Journal of Chromatography B, 879 (2011) 2229–2233

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of temozolomide in serum and brain tumor with micellar electrokinetic capillary chromatography

Melinda Andrási[a], Brigitta Törzsök[a], Álmos Klekner[b], Attila Gáspár[a,*]

[a] Department of Inorganic and Analytical Chemistry, University of Debrecen, H-4010 Debrecen, POB 21, Hungary
[b] Department of Neurosurgery, Medical and Health Science Center, University of Debrecen, 4012 Debrecen, POB 7, Hungary

ARTICLE INFO

Article history:
Received 7 January 2011
Accepted 6 June 2011
Available online 13 June 2011

Keywords:
Temozolomide
MEKC
Serum
Brain
Tumor

ABSTRACT

Micellar electrokinetic capillary chromatographic (MEKC) with photodiode-array detection was applied to determine temozolomide (TMZ) in human serum and brain tumor. The limit of quantitation (LOQ) was 0.096 μg/mL using 325 nm as detection wavelength. The method made possible that the TMZ could be detected in *in vivo* serum samples without sample pretreatment. In order to detect TMZ at lower concentration, an extraction with ethyl acetate was applied to preconcentrate the analyte. Small amount of brain tumor tissues (less than 1 g) were lyophilized and pretreated using extraction as a clean up and concentrating step. After removing the organic solvent a final sample volume of only 10 μL was analyzed. The obtained peak concentrations (8.2–10.1 μg/mL) and T_{max} (44–65 min) of TMZ in serum were similar to the data reported by others, the *in vivo* TMZ concentrations found in brain tumor ranged between 0.061 and 0.117 μg/g.

1. Introduction

Glioblastoma is a highly proliferating malignant brain tumor that tends to invade the normal brain and in spite of the combinant use of irradiation and chemotherapy it recurs in every case. The main problem regarding the chemotherapy against brain tumors is the low drug effectiveness due to the moderate penetration rate through the blood–brain barrier. The overall median survival time in glioblastoma patients is less then 18 months [1], so there are exhaustive efforts for developing effective chemotherapeutics. Recently the only drug routinously administered as first line therapy in the treatment of glioblastoma is the temozolomide, but its exact local concentration in the human brain tissue or brain tumor has not been directly determined [2].

Temozolomide (TMZ) is an anticancer prodrug that undergoes spontaneous chemical degradation at physiologic pH to form the highly reactive alkylating agent, methyl-triazenyl imidazole carboxamide (MTIC). The TMZ readily crosses the blood–brain barrier and it is one of the most often used compounds for the treatment of malignant primary brain tumors (e.g. glioblastoma) [3,4]. Temozolomide capsules are administered in combination with focal radiotherapy followed by several cycles of temozolomide monotherapy. Temozolomide demonstrated activity against experimentally induced tumors in mice and rats [5,6]. Positron emission tomography (PET) scanning in patients showed that intravenous ^{11}C-labeled temozolomide readily crossed the blood–brain barrier and entered brain tissue [7]. A predictive pharmacokinetic model was developed by Gallo et al. [2], because the direct measurement of drug concentrations in human tumors was found difficult to achieve.

Relatively few methods have been described for the determination of temozolomide and these methods were limited to blood. The most often used technique for the determination of TMZ is the reversed-phase high-performance liquid chromatography (HPLC) with UV [8–10], MS/MS [11,12] or radioanalytical (^{14}C-TMZ was applied) [4] detection. For sample pretreatment the blood plasma samples were acidified and extracted with methanol [13] or ethyl acetate [14] or cleaned by solid-phase extraction (SPE) [8].

The capillary electrophoresis (CE) has proven to be a powerful technique for the analysis of pharmaceuticals and ability to detect minute quantities of samples even in complex (biological) matrices [15,16]. Micellar elektrokinetic capillary chromatography 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 [17,18]. In a recent publication [19] we have demonstrated that the MEKC is a useful technique to simultaneously determine the TMZ and its degradants in model solution. Special advantages of MEKC were utilized to study the solution stability of the TMZ at different pH values. It was shown that at low pH, the TMZ is stable for weeks, but at physiological pH and above, the TMZ quickly hydrolizes.

This paper focuses on the applicability of micellar electrokinetic capillary chromatography for the *in vivo* determination of temozolomide in serum and brain tumor samples. It is not often

* Corresponding author. Fax: +36 52 518660.
E-mail address: gaspar@science.unideb.hu (A. Gáspár).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.06.005

possible to monitor a drug in clinical samples in a therapeutic concentration level using UV detection. It was possible in the case of TMZ, because it has strong absorbance both at 200 and 325 nm. The necessity of sample pretreatment and the possibility of direct injection were also studied. For this study we obtained human brain tumor samples due to an urgent operation after drug administration.

2. Experimental

2.1. Instrumentation

The capillary electrophoresis instruments were HP ^{3D}CE and 7100 models (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 a polyimide-coated fused-silica capillary of 72 cm × 50 μm i.d. with extended light path (Agilent, Waldbronn, Germany). The applied voltage was +25 kV. The detection was carried out by on-column diode array photometric measurement at 200 and 325 nm. The electropherograms were recorded and processed by ChemStation computer program of B.04.02 version (Agilent).

2.2. Chemicals

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 1.80 mg/mL sample stock solutions were prepared by dissolving the temozolomide (Temodal 20 mg, Schering-Plough, Brussels, Belgium) in 0.1 M HCl. Ethyl acetate was obtained from Merck (Darmstadt, Germany).

2.3. Clinical samples and their preparation for analysis

2.3.1. Serum samples

Serum samples of patients with glioblastoma were taken 30–250 min after receiving TMZ orally at a single dose of 400 mg. Additionally, serum samples were obtained from non-tumor patients for control samples and for matrix effects study, too. These samples were acidified with concentrated HCl to set their pH around 2 (1 mL serum was spiked with 10 μL concentrated HCl and vortex mixed) to avoid the hydrolysis of TMZ. All samples were stored at −20 °C before analysis. Prior CE analysis the serum samples centrifuged at 4 °C using 9000 × *g* for 15 min.

2.3.2. Brain tumor samples

2.3.2.1. Obtaining tumor samples. Brain tumor samples were obtained from a neurosurgical operation of a patient with recurrent glioblastoma in the right frontal lobe. The patient was on temozolomide monotherapy and last temozolomide dose (400 mg) was administered 1 h before the surgical intervention. During the operation three tumor samples (0.9915 g, 0.7803 g and 0.7647 g) and one piece of non-tumoral brain sample (peritumoral brain, frontal pole covering the tumor) (0.3782 g) were removed at 15 min intervals, rinsed with water and were immediately frozen on the surface of liquid nitrogen and stored at −80 °C until analysis. For analytical determinations tumor tissues were selected to disclose necrotic areas and to avoid additional blood presence, thus only serum free tumor tissues were forwarded for analytical procedures. To achieve radical tumor removal peritumoral brain areas were also evacuated, one of these tissue samples were studied as non-tumor brain tissue.

Table 1
Analytical parameters of determination of TMZ.

LOD (μg/mL)[a]	200 nm	0.042
	325 nm	0.029
LOQ (μg/mL)[b]	200 nm	0.14
	325 nm	0.096
RSD%[c] in serum	Migr. time	1.07
	Peak area	1.48

[a] $S/N = 3$.
[b] $S/N = 10$.
[c] Intra-day reproducibility, $c = 20$ μg/ml, $n = 10$.

2.3.2.2. Sample pretreatment. The non-tumor brain and brain tumor samples were lyophilized and homogenized. Then the homogenates (0.075–0.12 g) were carefully dissolved in minimal volume (300–600 μL) of 0.1 M HCl. The pH of the aquatic solutions used in the pretreatment procedures should be acidic (below pH 2) to avoid the hydrolization of TMZ (shown in our earlier work [19]). The obtained dense, viscous solutions were chilled and centrifuged at 4 °C using 9000 × *g* for 15 min. The supernatant was directly injected into the CE instrument.

For preconcentration procedures 50 μL aliquots of the supernatants have been extracted with 3 × 300 μL ethyl acetate (10 min vortex mixing). The ethyl acetate phases were combined into a glass vial of 1 mL and evaporated dry in rotary vacuum evaporator (Büchi) at 30 °C under 10 mbar vacuum. The dried material was dissolved in ethyl acetate and washed into a smaller vial (100 μL) applicable for CE instrument. The solvent was removed in the evaporator, and the dried material was dissolved into 10 μL 0.1 M HCl. The sample was stored at −20 °C before analysis.

All clinical samples have been obtained from Department of Neurosurgery, University of Debrecen. All procedures were approved by the National Ethical Committee, and the patient signed an informed consent form.

2.4. Analysis of TMZ

The condition of direct determination of temozolomide in model solutions was described elsewhere [19], the same separation parameters were used for serum and brain tumor analysis. The buffer electrolyte was 25 mM phosphate and 40 mM SDS at pH 6.8. The capillaries were preconditioned with the buffer electrolyte for 5 min. In the case of the analysis of serum and tumor samples a post-conditioning (0.5 M NaOH (4 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.

In this work, in order to improve the LOQ for TMZ, the newest model of CE instrument was used, thus the obtained LOQ was about 10 times better than that achieved in Ref. [19]. It is not often possible to monitor a drug in clinical samples in a therapeutic concentration level using UV detection. Fortunately, it was possible in the case of TMZ, because it has strong absorbance both at 200 and 325 nm. The most important analytical performance data of TMZ determination were summarized in Table 1.

3. Results and discussion

3.1. Analysis of serum samples

In general, the pharmaceutical analysis is carried out in a serum sample after deproteinization (using solid phase cartridge [20] or perchloric acid [21] or acetonitrile [22]), but all of these methods require complicated and time-consuming sample preparation, which can also cause measurement errors to occur easily. Since the SDS can be considered as a protein solubilizer, it is known that

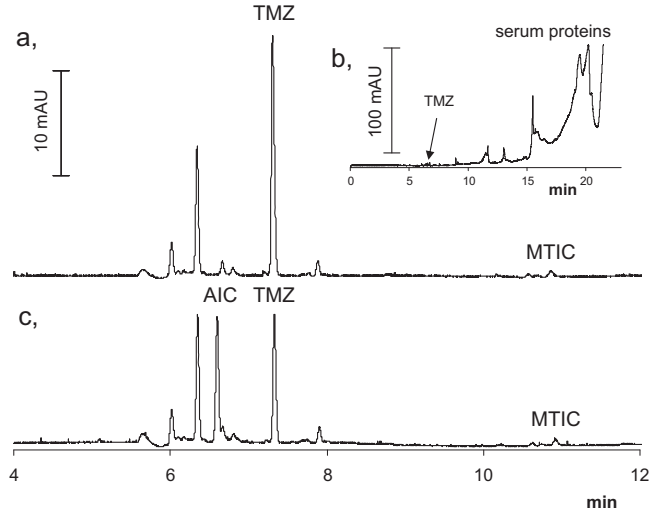

Fig. 1. The MEKC electropherograms of serum sample spiked with 20 μg/mL TMZ. The sample is injected in 2 min (a) and 30 min (c) after spiking with TMZ. MEKC electropherogram of serum including serum protein peaks (the position of TMZ is marked) (b) (conditions: 25 mM phosphate, 40 mM SDS, pH: 6.8, +25 kV, λ = 200 nm).

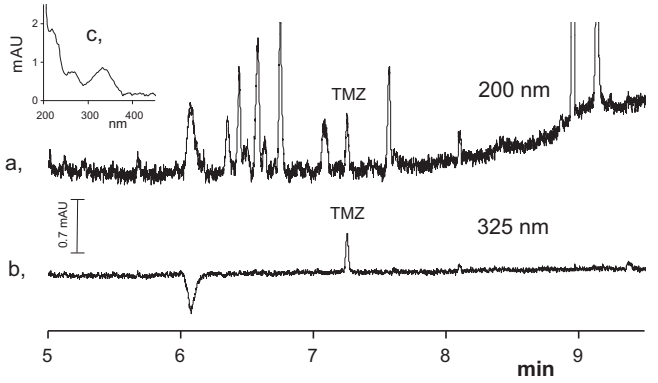

Fig. 2. MEKC electropherograms obtained for a serum of a cancer patient after 1 h having a single dose of 400 mg *per os* TMZ (the serum (1 mL) was treated by 10 μL concentrated HCl). The detection was carried out at 200 nm (a) and 325 nm (b). UV spectrum of TMZ is shown (c). (Separation conditions were as in Fig. 1, but using ^{3D}CE instrument with normal capillary (without extended light path).)

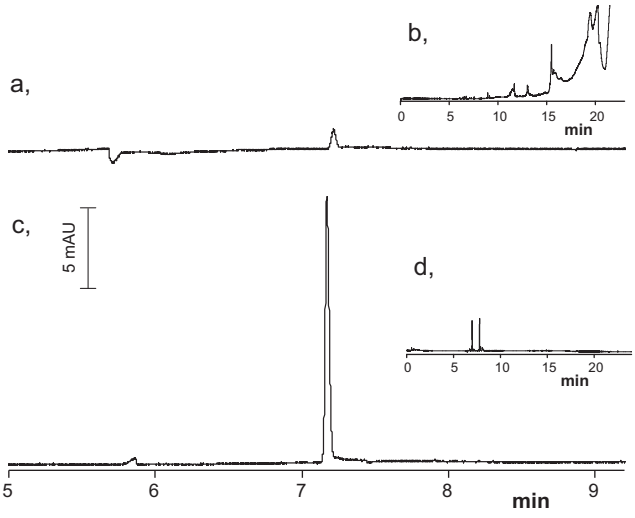

Fig. 3. MEKC electropherograms of serum spiked with 1 μg/mL TMZ with direct injection detected at 325 nm (a) and 200 nm (b); and after extraction to ethyl acetate and evaporation detected at 325 nm (c) and 200 nm (d). (Separation conditions were as in Fig. 1.)

the serum samples can be directly injected and the drugs can be analyzed, because the drugs often elute before the proteins [23]. Numerous MEKC works (where the SDS is included in the running electrolyte) can be found in literature, which report the direct injection of serum and other biofluids samples [24,25]. Therefore, in MEKC determination of TMZ, the serum samples were merely subjected to a centrifugation to remove particular materials avoiding clogging of the capillary.

In Fig. 1a the MEKC electropherogram of serum sample spiked with 20 μg/mL TMZ is analyzed. The TMZ reaches the detection window much faster than the SDS micelle protein complexes, therefore, although the proteins are present in a concentration with several orders of magnitude larger than the TMZ (Fig. 1b), no interferences were obtained.

The serum is spiked with a quite large amount of TMZ, to illustrate the fast conversion of TMZ to MTIC and 5-aminoimidazole-4-carboxamide (AIC) in neutral pH. In Fig. 1a beside the TMZ small amount of MTIC could be detected (sample is injected in 2 min after spiking with TMZ). In 30 min the 55% of TMZ was reduced to AIC (via MTIC) (Fig. 1c). In our earlier work the conversion of TMZ in water and serum was studied and it was shown that the clinical samples should be acidified to keep the TMZ content constant [19].

The conventional CE instruments with UV detection (HP ^{3D}CE instrument, without capillary with extended light path) are just able to detect the TMZ content in a serum of a cancer patient having a therapeutic dose of TMZ (Fig. 2). The peak of the TMZ can be very easily identified and integrated when 325 nm is used for the detection, because the TMZ has an absorption maximum at this wavelength (Fig. 2c), but the other components of serum do not have. A liquid–liquid extraction of TMZ as a preconcentration step makes possible the determination of TMZ at the level as small as 1% of the peak concentration. Ethyl acetate was used for extraction of TMZ in Ref. [14]. When 200 μL serum spiked with TMZ (20 μg/mL) was extracted to 3 mL ethyl acetate, the efficiency was around 50%, but repeating the extraction twice, the efficiency grew to 85%. The organic phase was evaporated and the dried material was dissolved in 10 μL 0.1 M HCl. With this extraction step the sensitivity could be improved by a factor of 17 (the peak heights of TMZ can be compared in Fig. 3a and c), and a large part of the matrix components were also removed (Figs. 3b and 4d). Using this procedure the concentrations of TMZ in the serum of two cancer patients (with similar age and weights) after having single doses of 400 mg *per os* (orally administrated) were monitored (Fig. 4). The concentration curves were relatively similar with C_{max} values (maximum plasma concentration) of 8.2 and 10.1 μg/mL, T_{max} values (time to maximum plasma concentration) of 44 and 65 min and $t_{1/2}$ values (elimination half-life) of 119 and 142 min. These values are close to the values of

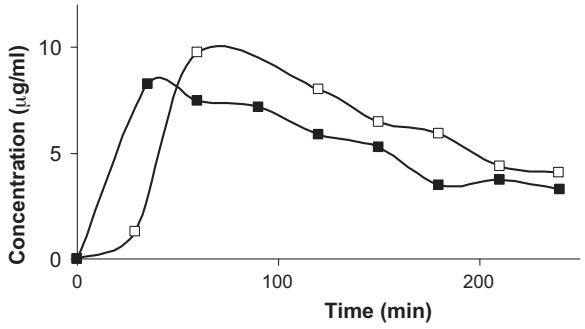

Fig. 4. Monitoring the concentration of TMZ in serum of two cancer patients in time after having a single dose of 400 mg *per os*.

Table 2
Temozolomide contents determined in human brain and brain tumor samples.

Sample	Time of sampling after having TMZ dose (min)	Mass before lyophilization (g)	Mass after lyophilization (g)	TMZ content (μg/g)
Non-tumoral brain	105	0.3782	0.0748	0.0476
Tumor 1	135	0.9915	0.1047	0.0852
Tumor 2	150	0.7803	0.1217	0.0614
Tumor 3	165	0.7647	0.1211	0.117

1.7–1.9 h obtained by others [26,27]). It is interesting to note that in the earlier *in vitro* serum studies the half-lives of TMZ were found to be only 15 min [14] and 33 min (and 28 min in water at pH 7.9) [19], which are much smaller than those obtained in this present *in vivo* analysis. Here further investigation is needed to find the explanation.

3.2. Analysis of brain tumor samples

Whereas the serum can be directly injected into the CE capillary, it is not possible in the case of the brain tumor samples due to their solid consistence. Additional difficulties are that the contents of the pharmaceuticals in the brain (tumor) are typically much smaller than in the serum, and the available amount of the *in vivo* tumor samples are small (less than 1 g). The lyophilization and subsequent dissolution of the dried material is a simple and effective procedure to ensure the homogeneity and to dissolve the analyte in a proper solution. The lyophilized tumor samples were dissolved in a minimal volume of 0.1 M HCl (300–600 μL) to prevent the hydrolization of the TMZ and to keep the dilution of the analyte small. Although the obtained solution was dense, its direct injection and determination was possible. The peak of the TMZ was just detectable (close to the LOQ) from a sample of a cancer patient after 165 min having a single dose of 400 mg, but it was already not detectable in other tumor samples.

In order to improve the sensitivity of the determination, the lyophilized and dissolved sample (50 μL) was extracted to ethyl acetate (85% extraction efficiency) and after removing the organic solvent a final sample volume of only 10 μL acidic solution (dried materials was dissolved in 0.1 M HCl) was analyzed. After the extraction preconcentration the TMZ could be well detected from the non-tumoral brain and the three brain tumor samples (Fig. 5). The confirmation of the identity of the TMZ peaks was obtained by comparing the spectra of the sample peaks and the TMZ standard, and by spiking the samples with TMZ.

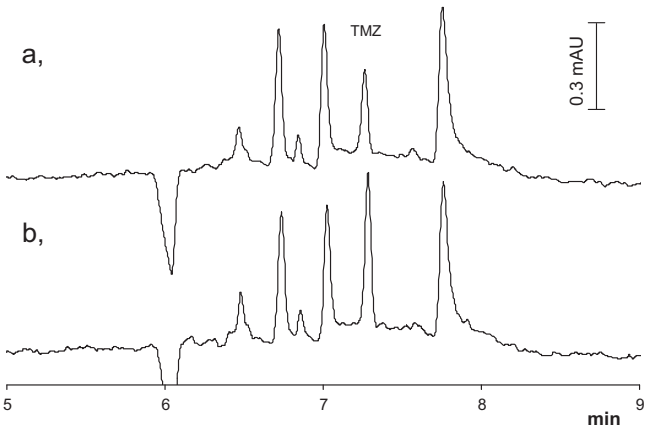

Fig. 5. MEKC electropherograms obtained for (a) a brain tumor (lyophilized and preconcentrated with extraction) of a cancer patient after 165 min having a single dose of 400 mg and (b) this tumor sample spiked with TMZ (0.4 μg/mL). (Separation conditions were as in Fig. 1, λ = 325 nm.)

The TMZ contents of the tumor samples are summarized in Table 2. The results of the few numbers of tumor samples are not enough to make a concentration curve of TMZ in time, and probably the analyte concentrations in the different tumor pieces cannot be compared due to their heterogeneities and vascularities. (The rapidly proliferating tumor tissue has areas of considerably different proliferating activity and vascularisation that makes glioblastoma inhomogeneous.) From the results of the obtained tumor samples (Table 2.) it can be concluded that the TMZ crosses the blood–brain barrier, but the peak concentration in the analyzed tumor tissue cannot be higher than 0.15 μg/g. This TMZ concentration found *in vivo* in the brain was smaller than the one reported by others. In the intracerebral microdialysis study the TMZ peak concentration in brain interstitium was found to be around 0.6 μg/mL [2,28]. Based on a predictive pharmacokinetic model 1.8–3.7 μg/mL predicted peak TMZ concentration was found in normal brain [7]. The differences are supposed to be primarily due to the difference in the physiologic compartment being sampled [28].

The smaller TMZ content obtained in our study can be the reason for the different vascularity of the tumor. (Tumor samples were originating from a recurrent tumor. The patient had concomitant radio-chemotherapy 6 months before tumor regrowth, so recent tissue samples were removed from a glioblastoma with relatively reduced vascularity and vitality due to the combinant oncotherapy.)

4. Conclusion

We have demonstrated that the MEKC technique with a common UV spectrophotometric detection is well applicable to direct determination of the *in vivo* TMZ content in the serum of cancer patients. Below 1 μg/mL (the 10% of the peak concentration of TMZ), an extraction is needed to apply to preconcentrate the analyte.

Knowledge of drug concentrations in tumors is critical for understanding the drug effectiveness and accumulation. The problem of measuring drug concentrations in human brain tumors highlights a void in data on drug disposition in human tumors. The MEKC seems advantageous for analysis of brain tumor investigations, too. In the present work relatively small work-up procedures were involved and as little as 0.8 g sample (tumor) was required for preconcentration and analysis. In the case of the MEKC determination of TMZ in serum or brain tumor samples, pretreatments (extraction or lyofilization) are needed to either improve the LOQ or obtain an injectable, liquid-state sample from a solid consistency material as brain tumor.

The obtained peak concentrations (8.2–10.1 μg/mL) and T_{max} (44–65 min) of TMZ in serum were similar to the reported serum data, but the *in vivo* TMZ concentrations found in brain and brain tumor (0.047–0.117 μg/g) using our direct analysis largely differ from the values obtained by others with indirect procedures. Here further investigations are needed.

Acknowledgements

The authors gratefully acknowledge financial support for this research by grants from the National Scientific Research Fund,

Hungary (K75286, F049050) and TÁMOP 4.2.1./B-09/1/KONV-2010-0007 project. We are thankful for the useful advices of Dr. G. Gyémánt.

References

[1] R. Stupp, W.P. Mason, M.J. van den Bent, et al., N. Engl. J. Med. 352 (2005) 987.
[2] Q. Zhou, P. Guo, G.D. Kruh, P. Vicini, X. Wang, J.M. Gallo, Clin. Cancer Res. 13 (2007) 4271.
[3] M.J.M. Darkes, G.L. Plosker, B. Jarvis, Am. J. Cancer 1 (2002) 55.
[4] S.D. Baker, M. Wirth, P. Statkevich, et al., Clin. Cancer Res. 5 (1999) 309.
[5] M.F. Stevens, J.A. Hickman, S.P. Langdon, et al., Cancer Res. 47 (1987) 5846.
[6] A.B. Heimberger, G.E. Archer, R.E. McLendon, et al., Clin. Cancer Res. 6 (2000) 4148.
[7] L. Rosso, C.S. Brock, J.M. Gallo, et al., Cancer Res. 69 (2009) 120.
[8] F. Shen, L.A. Decosterd, M. Gander, S. Leyvraz, J. Biollaz, F. Lejeune, J. Chromatogr. B 667 (1995) 291.
[9] M. Patel, C. McCully, K. Godwin, F.M. Balis, J. Neuro-Oncol. 61 (2003) 203.
[10] J.M. Reid, M.J. Kuffel, J.K. Miller, R. Rios, M.M. Ames, Clin. Cancer Res. 5 (1999) 2192.
[11] B.D. Diez, C.P. Statkevich, C. Yali, C. Zhu, et al., Cancer Chemother. Pharmacol. 65 (2010) 727.
[12] S.K. Chowdhury, D. Laudicina, N. Blumenkrantz, M. Wirth, K.B. Alton, J. Pharm. Biomed. Anal. 19 (1999) 659.
[13] H.K. Kim, C.C. Lin, D. Parker, et al., J. Chromatogr. B 703 (1997) 225.
[14] H. Kim, P. Likhari, D. Parker, J. Pharm. Biomed. Anal. 24 (2001) 461.
[15] K.D. Altria (Ed.), Analysis of Pharmaceuticals by Capillary Electrophoresis, Vieweg, Braunschweig, 1998.
[16] S.M. Palfrey (Ed.), Clinical Applications of Capillary Electrophoresis, Humana Press, Totowa, NJ, 1999.
[17] D.K. Lloyd, H. Watzig, J. Chromatogr. B 663 (1995) 400.
[18] M. Andrasi, A. Gaspar, A. Klekner, J. Chromatogr. B 846 (2007) 355.
[19] M. Andrasi, R. Bustos, A. Gaspar, F.A. Gomez, A. Klekner, J. Chromatogr. B 878 (2010) 1801.
[20] G.W. Peng, R.P. Stryd, S. Murata, M. Igarashi, K. Chiba, H. Aoyama, T. Zenki, N. Ozawa, J. Pharm. Biomed. Anal. 20 (1999) 65.
[21] K. Borner, E. Borner, H. Lode, Int. J. Antimicrob. Agents 18 (2001) 253.
[22] C.M. Tobin, J. Sunderland, L.O. White, A.P. MacGowan, J. Antimicrob. Chemother. 48 (2001) 605.
[23] T. Kitahashi, I. Furuta, J. Pharm. Biomed. Anal. 35 (2004) 615.
[24] D.K. Lloyd, J. Chromatogr. A 735 (1996) 29.
[25] S.E. Deeb, M.A. Iriban, R. Gust, Electrophoresis 32 (2011) 166.
[26] C.D. Britten, S.D.B. Rowinsky, et al., Clin. Cancer Res. 5 (1999) 1629.
[27] D.M. Park, D.D. Shah, M.J. Egorin, J.H. Beumer, J. Neuro-Oncol. 93 (2009) 279.
[28] J. Portnow, B. Badie, M. Chen, A. Liu, S. Blanchard, T.W. Synold, Clin. Cancer Res. 15 (2009) 7092.