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Analysis and stability study of temozolomide using capillary electrophoresis

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ABSTRACT

The applicability of micellar electrokinetic capillary chromatography (MEKC) for the analysis of temozolomide (TMZ) and its degradants, 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) and 5-amino-imidazole-4-carboxamide (AIC) has been studied. Using short-end injection, the analysis of TMZ and its degradants could be performed within 1.2 min. The obtained precision of migration times was better than 1.6 RSD%, and the limit of quantitation (LOQ) was 0.31–0.93 μg/mL. The therapeutic concentration of TMZ in blood samples can be determined after direct sample injection and conventional on-capillary UV detection. The proposed MEKC method was applied to study the stability of TMZ in water and serum at different pH values. It was established that the half-life of the TMZ *in vitro* serum at room temperature was 33 min, close to the half-life (28 min) obtained in water at pH 7.9.

1. Introduction

Temozolomide (TMZ) is an oral alkylating agent that readily crosses the blood–brain barrier that can be used for the treatment of malignant primary brain tumors (e.g. glioblastoma). TMZ is spontaneously hydrolyzed at physiologic pH to the active component 3-methyl-(triazen-1-yl)imidazole-4-car-boxamide (MTIC) (Fig. 1). MTIC is further hydrolyzed to 5-amino-imidazole-4-carboxamide (AIC) which is known to be an intermediate in purine and nucleic acid biosynthesis and to methylhydrazine which is the active alkylating species. This highly reactive cation methylates guanines in DNA at the *O*6 position and causes base pair mismatch. Unsuccessful cycles of mismatch repair eventually lead to breaks in the daughter strand of DNA and the cell undergoes apoptosis [1].

The decomposition of temozolomide is caused mainly by the pH-dependent hydrolysis to MTIC and hepatic metabolism only plays a minor role. TMZ is stable under acidic conditions but rapidly decomposes under neutral and basic conditions [2]. It has been reported that only negligible amounts of MTIC are detected in plasma or tumor tissues but the lack of MTIC might be caused by degradation during the sample pretreatment [3]. Because TMZ exerts its antitumor activity via its degradation product MTIC, monitoring the plasma concentration of MTIC during administration of TMZ would be essential to evaluate its effectiveness and pharmacokinetics [1].

TMZ is rapidly and completely absorbed after oral administration; peak plasma concentrations occur in 1 h. It is rapidly eliminated with a half-life ($t_{1/2} = \ln 2/k$, where k is the elimination rate constant) of 1.7–1.9 h and exhibits linear kinetics over the therapeutic dosing range [4,5]. The half-life for MTIC was calculated to be 2.5 min [2,6] but has been determined to be 1.9 h [7,8] and 25 min [7,9] in human plasma *in vivo* and *in vitro*, respectively. AIC is stable in human plasma at room temperature [7].

Until now, reversed-phase high-performance liquid chromatography (HPLC) with UV [10–12] or MS/MS [8,13] detection was used in the analysis of TMZ. Baker et al. combined HPLC analysis with radioanalysis (^{14}C-TMZ was applied) [2]. Recently, positron emission tomography (PET) was involved to determine TMZ in patients [14]. Several authors followed the procedure of sample pretreatment and HPLC analysis published by Kim et al. [7,9]. Here, blood samples were acidified and extracted with methanol [7] or ethyl acetate [9] or cleaned by solid-phase extraction (SPE) [10]. The organic layer was evaporated and dissolved in the mobile phase prior injection. Only a few papers have detailed the analysis of all three analytes (TMZ, MTIC and AIC) (analysis times were long; 20–30 min [9,2]).

Over the past two decades, capillary electrophoresis (CE) has emerged as an efficient and versatile separation tool due to its high resolution and ability to detect minute quantities of samples even in complex (biological) matrices [15,16]. CE has proven to be a powerful technique for the analysis of pharmaceuticals. Micellar electrokinetic capillary chromatography (MEKC) can be especially useful for determination of drugs in samples having high protein content (clinical samples, biofluids) reducing disadvantageous matrix effects caused by organic materials and proteins [16–18]. In

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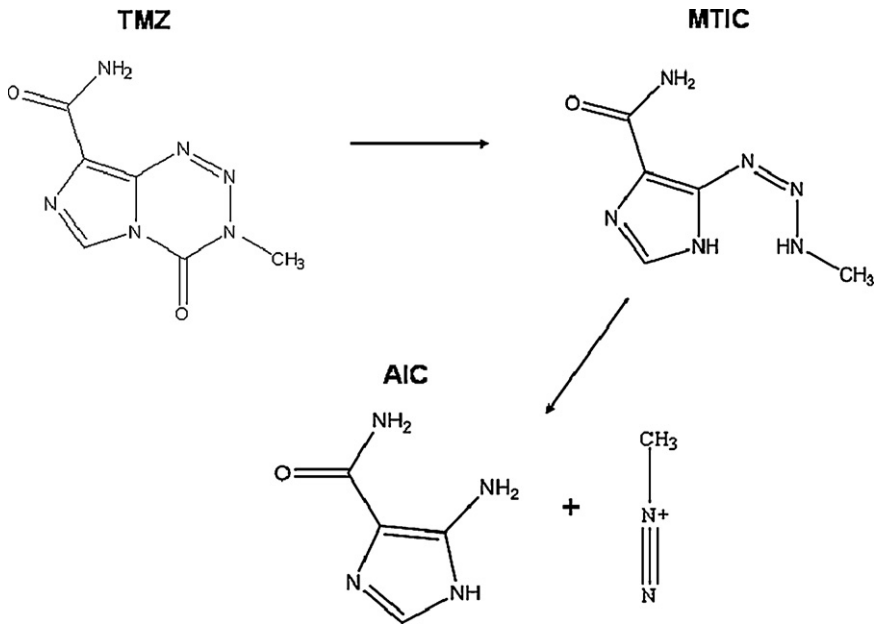

Fig. 1. Chemical structures of TMZ, MTIC and AIC.

spite of the expected advantages of CE, no study has detailed the analysis of TMZ and its degradants using this technique.

The aim of this work was to study the applicability of CE for the simultaneous determination of TMZ and its degradants; MTIC and AIC. To study the solution stability of these components, some special merits of CE were utilized and detailed herein. The potential of analyzing TMZ in serum samples without the use of complicated sample pretreatment was also investigated.

2. Experimental

2.1. Instrumentation

The capillary electrophoresis instrument was a HP3D CE model (Agilent, Waldbronn, Germany). In all measurements hydrodynamic sample introduction (50 mbar, 2 s) was used for injecting samples. The sample solutions were introduced at the anodic end of the capillary. Separations were performed using polyimide-coated fused-silica capillaries of 68 cm × 50 μm i.d. (effective lengths were 60 cm and 8 cm for normal and "short-end" injections, respectively) (Polymicro Technology, Phoenix, AZ, USA). The applied voltage was 25 kV. The detection was carried out by on-column diode array photometric measurement at 200 nm, 214 nm, 260 nm and 325 nm. The electropherograms were recorded and processed by ChemStation computer program of 7.01 version (Agilent). The execution of the stability investigation was convenient due to the possibility of the automatic measurement repetition/time programming mode of the CE instrument.

2.2. Chemicals and samples

Reagents of analytical grade were obtained from various distributors. Sodium dihydrogen phosphate, disodium hydrogen phosphate, HCl, NaOH and sodium dodecyl sulphate (SDS) for preparing buffer electrolytes were purchased from Reanal (Hungary). The 180 μg/mL sample stock solutions were prepared by dissolving the temozolomide (Temodal 20 mg, Schering–Plough) in water.

A serum sample of a patient with glioblastoma (blood samples were taken 60 min after receiving TMZ orally at a single dose of 400 mg) and serum samples were obtained from non-tumor patients (Department of Neurosurgery, University of Debrecen). The plasma samples were obtained by centrifugation (3000 rpm). All sample solutions were stored at −80 °C before analysis. The serum samples were injected into the capillary directly without sample pretreatment (those were even not diluted, only filtered through 0.45 μm syringe filter). All procedures were approved by the competent Ethical Committee and every patient signed an informed consent form.

The capillaries were preconditioned with the buffer electrolyte for 5 min. Using a new capillary it was washed with 1 M NaOH (10 min), 0.1 M NaOH (10 min), water (5 min) and buffer (20 min). In case of analysis of serum samples postcondition 0.5 M NaOH (3 min), 0.3 M SDS (3 min) and buffer (3 min) was applied to remove all possibly adsorbed materials from the capillary. Prior to CE analysis all buffers were filtered through a 0.45 μm syringe filter and stored in refrigerator at +4 °C.

2.3. Separation and detection conditions

Since the charge of TMZ and its degradants are minimal, MEKC had to be used to resolve them from the neutral components. Below 20 mM SDS content of electrolyte, resolution between the neutral components could not be achieved, but the use of 100 mM SDS concentration in the buffer electrolyte resulted in excess of Joule heat generation. The optimal SDS content of the electrolyte was found to be 40 mM. The pH or the ionic strength of the electrolyte had little to no effect on the effective mobility of these components; only the electroosmotic flow (EOF) was changed. At pH 9 the analysis time was the shortest, but the resolution was poorer than that at lower pH values. Using acidic buffer the degradation of temozolomide was larger during the electrophoretic run. The optimum pH value for the separation was about 7 because the work at this pH provides acceptable resolution of the components with a relatively short analysis time. The effect on changing buffer ionic strength (5–100 mM phosphate) on resolution was examined and it was determined that it had little effect on the separation. When the buffer concentration increased, migration times increased slightly. Using a phosphate buffer above 75 mM (with the 40 mM SDS in the running buffer), the current exceeded 75 μA causing broad-

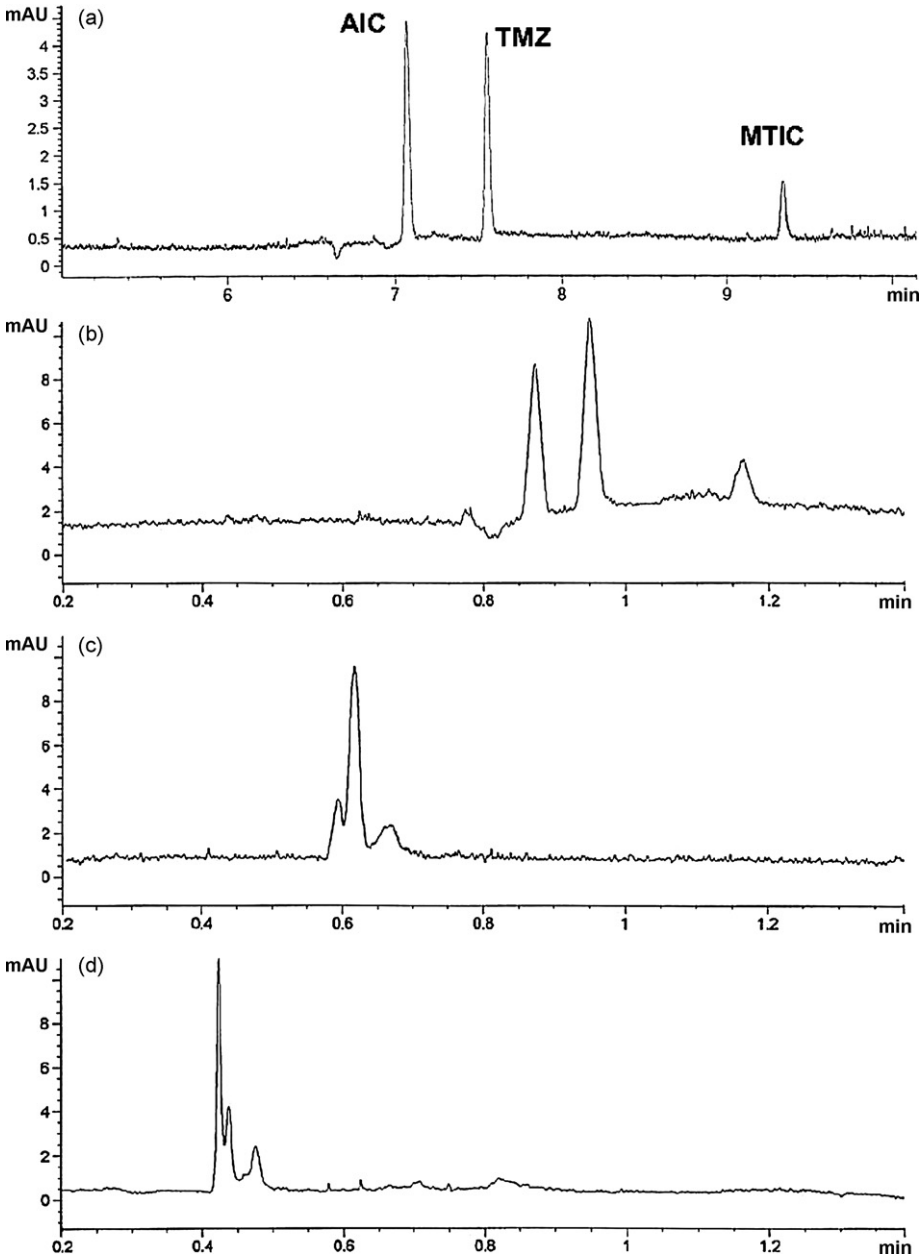

Fig. 2. MEKC electropherograms of TMZ, MTIC and AIC using (a) conventional injection mode (sample was injected at the capillary end farther from the detection, effective length: 60 cm), (b) short-end injection (sample was injected at the capillary end closer to the detection, effective length: 8 cm), (c) short-end injection with extra 50 mbar pressure applied at the injection end of the capillary, (d) short-end injection with extra 50 mbar pressure applied at the injection end of the capillary using 40 °C. (Conditions of the separation: 25 mM phosphate, 45 mM SDS, pH: 6.8, U = 25 kV, 100 mbar s, λ = 214 nm, sample: 180 μg/mL TMZ, pH of the sample: 7.9 [in the samples the actual concentrations of the 3 components were not equals due to the fast decomposition processes]).

ened peaks. After the optimization of the MEKC method the buffer electrolyte was 25 mM phosphate and 40 mM SDS at pH 6.8. Each component was identified on the basis of their migration times and their characteristic UV spectra.

3. Results and discussion

3.1. Fast analysis with conventional CE instrument

Because the half-lives of TMZ and its degradants under physiological pH and temperature are short (often less than 10 min in "non-extreme" conditions [e.g. at pH 6 or at pH 9]), analysis within 1 min is encouraged. Although one merit of CE is rapid analysis (compared to the other conventional separation techniques like GC and HPLC), MEKC generally requires more time than CZE. At the beginning of this study efforts were made to achieve the fastest separation of TMZ using a conventional CE instrument in order to study the fast decomposition of TMZ.

To decrease the analysis time there are several experimental modifications that can be tried. For example, the applied running buffer yielded a high rate of EOF (the pH was 7, the content of the phosphate and the SDS was relatively small to keep the ionic strength of the solution low), the components could not be made more ionized with higher pH. The applied voltage was 25 kV and the obtained current (60 μA) did not generate appreciable Joule heating (dispersion of the sample zones).

The analysis time can be decreased by shortening the effective length or adding extra pressure during the electrophoretic run

although both these options decrease the attainable resolution. The minimal effective length in a conventional CE instrument is the distance between the detection cell and the closest capillary end ("short-end" injection [19–21]). Compared to the conventional ("long-end") way (Fig. 2a), the use of the short-end injection (Fig. 2b) provided about 8× faster separation and the three analytes were still completely resolved.

The applied CE instrument was able to keep a constant low pressure (10–50 mbar) at the injection end of the capillary maintaining an extra laminar flow during the electrophoretic run. The 50 mbar extra pressure increased the speed of the components to almost double but with a small resolution (Fig. 2c).

Another way to hasten the separation is to elevate the temperature (40 °C) during the electrophoretic run. Although all three components reached the detector within 30 s (Fig. 2d), which is remarkable in MEKC even with use of chip technology [22], complete resolution could not be achieved. Furthermore, decomposition of the components is probably more considerable at this elevated temperature. (In the electropherograms in Fig. 2 the concentration ratios of the components were different due to the fast decomposition processes. The three components are present in the solution only in this fast decomposition period.).

According to the aforementioned results, when fast analysis was required (e.g. the solution stability of the component has a half-time of less than 10 min) and the resolving power was moderate (e.g. in model solution, which includes only 3–4 components), then short-end injection with or without extra laminar flow should be applied. However, when the resolving power has to be kept high (e.g. serum analysis), the conventional injection mode should be used.

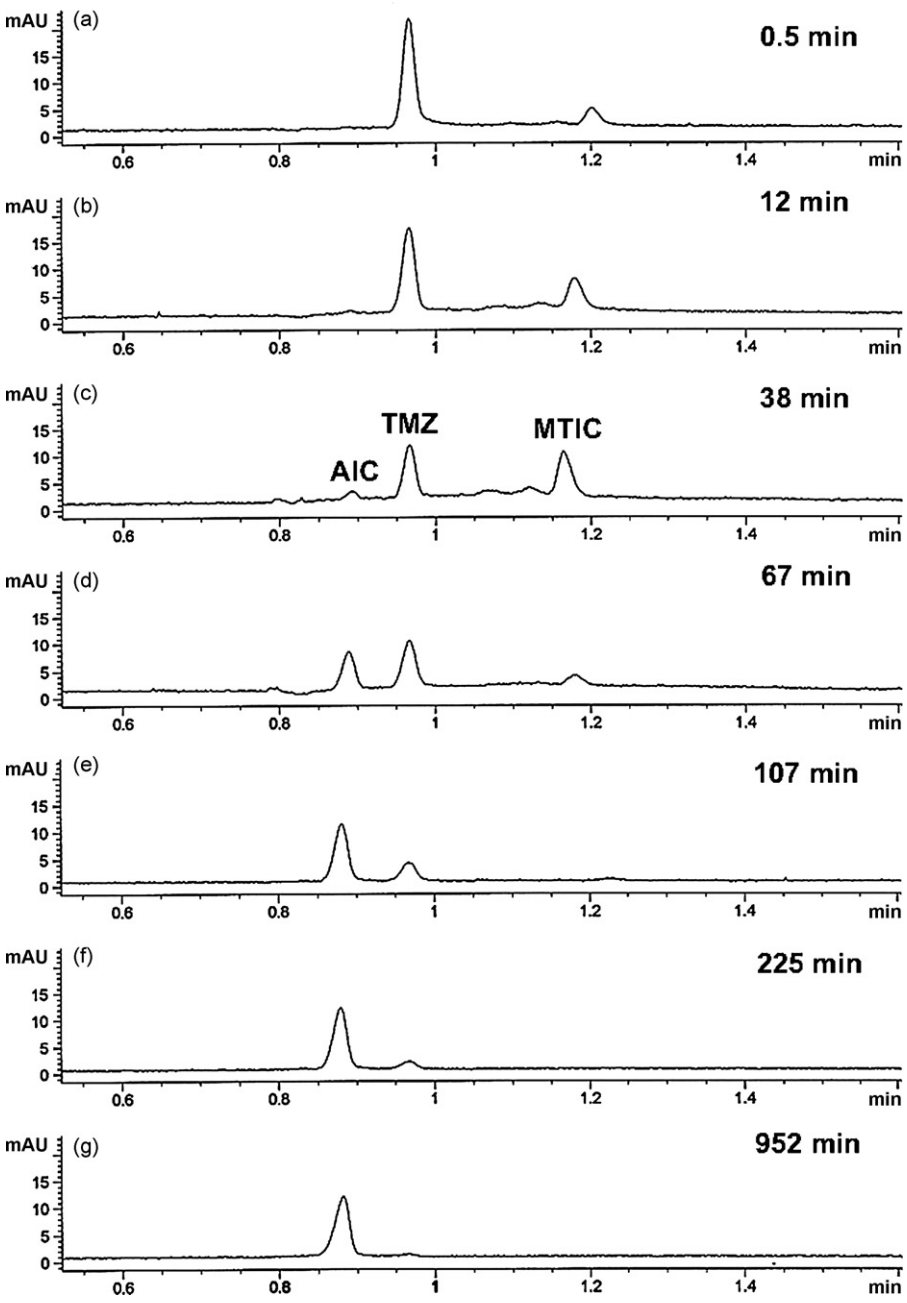

Fig. 3. MEKC electropherograms obtained for sample solution injected in (a) 0.5 min, (b) 12 min, (c) 38 min, (d) 67 min, (e) 107 min, (f) 225 min and (g) 952 min after dissolution of TMZ in water of pH 7.9. Initial concentration of TMZ was 180 μg/mL. Separation conditions were as in Fig. 2b.

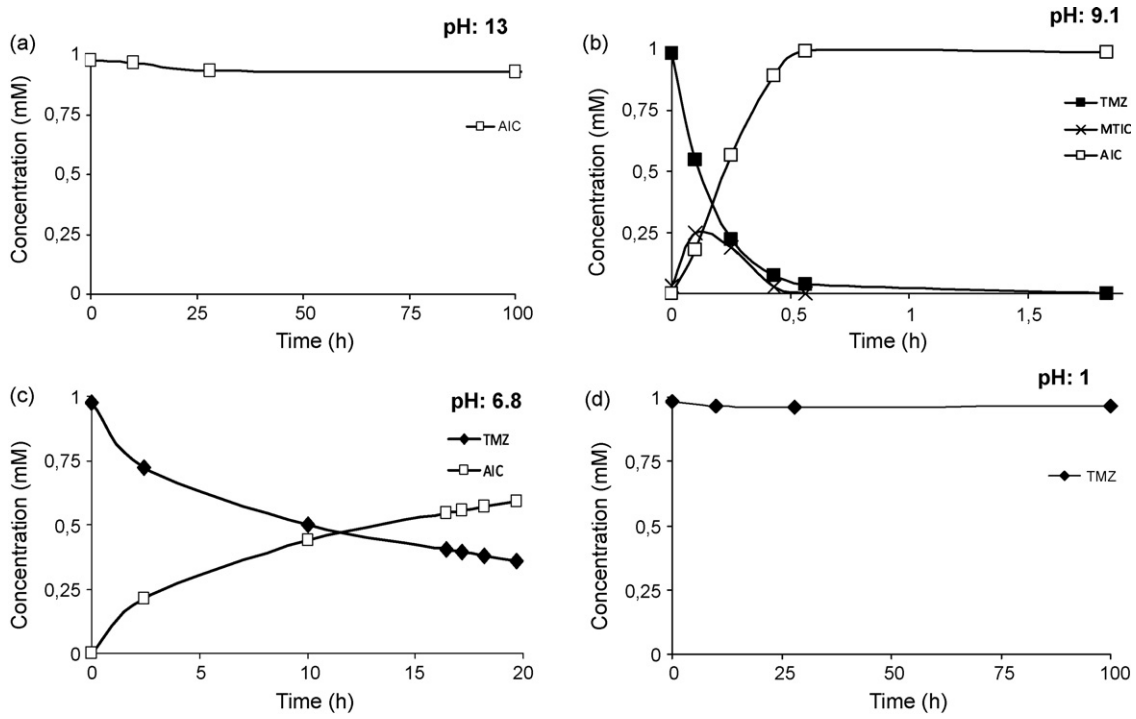

Fig. 4. Monitoring the amount of TMZ, MTIC and AIC in time after the dissolution of TMZ in solution of (a) pH 13, (b) pH 9.1, (c) pH 6.8 and (d) pH 1. The initial concentration of TMZ was 180 μg/mL. Separation conditions were as in Fig. 2b. The samples between the injections were stored at room temperature.

3.2. Solution stability of temozolomide

The concentration value of TMZ determined in a given sample is valid only at the time of injection into the CE instrument and rarely provides relevant information regarding its true concentration during the sampling step (e.g. the point in time when the blood sample is taken from the patient). Few works have detailed the study of all three analytes (TMZ, MTIC, AIC) simultaneously and no published work could be found where the concentration of AIC was followed in time.

Using the MEKC method all three analytes could be determined in single runs within a short period of time. The concentration of the components was determined after the dissolution of TMZ in aqueous solution of pH 1–13 (pH was adjusted by HCl, NaOH and phosphate buffer). The time from preparation of the solutions until their injection into the CE capillary was less than 1 min (temperature of solution should be kept at about 4 °C during the sample preparation). Between pH 6 and 8 a small change in the pH caused a large effect on the concentration distribution of the three components. Several electropherograms obtained for a TMZ solution of pH 7.9 at different times after the dissolution of the solid TMZ are shown in Fig. 3. While at this pH the half-lives were 28 min and 13 min for TMZ and MTIC, respectively, at pH 9.1 these values were 9 min and 11 min. Fig. 3 shows the variability in TMZ and MTIC on run to run while the AIC remains constant after 4 h. From this data we conclude that the constant amount of AIC equals the initial concentration of TMZ and is much larger than the maximal concentration of MTIC.

The concentration-time profiles of TMZ and its degradants in solutions of four different pH values are plotted in Fig. 4. Near physiological pH, all three components could be detected; at more extreme pH, only a single component existed. At low pH, TMZ is stable; above pH 12, the conversion of TMZ to AIC is immediate and the concentration of AIC remains constant for at least a week at room temperature.

The stability of MTIC cannot be improved using organic solvents. Although the solubility of MTIC in 20/80 isopropanol/methylene chloride (v/v) is high, its stability was very poor [7]. With a more hydrophobic solvent the analytes cannot be well extracted, and its use is impractical in CE.

The degradation can be minimized at low temperature. In case of storage at −20 °C the concentrations of all three components was constant for at least 90 days. These results demonstrate that when using neutral solutions, sampling, any type of treatment or sample storing at room temperature (even for only a few minutes) can dramatically change the concentration of the analytes. Therefore, the samples should not be kept in the autosampler but should be placed into the instrument shortly before the injection. The analysis time should also be short.

3.3. Analytical performance

The proposed MEKC method was evaluated on the basis of precision (migration time and peak area), linearity, limit of detection (LOD) and limit of quantitation (LOQ). For these investigations stable standards were needed. TMZ standard solutions were prepared in 0.1 M HCl and the AIC standard solutions were prepared in 0.1 M NaOH. Because the MTIC in all known aqueous media are unstable, exact performance data could not be determined. The precision data, the LOD and LOQ values at three wavelengths (all three components could be detected at 200 nm; λ = 260 nm and 325 nm were optimal for AIC and TMZ, respectively) are summarized in Table 1. The obtained LOQ values and the linear ranges (Table 2) demonstrate that the method with direct sample injection is able to determine the peak or the therapeutic concentration of TMZ in serum or some biological samples. (The therapeutic concentration of TMZ can be about 1–15 μg/mL because the patients are generally given a standard regimen of 150 mg/m^2/day or 200 mg/m^2/day TMZ for five consecutive days and during this treatment the peak plasma concentrations measured range from 10 μg/mL to 15 μg/mL [1,2,23,24].)

Table 1
Analytical parameters of determination of TMZ and AIC.

		TMZ	AIC
LOD (μg/mL)[a]	200 nm	0.33	0.22
	325 nm	0.28	
	260 nm		0.094
LOQ (μg/mL)[b]	200 nm	1.10	0.73
	325 nm	0.93	
	260 nm		0.31
RSD%[c] in water	Migr. time	1.63	0.84
	Peak area	2.15	0.86
RSD%[c] in serum	Migr. time	1.22	0.67
	Peak area	1.34	1.45
Recovery (%)	In serum	97.6	101.2

[a] $S/N = 3$.
[b] $S/N = 10$.
[c] Intra-day reproducibility, $c = 10$ μg/mL, $n = 10$.

The accuracy was studied by performing recovery test in serum sample (known amounts of TMZ and AIC are added to a serum obtained from people having no drug, and the spiked samples were analyzed and the concentration of the added components were determined). The obtained recovery data (Table 1) show that the developed method is reliable in analysis of serum samples.

The precision data (migration times, peak areas) obtained in water were less than 1 RSD% for the AIC and about 2 RSD% for TMZ which are in the usual range achievable with MEKC. Similar precision data have been found in serum. The individual linear regression equations for AIC and TMZ were calculated using ten concentrations (Table 2). The response was found to be linearly dependent on concentration in each experiment (response-concentration, $r^2 > 0.99$); precision at each concentration was better than 5 RSD%. The calibration graphs are shown in Fig. 5. Due to the good precision data and the lack of sample pretreatment steps the internal standard was not used during the measurements.

The selectivity of the method is made even higher by the applied detection wavelength (260 nm and 325 nm for AIC and TMZ, respectively) because the components of serum (proteins, peptides, inorganic compounds) have no absorbance in this range.

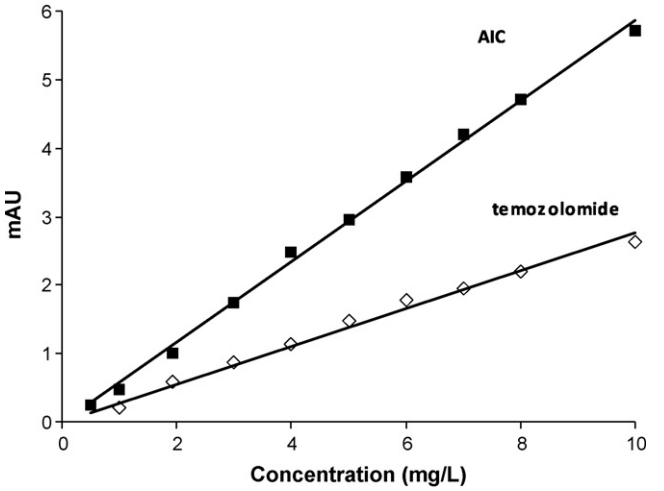

Fig. 5. Calibration graphs of TMZ and AIC. TMZ and AIC were detected at 325 nm and 260 nm, respectively. The standard solutions of TMZ were prepared in 0.1 M HCl, the AIC standards were obtained by dissolving equal molar TMZ in 0.1 M NaOH. Separation conditions were as in Fig. 2b.

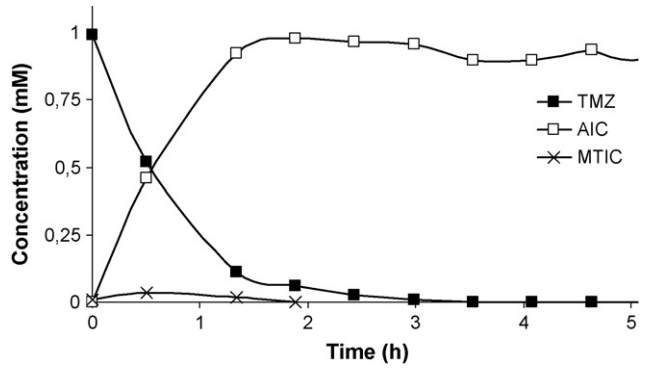

Fig. 6. Monitoring the amount of TMZ, MTIC and AIC in time after the dissolution of TMZ in human serum. The initial concentration of TMZ was 180 μg/mL. Separation conditions were as in Fig. 2b.

3.4. Stability and analysis of temozolomide in serum

TMZ is rapidly and completely absorbed after oral administration; the peak plasma concentrations occur in 1 h [1,2]. TMZ undergoes rapid nonenzymatic conversion at physiologic pH to the reactive compound MTIC which then converts to AIC.

On monitoring the amount of TMZ, MTIC and AIC in time, similar tendencies can be found than in case of the solution stability study in water (Fig. 3). Also the concentration-time profile of the TMZ and its degradants in human serum (Fig. 6) fit well to the results obtained in water at pH 6.8–9.1 (Fig. 4b and c). These similarities of the results prove that both the TMZ-MTIC and the MTIC-AIC conversions are mainly pH-dependent and the components of serum (proteins, DNA, smaller serum constituents) have only a negligible role in the degradation of the TMZ. At the pH of the serum (7.3–7.5), only a very small amount of MTIC could be detected. In this *in vitro* serum study at room temperature the half-life of the TMZ was 33 min, close to the half-life (28 min) obtained in water at pH 7.9. In a similar *in vitro* determination at 37 °C, a 15 min half-life was reported [9]. (In *in vivo* studies the half-lives were determined to be 1.7–1.9 h [4,5]. Here, further investigation is needed to clear the reason of the larger $t_{1/2}$ values obtained *in vivo*. Very similar differences were obtained for MTIC: the $t_{1/2}$ values were 1.9 h and 25 min for human plasma *in vivo* and *in vitro*, respectively [4].)

The MEKC method was used to determine TMZ in a serum sample of an advanced cancer patient that received TMZ orally at a single dose of 400 mg. The blood samples were taken 60 min after the administration of the drug (around the expected time to get

Table 2
Linearity regression data for TMZ and AIC obtained at 200 nm and their optimal wavelengths (325 nm and 260 nm for TMZ and AIC, respectively).

	Regression equation	Correlation coefficient	Range (μg/mL)
TMZ (200 nm)	$y = 0.4136x - 0.0174$	0.997	2–50
TMZ (325 nm)	$y = 0.2692x + 0.0509$	0.991	1–50
AIC (200 nm)	$y = 0.6188x + 0.0537$	0.993	2–50
AIC (260 nm)	$y = 0.592x - 0.0368$	0.997	1–50

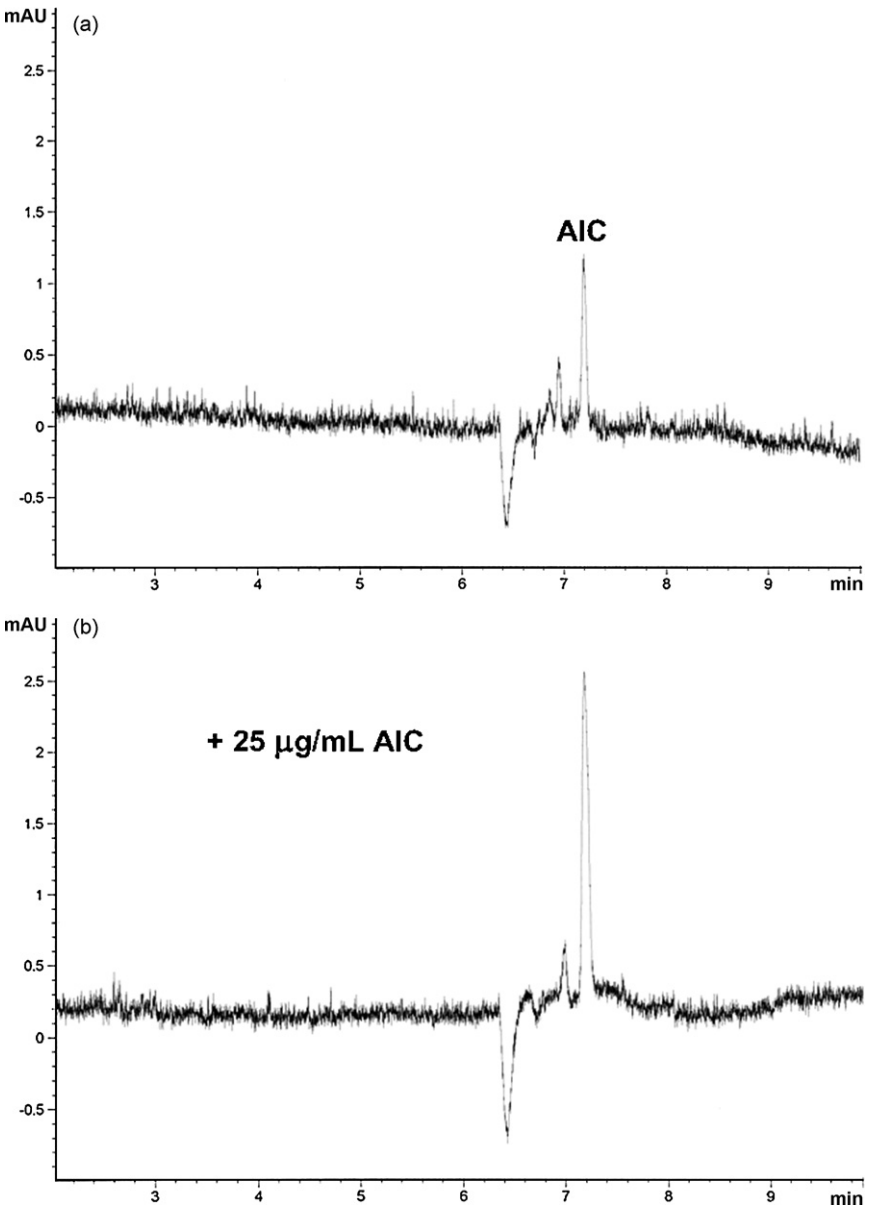

Fig. 7. MEKC electropherograms obtained for (a) a serum of a cancer patient after 1 h having a single dose of 400 mg per os, and (b) this serum spiked with AIC (25 μg/mL). (The serum was not treated by acid or base.) Separation conditions were as in Fig. 2a, but λ = 260 nm.

the peak plasma concentration). When the blood sample was put into the freezer (−20 °C) only in a few hours after taking the blood, the entire amount of TMZ was supposed to convert to AIC and the amount of AIC remained constant for months at −20 °C. In *in vitro* serum experiments the amount of the AIC determinable in the sample would be equal with the initial (absorbed) TMZ content; but in *in vivo* serum studies, since the AIC is included in biological processes (urinary excretion, purine and nucleic acid biosynthesis) the measured AIC should be less than the absorbed TMZ. In Fig. 7 the MEKC electropherograms obtained for the serum of this cancer patient and the serum spiked with AIC (25 μg/mL) are shown. In the electropherogram, only the AIC could be detected.

Based on the stability studies, the proper way to determine the concentration of TMZ at the time of the blood sampling is if the blood is immediately acidified to pH 1.

When the blood sample of a patient who received TMZ orally at a single dose of 400 mg was taken 60 min after the administration of the drug, the blood was immediately acidified with cc. HCl to pH 1 and put it into the freezer (−20 °C), the decomposition processes were stopped and the TMZ content of blood in the moment of the taking sample could be determined (Fig. 8). In the electropherogram only the peak of TMZ can be observed in a wide time window. The concentration of TMZ in the analyzed sample was 14.2 μg/mL; this concentration was expected near the c_{max}. The MTIC and AIC was probably not detected because the sampling was near the complete absorption of TMZ into the blood and degradation of TMZ had yet to begin and the acidic conditions in the gastrointestinal tract favors its stability. The TMZ and the degradants content of blood remained constant for at least 2 months after acidifying and storing at −20 °C.

The electropherograms recorded at 260 nm and 325 nm demonstrate that practically only the AIC and TMZ show light absorbance, respectively, and the components of the serum do not interfere, therefore, achievable detection sensitivity is remarkable.

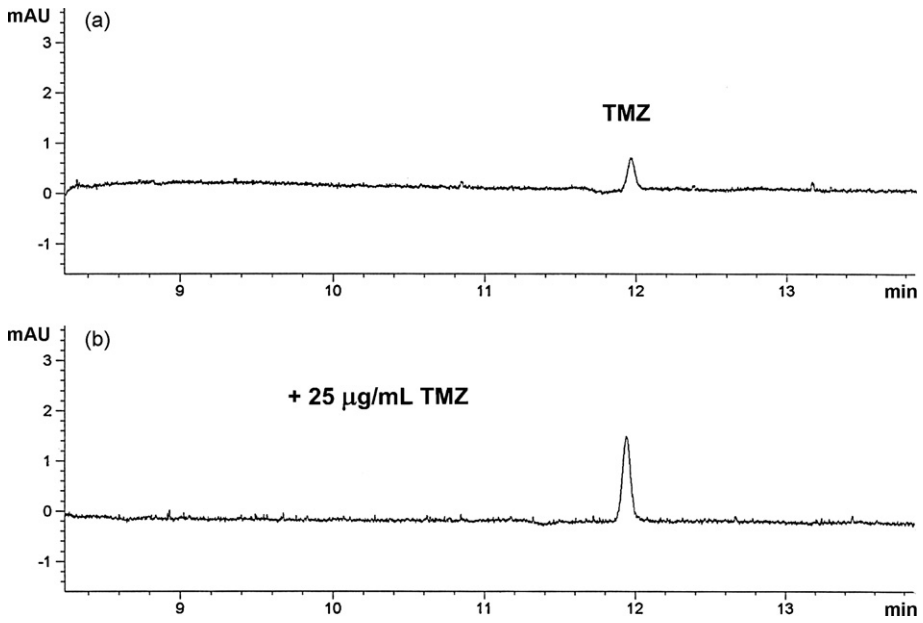

Fig. 8. MEKC electropherograms obtained for (a) a serum of a cancer patient after 1 h having a single dose of 400 mg per os, and (b) this serum spiked with TMZ (25 μg/mL). (The serum was treated by acid (50 μL cc. HCl for 5 mL blood), and it was stored in −20 °C for 1 month.) Separation conditions were as in Fig. 2a, but λ = 325 nm.

4. Conclusion

We have demonstrated that the MEKC technique is a simple and alternative method to simultaneously determine TMZ and its degradants MTIC and AIC. Some special merits of CE were utilized to study the solution stability of these components in water at different pH and in human serum.

Application of short-end injection made it possible to determine TMZ within 1 min with proper resolution. The obtained LOQ values (0.31 μg/mL and 0.93 μg/mL for AIC and TMZ, respectively) and analysis of serum sample obtained from a patient demonstrate that the method with direct sample injection and simple on-capillary UV detection is able to determine the peak or the therapeutic concentration of TMZ in serum samples.

Near the physiological pH, all three components could be detected in water and in serum. The amounts of TMZ and MTIC are highly volatile while the formed AIC remains constant after 4 h. In low pH, TMZ is stable, while above pH 12, TMZ immediately converts to AIC. The amount of AIC in a sample can provide exact, yet indirect information about the amount of MTIC that was present in the system previously.

The analysis of TMZ and its degradants in serum samples does not require a complicated sample pretreatment, thereby, saving in analysis time, cost and loss of analytes. This feature of MEKC makes it a very attractive alternative to HPLC.

Acknowledgements

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