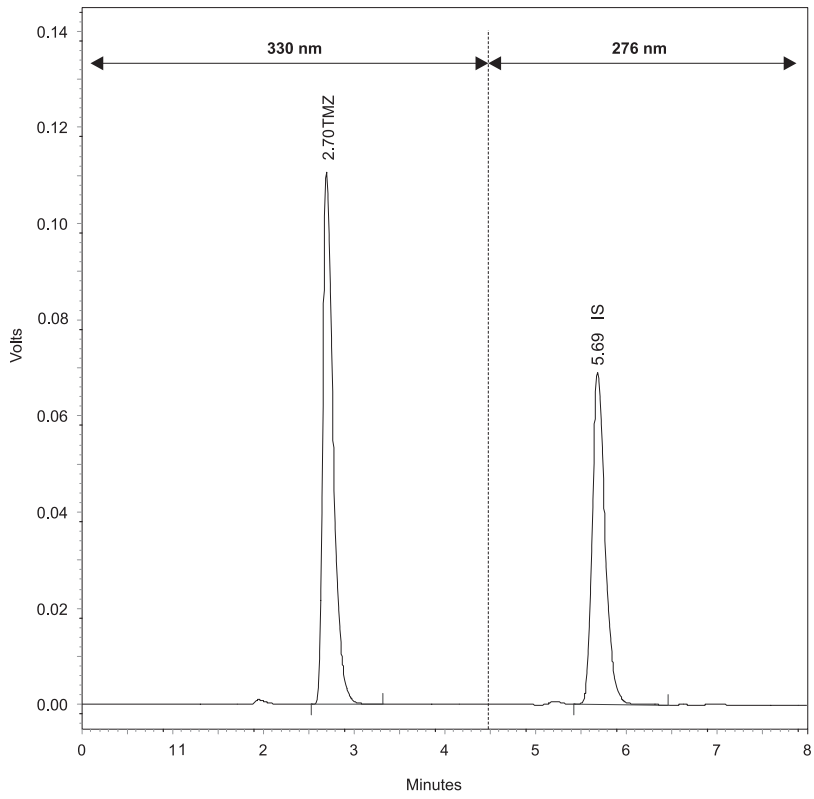

Figure 3. HPLC chromatogram of the extracted plasma sample containing temozolomide at 20.00 µg/mL (RT = 2.70 min) and the working concentration of the I.S. (RT = 5.69 min); the change of the wavelength from 330 nm to 276 nm after 4.5 min

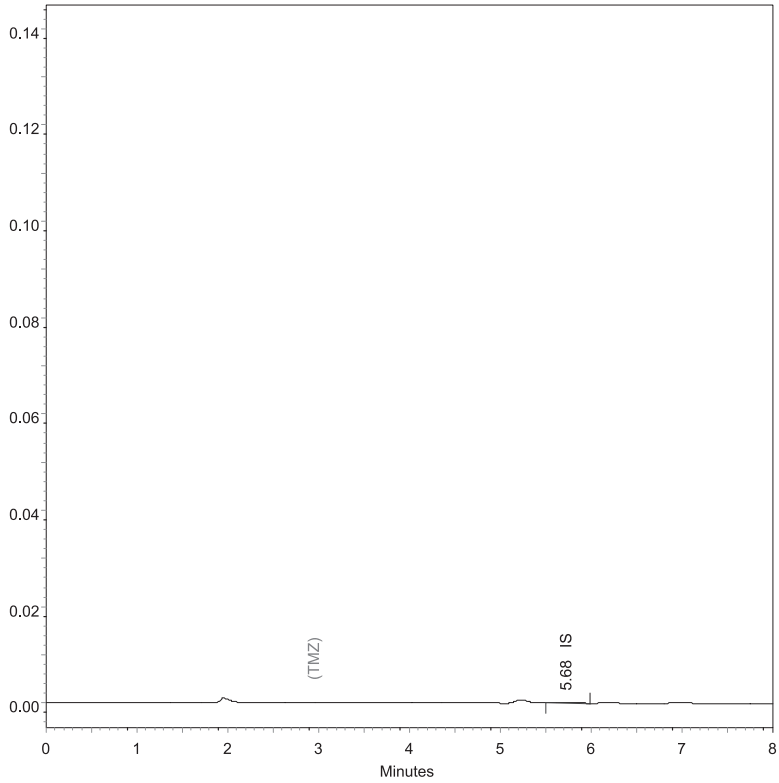

Figure 4. HPLC chromatogram of extracted blank plasma sample

Table 1. Method selectivity.

Blank plasma source	Peak height	
	Interferences with TMZ	Interferences with I.S. (%)*
Blank plasma No. 1	0	135 (0.3%)
Blank plasma No. 2	0	0
Blank plasma No. 3	0	543 (1.1%)
Blank plasma No. 4	0	279 (0.6%)
Blank plasma No. 5	0	405 (0.9%)
Blank plasma No. 6	0	21 (0.1%)

* % of I.S. working concentration peak height, acceptance limit 5%.

sample volume of 20 µL was injected onto the column whose temperature was maintained at 35 ± 2°C. The retention times of TMZ and the I.S. were around 2.7 min and 5.7 min, respectively, while the analyses run time was set at 8 min. The UV wavelength λ = 330 nm for TMZ and λ = 276 nm for the I.S. were selected (Fig. 3).

Calibration and quality standards

The stock solution TMZ and the I.S. were prepared in ACN / water (50:50, v/v) at 1000 µg/mL and stored at ≤ –20°C. The working solution TMZ and the I.S. were prepared in mobile phase and stored in a refrigerator at ≤ 15°C. Calibration standards of TMZ (CS) were prepared immediately before analysis at following concentration levels: 0.10, 0.30, 1.00, 2.50, 5.00, 10.00, 15.00 and 20.00 µg/mL. Quality control (QC) samples contained TMZ at 4 concentration levels: 0.30, 2.50, 10.00 and 15.00 µg/mL. CS and QC were prepared by spiking the blank plasma with the standard solution of TMZ in an appropriate proportion. Then the aliquot of

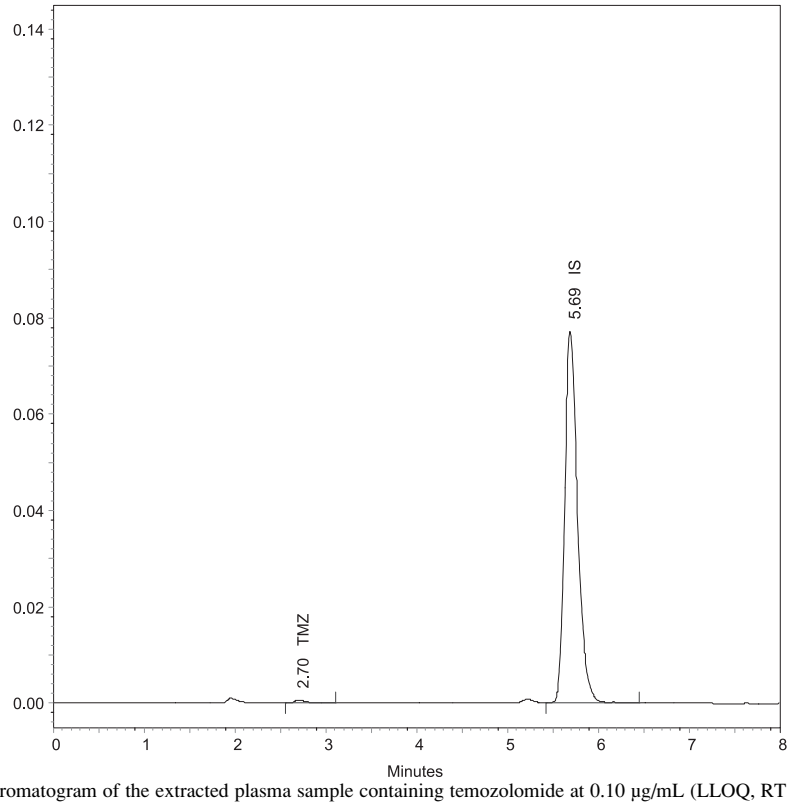

Figure 5. HPLC chromatogram of the extracted plasma sample containing temozolomide at 0.10 µg/mL (LLOQ, RT = 2.70 min) and the working concentration of the I.S. (RT = 5.69 min)

Table 2. Accuracy and precision of the assay for TMZ in plasma (n = 6)

Nominal concentration (µg/mL)	Concentration found (mean ± SD, µg/mL)	Accuracy (mean, %)	Precision (RSD, %)
Day 1			
0.10	0.110 ± 0.004	109.7	3.7
0.30	0.294 ± 0.004	97.9	1.3
2.50	2.437 ± 0.067	97.1	2.8
10.00	9.869 ± 0.314	98.7	3.2
15.00	14.867 ± 0.180	99.1	1.2
Day 2			
0.10	0.096 ± 0.003	96.3	2.6
0.30	0.280 ± 0.008	93.2	2.9
2.50	2.514 ± 0.085	100.6	3.4
10.00	10.239 ± 0.353	102.4	3.5
15.00	14.695 ± 0.376	98.0	2.6
Day 3			
0.10	0.103 ± 0.003	102.8	2.8
0.30	0.293 ± 0.004	97.6	1.3
2.50	2.598 ± 0.048	103.9	1.9
10.00	10.485 ± 0.210	104.9	2.0
15.00	15.034 ± 0.273	100.2	1.8

human plasma sample was acidified with 1 M HCl. The QC samples were stored in a freezer at ≤ –65°C.

Sample preparation

A 525 µL aliquot of acidified human plasma sample was transferred to the extraction tube. Following addition of 50 mL of 1 M HCl and 50 mL of the working solution I.S. (100 µg/mL) the sample was vortex mixed for 10 s. Then 4 mL of ethyl acetate was added and mixed for 15 min on a vibrax mixer at 1600 rpm. The sample was centrifuged at 3500 × g for 5 min and frozen for 20 min at ≤ –14°C. Then, the organic layer was transferred to the glass tube and evaporated to dryness under a stream of

Table 3. Extraction recovery of TMZ and the I.S. (n = 6).

TMZ nominal concentration (µg/mL)	I.S. recovery* (mean, %)	TMZ recovery (mean, %)
0.30	38.3	49.5
10.00	37.1	49.4
15.00	41.4	53.9

* at the I.S. nominal concentration of 10.00 µg/mL

Table 4. The stability of TMZ in acidified plasma (n = 6).

Validation parameters	Storage period	Temperature	Stability (mean and 90% C.I.,%)	
			0.30 µg/mL	15.00 µg/mL
Short-term stability	4 hours	room temperature	103.0 (101.7–104.2)	100.0 (98.4–101.7)
Freeze and thaw stability	3 cycles	≤ –65°C	100.9 (99.8–102.0)	102.3 (100.3–104.4)
Long-term stability	50 days	≤ –14°C	92.5 (90.4–94.6)	94.3 (92.4–96.3)
Long-term stability	50 days	≤ –65°C	96.8 (93.9–99.6)	98.6 (94.6–102.6)

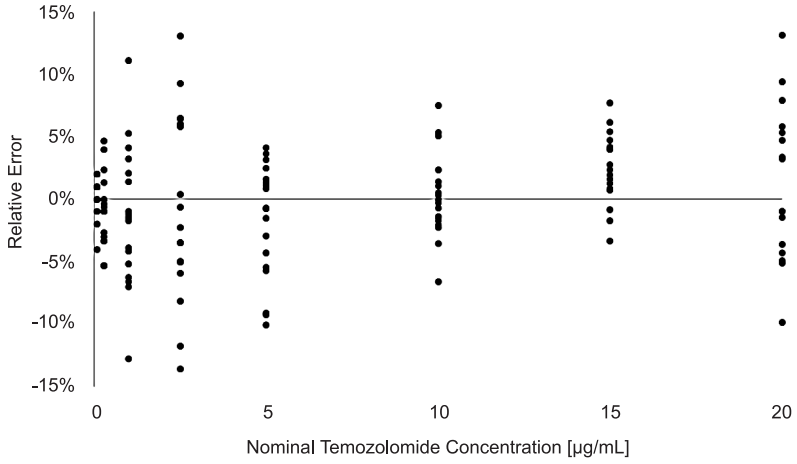

Figure 6. Percentage relative error plotted against TMZ nominal concentration; weighting factor $w = 1/x$ (n = 18)

nitrogen at 45 ± 2°C. Afterwards, 250 µL of mobile phase was added to the dry residue and the glass tube was shaken for 15 s on a vibrax mixer at 1000 rpm. The sample was transferred to the Eppendorf tube and centrifuged at 13000 × g for 5 min. The solution was transferred to an autosampler vial.

Method validation

The validation of the bioanalytical method of temozolomide determination in human plasma was performed according to EMA (17) and FDA (18) guidelines. The main characteristics of the bioanalytical method, essential to ensure the reliability of analytical results, were: selectivity, lower limit of quantification, the response function and calibration range, accuracy, precision, stability of TMZ in the biological matrix, stability of TMZ and the I.S. in the stock and working solutions as well as in the extracts under the entire period of storage and processing conditions. The outliers were detected with Dixon Q-test ($\alpha = 0.05$) and discarded from the calculations of stability as well as the calibration curve parameters. The statistical analysis of stability included the comparison of two sets of experimental data, assumed log-normal distribution of measurements results and it was based on the application of confidence intervals (C.I.) (19, 20). The construction of C.I. depends on variance equality, therefore, the F-Snedecor test at $\alpha = 0.01$ was applied to test the hypothesis on variance equality. The study was performed in compliance with the principles of Good Laboratory Practice (GLP).

RESULTS

Selectivity

Blank human plasma samples from six different sources were analyzed. The chromatograms of the blank plasma samples stored in the Pharmacology Department did not show any interferences in the retention time of TMZ. The peak height of interferences in the retention time of the I.S. did not exceed 1.1% of the working concentration for the I.S., (Fig. 4, Table 1). Normally, the absence of interfering components is accepted if the response is less than 20% of the lower limit of quantification for the analyte and 5% for the internal standard.

Limit of quantification

The lower limit of quantification (LLOQ) was determined at 0.10 µg/mL (Fig. 5). The 90% C.I. for the accuracy within one day and within three days at LLOQ were within the ranges of 106.3–113.0 and 94.3–113.0%, respectively. The 90% C.I. for within one day and within three days' precision at LLOQ were within the ranges of 3.00–7.19 and 1.84–7.19%, respectively. The accuracy and precision for LLOQ is presented in Table 2. For each LLOQ chromatogram S/N > 5 was observed.

Linearity

The calibration curve, constructed by plotting the peak height ratios of TMZ to the I.S. against the nominal concentrations of TMZ, was linear within the range of 0.10–20.00 µg/mL. The calibration curves for TMZ were obtained by a linear regression analysis with a weighting factor ($1/x^2$) chosen according to the minimum sum of percentage relative errors (RE%) (21). The values of regression parameters for the curve, described by the equation: $y = ax + b$, were: $a = 0.0688$, $b = 0.0007$ and $r = 0.9951$ (n = 6). All regression parameters were statistically significant ($\alpha = 0.05$, $df = n - 2$).

Accuracy and precision

The precision and accuracy of the method were determined within one day and within three days using four QC concentrations of TMZ (0.30, 2.50, 10.00 and 15.00 µg/mL), each in six replicates. The 90% C.I. for within one day and within three days' accuracy at QC were within the ranges of 94.9–101.3 and 90.9–106.6%, respectively. The 90% C.I. for within one day and within three days' precision at QC were within the ranges of 0.88–5.53 and 0.88–6.23 %, respectively. The results of accuracy and precision, expressed as mean values, for 4 QC levels are presented in Table 2.

Extraction recovery

The recovery was determined by the comparison of the peak height of TMZ from the spiked plasma following the extraction to the peak height of the standard aqueous solutions of TMZ injected directly onto the HPLC system. The extraction recovery of TMZ from human plasma ranged of 37.1–41.4% and was consistent for all QC levels. The extraction recovery of the I.S. ranged from 49.4 to 53.9% and was not affected by TMZ concentration. The results are presented in Table 3.

Stability

No effect of 3 freeze-thaw cycles at ≤ –65°C on stability of TMZ was observed (90 % C.I. for mean stability was within the ranges of 99.8–104.4%). TMZ was found to be stable in acidified human plasma for at least 4 h at room temperature (90% C.I. within the ranges of 98.4–104.2%). The results

Table 5. TMZ and the I.S. stock solution stability (n = 6).

Storage period	Temperature	Stability (mean and 90% C.I., %)	
		TMZ	I.S.
24 hours	room temperature	93.8 (93.3–94.3)	99.4 (98.9–99.9)
20 days	≤ –6°C	102.1 (101.7–102.4)	96.6 (96.2–97.0)

Table 6. TMZ and the I.S. working solutions stability (n = 6).

Storage period	Temperature	Working solution	Stability (mean and 90% C.I., %)
24 hours	room temperature	TMZ 2.0 µg/mL	101.2 (100.9–101.5)
		TMZ 10.0 µg/mL	99.1 (98.3–99.8)
		I.S. 100.0 µg/mL	99.4 (98.9–100.0)
15 days	refrigerator ≤ 15°C	TMZ 2.0 µg/mL	102.4 (101.9–102.8)
		TMZ 10.0 µg/mL	101.9 (101.5–102.3)
		I.S. 100.0 µg/mL	101.1 (100.7–101.4)

of the long-term stability test confirmed the stability of TMZ in acidified human plasma after storage at ≤ –14°C and ≤ –65°C for at least 50 days. (90% C.I. for the mean stability were within the ranges of 90.4–96.3 and 93.9–102.6%, respectively). For each concentration 90% C.I. for the mean stability fell within the acceptance criteria of 85–115%. The results of the test and reference samples fell within 85–115% of their nominal concentration. The results of the stability tests: freeze and thaw, short-term and preliminary long-term at two storage temperatures are presented in Table 4.

The results confirmed the stability of temozolomide and the I.S. in the stock solutions after storage in a freezer at ≤ –6°C for at least 20 days and after storage for 24 h at room temperature (Table 5). The results confirmed the stability of TMZ working solutions and I.S. working solution after storage in a refrigerator at ≤ 15°C for at least 15 days and after storage at room temperature for at least 24 h. For all compounds the 90% C.I. for the mean stability fell within the acceptance criteria of 90–110 %. (Table 6).

DISCUSSION AND CONCLUSIONS

The validated method is based on previously reported high performance liquid chromatography (HPLC) methods with ultraviolet (UV) detection. The mobile phase consisting of methanol and acetic acid but in different ratio was used by Shen et al. (15), whereas the plasma sample preparation is based on the slightly modified procedure described by Estlin et al. and Kim et al. (5, 6). However, an important innovation was introduced. The bioanalytical methods described in the literature made use of ethazolastone (2, 5, 6, 8, 11, 13–16), which is hardly available. In this study, theophylline, which is a widely available and low-cost substance, was used as the I.S. The change of the detected wave length from 330 nm to 276 nm during the analysis made the use of theophylline possible. It has not been applied yet as the I.S. for temozolomide determination in human plasma. The results obtained during the study confirmed the proper selection of the I.S.

As expected, the LC-MS methods used for the determination of TMZ in human plasma (8–10, 12) were substantially more sensitive and allowed for the temozolomide quantification from the level 0.02 µg/mL (10). Still, much less expensive the HPLC-UV methods (1, 2, 5–7, 11, 14) enable sufficient sensitivity. For the most sensitive HPLC-UV methods the limit of quantification (LLOQ) of 0.1 µg/mL was reported (1, 5, 6), which seems to be sufficient for the pharmacokinetic studies of TMZ administered in standard doses (1, 5, 7, 8, 10, 12). The previously reported efficiency of the solid phase extraction (SPE) was about 90% (15, 16). In the presented study, the recovery of temozolomide after liquid-liquid extraction was low (37.1–41.4%). However, it was sufficient to quantify the lowest concentration of TMZ reliably, with the acceptable accuracy, precision and S/N ratio (Table 2). By virtue of the analogy with the extraction procedure, the presented recovery was similar to the recovery reported by Kim et al. (5) (47–50 %). In spite of the analogy with the extraction procedure, the results presented by Meany et al. (9) were considerably different (83%). To maximize recovery, the authors performed laborious procedure of second extraction with the remaining aqueous layer in the same manner as the first one. It enabled them to perform the extraction with smaller values of the plasma (100 µL) followed by the HPLC-MS/MS analysis without adding HCl during sample preparation. A plasma acidification during the extraction followed by the HPLC-UV analysis seems to be an essential element that provides reproducibly of prepared samples and enables to obtain required selectivity (Table 1).

In the presented study, linearity was assured in the wide range 0.10–20.00 µg/mL. During validation, 18 calibration curves were prepared; six of them were used in the linearity test (first sequence) and then, one curve for each sequence was used. All calibration curves met the acceptance criteria and, what is important, none of the calibration standards was rejected during the whole study (Fig. 6).

The method was validated according to recent European Medicines Agency (EMA) guideline (17), which requires calibration curves to be prepared using freshly spiked samples. Previously prepared quality control (QC) samples, stored in a freezer at ≤ –65°C, were thawed successively during the validation to perform validation tests. In a situation where the long-term stability of temozolomide was not yet known, the application of freshly prepared calibration curves enabled to keep track of temozolomide concentration in the frozen QC samples. It was very important in the case of temozolomide because of frequently reported instability of the drug in plasma (1, 2, 5–16).

As was already mentioned above, the stability of temozolomide in human plasma was achieved by acidification with phosphoric acid (1, 5–10) or hydrochloric acid (2, 11–16). In this study 1 M hydrochloric acid was used in the proportion 0.025 mL of 1 M HCl to 0.5 mL of plasma. Under these conditions, the stability of temozolomide was confirmed after storage at ≤ –14°C and ≤ –65°C for at least 50 days. Only Shen et al. (15) reported longer stability period of temozolomide in acidified plasma (six months at –20°C), whereas in most publications the amount of temozolomide was found to be unchanged for at least one month. The acidification with 1 M HCl seems to be a reliable method to keep temozolomide stable in frozen plasma samples.

There is no information about the stability of temozolomide in the stock and working solutions used in the bioanalytical methods for the determination of temozolomide in human plasma and the composition of solution medium was rarely reported. The temozolomide stock solution was prepared in the mixture of MeOH and HAc (5, 6) or in 0.1 M HCl (15). Portnow et al. (12) reported that the working standard solutions were prepared in 0.5% HAc. In the presented study, stock and working solutions were prepared in 50% (v/v) ACN in water and in the mobile phase, respectively. The lack of stability data in the literature astounds because it is an important issue raised by the validation guideline (17, 18) and requested in validation reports.

The full validation of the bioanalytical method of temozolomide determination in human plasma in the range of 0.10–20.00 µg/mL was performed according to EMA (17) and FDA (18) guidances. The study was performed in compliance with the OECD Principles of Good Laboratory Practice (GLP). All validation parameters met the acceptance criteria, which proves that the method for the determination of temozolomide in human plasma leads to reliable results. The method described above may be applied to pharmacokinetic studies in humans after the administration of temozolomide.

Acknowledgments

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