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Determination of temozolomide in human plasma and urine by high-performance liquid chromatography after solid-phase extraction

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Abstract

As a part of a pilot clinical study, a high-performance reversed-phase liquid chromatography analysis was developed to quantify temozolomide in plasma and urine of patients undergoing a chemotherapy cycle with temozolomide. All samples were immediately stabilized with 1 *M* HCl (1 + 10 of biological sample), frozen and stored at −20°C prior to analysis. The clean-up procedure involved a solid-phase extraction (SPE) of clinical sample (100 μl) on a 100-mg C_{18}-endcapped cartridge. Matrix components were eliminated with 750 μl of 0.5% acetic acid (AcOH). Temozolomide was subsequently eluted with 1250 μl of methanol (MeOH). The resulting eluate was evaporated under nitrogen at RT and reconstituted in 200 μl of 0.5% AcOH and subjected to HPLC analysis on an ODS-column (MeOH–0.5% AcOH, 10:90) with UV detection at 330 nm. The calibration curves were linear over the concentration range 0.4–20 μg/ml and 2–150 μg/ml for plasma and urine, respectively. The extraction recovery of temozolomide was 86–90% from plasma and 103–105% from urine over the range of concentrations considered. The stability of temozolomide was studied in vitro in buffered solutions at RT, and in plasma and urine at 37°C. An acidic pH (<5–6) should be maintained throughout the collection, the processing and the analysis of the sample to preserve the integrity of the drug. The method reported here was validated for use in a clinical study of temozolomide for the treatment of metastatic melanoma and high grade glioma.

1. Introduction

The methylating agent temozolomide (CCRG 81045, M&B 39831, NSC 362856) (Fig. 1) is a member of a series of synthetic imidazotetrazinone derivatives which have a broad-spectrum antitumour activity in preclinical screening [1]. Preliminary clinical investigations have shown that temozolomide has some antitumour activity against malignant melanoma and high-grade glioma with dose-limiting myelosuppression [2–4]. Temozolomide is structurally related to the clinically used antineoplastic agent dacarbazine (DTIC) (Fig. 1) and acts presumably as a prodrug of the cytotoxic triazene MTIC, the active metabolite of DTIC. However, while

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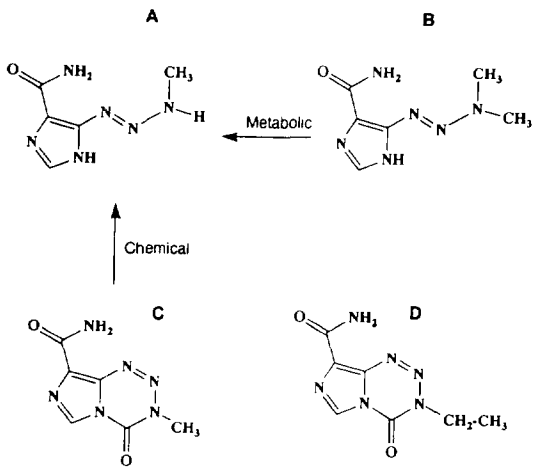

Fig. 1. Chemical structure of MTIC (A), dacarbazine (B), temozolomide (C) and ethazolastone (D).

DTIC generates MTIC via hepatic metabolic oxidation, temozolomide undergoes chemical degradation to MTIC at physiological pH and may represent a more favorable prodrug than DTIC [1].

The mechanism of the antitumour activity of temozolomide is still unclear. This drug is thought to act through the specific methylation of the O^6-position of guanine which in turn depletes the cellular repair enzyme O^6-alkylguanine alkyltransferase (O^6-AT). This "suicide enzyme" is capable of inactivating itself irreversibly by transferring the alkyl group of the O^6-position of guanine to one of its cysteine residue. The cellular level of O^6-AT seems to be involved in the tumour resistance to nitrosourea agents. Temozolomide has indeed been shown to reduce the level of this enzyme in vitro and in vivo [5]. This is the rationale for a combination therapy of temozolomide with the nitrosourea fotemustine [6,12]. In order to determine a possible correlation between clinical response, O^6-AT depletion and plasma level of temozolomide, a rapid and reliable HPLC method was developed to measure temozolomide in biological samples.

An HPLC analytical method based on liquid–liquid partition has been described for temozolomide extraction from a biological matrix [2,7]. It does not overcome some of the problems inherent to the recovery of the target compound, i.e. repetitive solvent extractions and low extraction yield. Furthermore, the analytical method should be fast enough to be applied to a large number of samples, while being in full compliance with the requirements contained in the Conference Report on Bioanalytical Methods Validation [8]. This prompted us to develop and validate an analytical method based on solid-phase extraction (SPE) as a clean-up procedure.

In contrast to mitozolomide, a member of the first generation of antitumor imidazotetrazones [9], only few data are available on the stability of temozolomide in biological fluids, especially in urine [1]. Such an assessment is of particular interest in studying the urinary excretion of temozolomide due to its potential degradation in the patient's bladder.

2. Experimental

2.1. Chemicals

Temozolomide (8-carbamoyl-3-methyl-imidazo[5, 1 - *d*] - 1, 2, 3, 5, - tetrazin - 4[3H] - one) was supplied by Schering Plough (Kennilworth, NJ, USA). Internal standard, ethazolastone (8-carbamoyl - 3 - ethyl - imidazo[5,1 - *d*] - 1,2,3,5, - tetrazin-4[3H]-one) (Fig. 1) was kindly supplied by Dr. D'Incalci, Instituto di Ricerche Farmacologiche "Mario Negri" (Milan, Italy). HPLC-grade methanol (MeOH) and acetic acid (AcOH) were obtained from Merck (Darmstadt, Germany) and J.T. Baker (Deventer, Netherlands), respectively. All other chemicals were of analytical grade and used as received. Temozolomide for intravenous administration to patients was kindly given by the Cancer Research Campaign (UK).

2.2. Standard stock solutions, calibration and control samples

Temozolomide stock solutions (4, 0.4 and 0.04 mg/ml) and internal standard (I.S.) solution (20 μg/ml for plasma samples; 160 μg/ml for urine samples) were prepared in 0.1 *M* hydrochloric acid (HCl). The plasma calibration standards were prepared in batches of 5.5 ml:5 ml blank

human plasma spiked with the proper temozolomide stock solution, acidified with 250 μl of 2 *M* HCl and completed to 5.5 ml with 0.1 *M* HCl (total added volume ≤10% of the biological sample volume). The 5 calibration standards (0.4, 2, 4, 10 and 20 μg/ml of temozolomide) were stored as 280-μl aliquots in polypropylene Eppendorf tubes at −20°C until use. The urine calibration standards (2, 10, 40, 100 and 150 μg/ml) were prepared as above in batches of 11 ml.

Plasma control samples at low (1 μg/ml), medium (10 μg/ml) and high (18 μg/ml) temozolomide concentrations were similarly prepared as above in batches of 11 ml and distributed in polypropylene Eppendorf vials which were individually thawed the day of analysis. The urine controls were prepared similarly at 4, 70 and 130 μg/ml.

2.3. Chromatographic system

The chromatographic system consisted of an HP 1050 Isocratic/Quaternary pump (Hewlett Packard, Germany) connected to an HP 1050 autosampler and an HP 1050 multiwavelength detector set at UV 330 nm. Separations were performed on a reversed-phase HP ODS-Hypersil 5 μm column (100 × 4.6 mm I.D) equipped with an HP ODS-Hypersil 5 μm (20 × 4.0 mm) guard column. The HPLC mobile phase was MeOH–0.5% AcOH (10:90, v/v). The flow-rate was 1 ml/min. HPChemStation A-00-33 software loaded on an HP Vectra 486/33N was used for data processing of the chromatograms.

For the optimization of the HPLC conditions, UV diode-array detection of temozolomide and I.S. in clinical samples were performed on an HP 1090 diode-array detector. Three-dimensional data were processed with the 3D programm of the HPChemStation software version B-02-04.

2.4. Plasma and urine collection

Blood and urine samples were obtained from melanoma and glioma patients undergoing chemotherapy according to a pilot protocol approved by the institution ethical committee. Blood samples were drawn into lithium heparinised syringes and immediately centrifuged at 900 *g* for 10 min at 4°C. A 1-ml volume of plasma was acidified with 1 *M* HCl (100 μl) and stored at −20°C until analysis. Throughout the chemotherapy, patient urine was collected and processed immediately after voiding. An aliquot of urine (10 ml) was acidified with 1 *M* HCl (1 ml) and stored at −20°C prior to analysis.

2.5. Sample preparation

C_{18} endcapped cartridges (100 mg) (Chromabond, Macherey-Nagel, Düren, Germany) were conditioned with 2 × 1 ml of MeOH followed by 2 × 1 ml of 0.5% AcOH. An aliquot (253 μl) of acidified plasma or urine samples was mixed in a polypropylene Eppendorf vial with 115 μl of I.S. solution (20 μg/ml for plasma, 160 μg/ml for urine). A 160-μl volume of the resulting solution was loaded on the cartridges in duplicate and drawn through completely under light vacuum. The cartridges were allowed to stand for 1 min and were subsequently washed with 750 μl of 0.5% AcOH, and finally dried under vacuum for 5 min. Temozolomide was desorbed with 1.25 ml of MeOH. The eluted solutions were evaporated under a stream of nitrogen at room temperature (RT) and reconstituted in 200 μl of 0.5% AcOH. Samples were transferred into Eppendorf vials and centrifuged with an Eppendorf centrifuge 5413 for 5 min. The supernatants were introduced into HPLC microvials and a volume of 30 μl was used for HPLC analysis.

2.6. Calculations

Calibration curves were obtained by unweighted least-squares linear regression analysis of the peak-area ratio of temozolomide to I.S. versus the ratio of the injected amount of temozolomide to I.S. in each standard solution.

2.7. Validation of the method

Each level of the calibration curve was measured with two sets of calibration standards: one set at the beginning and a second set at the end of the HPLC run. Throughout clinical sample

analysis, control samples at the three concentration levels (1, 10 and 18 μg/ml for plasma; 4, 70 and 130 μg/ml for urine) were assayed every 5 samples. Three injections were performed for each sample.

The control samples were used for determination of the precision and accuracy of the method, precision being calculated as the coefficient of variation (C.V.%) within a single run and between different assays, and accuracy as the percentage of deviation between nominal and found concentration with the established calibration curves.

The recovery was determined by comparison of the peak area of temozolomide from spiked plasma or urine samples after SPE to the peak area of standard aqueous solutions of temozolomide directly injected onto the HPLC apparatus.

The limit of quantitation (LOQ) was determined by analysing spiked samples ($n = 5$) at concentrations of 0.4 down to 0.1 μg/ml for plasma and of 2 down to 0.1 μg/ml for urine. The LOQ value was estimated to be the minimal concentration with an accuracy and a precision within +/−20% (range recommended by the Conference Report on Bioanalytical Methods Validation [8]).

2.8. Stability

The stability of temozolomide in buffers and in physiological fluids was determined as follows.

(a) Stability in phosphate buffers (0.067 *M*) at various pH at RT: series of temozolomide solutions (8 μg/ml) prepared in 0.067 *M* phosphate buffers [10] at pH 5, 6, 7.4 and 8 were left at RT for 5.5 h; aliquots of each solution were injected directly onto the HPLC system at selected time intervals.

(b) Stability in human plasma at 37°C in vitro: plasma spiked with temozolomide (8 μg/ml) was incubated in a water bath at 37°C for 3 h; an aliquot (100 μl) was taken in duplicate at 0, 20, 40, 60, 90, 120 and 180 min and mixed with 10 μl of 1 *M* HCl in an Eppendorf vial precooled at 4°C; subsequent sample processing was performed as previously mentioned.

(c) Stability in human urine at various pH at 37°C in vitro: series of urine were adjusted to pH 5, 6, 7 and 8 with 0.1 *M* HCl or 0.5 *M* KOH and spiked with temozolomide (8 μg/ml); they were incubated for 6 h in a water bath at 37°C; an aliquot (100 μl) was taken in duplicate every hour and mixed with 10 μl 1 *M* HCl in an Eppendorf vial precooled at 4°C; further sample preparations were done as above.

(d) Stability in reconstituted samples: plasma spiked with temozolomide (6 μg/ml) was subjected to SPE; after evaporation under nitrogen at RT, the residue was reconstituted with 0.5% AcOH, stored at RT in HPLC microvials and injected in triplicate at selected intervals over 70 h.

3. Results

Typical HPLC profiles of temozolomide and I.S. in plasma are shown in Fig. 2. The retention time for temozolomide and I.S. were 3.2 and 7.4 min, respectively. In order to optimize the chromatographic conditions and to control that no interfering peaks were present at the retention time of the analyte and I.S., three-dimensional chromatography of selected clinical samples was performed using an UV diode-array detector (plasma data shown in Fig. 3). The detection at 330 nm was found to be optimal for increasing the selectivity and sensitivity of the analysis due to the strong chromophore of the triazene linkage of temozolomide near 320 nm. Under the above chromatographic conditions, only the peak of temozolomide was detectable: none of its metabolites were observed in the plasma at 330 nm. However, an additional peak was visible in some urine samples (data not shown), which may be one of the urinary metabolites characterized previously [7].

The clean-up procedure by SPE applied to plasma and urine samples was found to be a reliable method for eliminating interfering material from the biological matrix. With an appropriate vacuum manifold, 24 samples could be processed at the same time. After loading the sample onto the cartridge, a one-minute delay

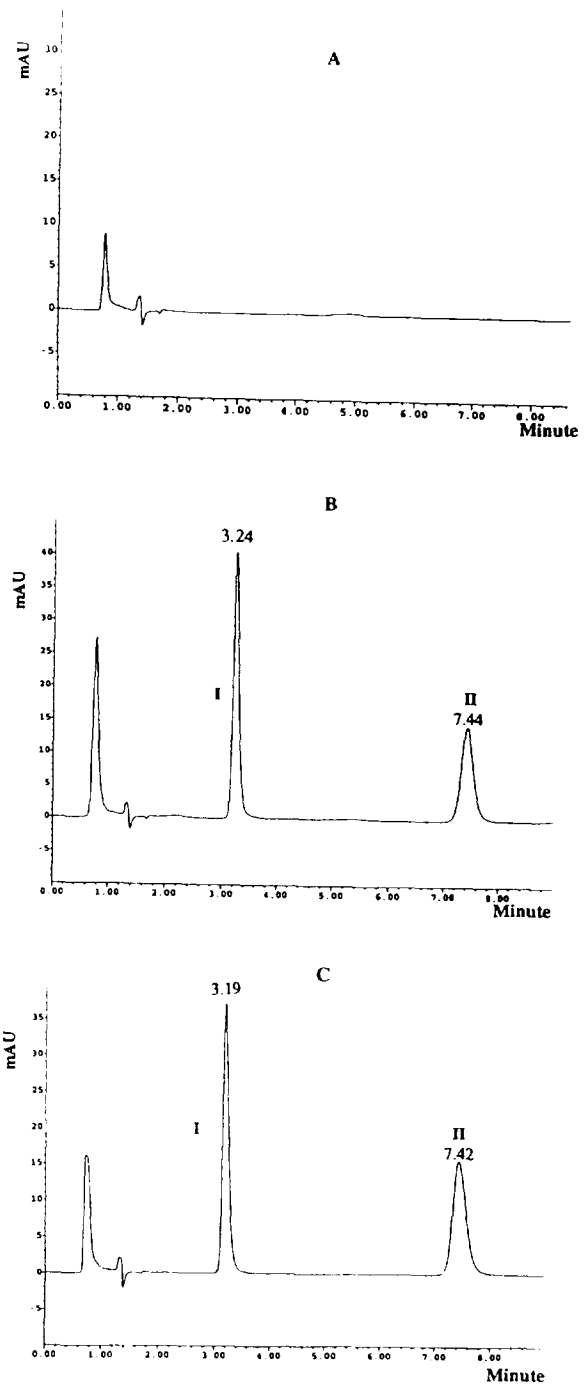

Fig. 2. Chromatograms obtained from (A) blank plasma, (B) plasma spiked with 10 μg/ml temozolomide (I) and 10 μg/ml internal standard (II), and (C) clinical plasma obtained 1 h after stopping the 60-min constant-rate infusion of temozolomide (250 mg/m^2).

before washing the column was mandatory: the non-observance of this lapse of time could result in a loss of the analytes. The components of the matrix were efficiently eliminated by washing the cartridges with 750 μl of 0.5% AcOH: a larger volume caused some bleeding of temozolomide with the washing solutions. In our hands, the solvent suggested by Newlands et al. [2] (MeOH–0.5% AcOH, 1:1) for dissolving the residue did not adequately solubilize temozolomide, giving erratic chromatographic profiles. Temozolomide was readily solubilized in 0.5% AcOH, generating excellent chromatograms.

The standard curves were linear over the concentration range 0.4–20 μg/ml in plasma and 2–150 μg/ml in urine. Typical standard curves were described by $y = 1.66x + 0.0629$ for plasma and $y = 1.21x + 0.00669$ for urine, in which y is the peak-area ratio of temozolomide to I.S., and x is the amount ratio of temozolomide to I.S. injected onto the column. In all cases the regression coefficients were $0.994 < r^2 \leq 1.000$, either for the plasma ($n = 20$) or urine ($n = 10$) calibrations curves.

The precision and accuracy determined for the validation of the analytical method are given in Tables 1 and 2. At low, medium and high concentrations, the overall intra-assay precision was 1.6–6.0% for both plasma and urine samples. The inter-assay precision for medium and high levels was in the range 5.4–7.5%, while the C.V.% was significantly higher at low concentrations either in plasma or urine. These values, however, do not fall outside the +/−20% allowance for the levels close to the LOQ [8]. The intra- and inter-assay accuracy at medium and high concentrations was excellent ($\leq 3.2\%$ of deviation from nominal values, both in plasma and urine). At low concentrations the accuracy was reduced, but the measured deviations did not exceed the +/−20% range limits [8].

The efficiency of the SPE has been determined for plasma and urine (Table 3). The overall recovery rates were 86–90% in plasma and 103–105% in urine. The urine recovery rate was substantially higher than that obtained (58.4% in urine) with the previously described method

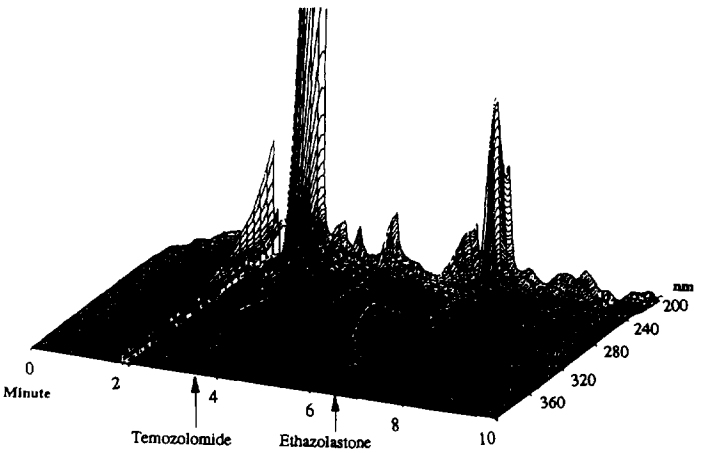

Fig. 3. Three-dimensional chromatogram of a clinical plasma sample with addition of internal standard. The chromatography was carried out on an HP1090 HPLC. Column: Nucleosil 100-5 μm C_{18}AB (250 × 8 × 4 mm); solvent: MeOH–0.5% AcOH (85:15); flow-rate: 1 ml/min.

using liquid–liquid extraction with ethylacetate [7].

The LOQ was 0.2 μg/ml in plasma and 2.0 μg/ml in urine for the described analytical procedure. A lower LOQ can be expected by loading a larger sample volume on the cartridges and/or injecting a larger volume of sample onto the HPLC column.

As temozolomide has been shown to undergo chemical degradation, the stability of temozolomide in biological matrix and at various steps of the analytical procedure had to be explored for assessing the integrity of the drug from the time of sampling from patients to the processing in the laboratory. In phosphate buffer (0.067 *M*) at pH 7.4, only 60% of the starting concentration of temozolomide could be detected after 5.5 h at RT, while a negligible reduction (<0.5%) was observed at pH 5 over the same period of time (Fig. 4). With our procedure, the half-life of temozolomide in vitro in human plasma at 37°C was 2.4 h (Fig. 5). This differs appreciably from the previously reported data ($t_{1/2}$ in vitro of 0.42 h in human plasma at

Table 1
Accuracy and precision of the assay for temozolomide in plasma

Nominal concentration (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Precision (C.V., %)[a]	Accuracy (deviation, %)[b]
Intra-assay (n = 8)			
1.00	0.83 ± 0.05	6.0	17.0
10.00	10.07 ± 0.16	1.6	0.7
18.00	18.34 ± 0.67	3.7	1.9
Inter-assay (n = 16)			
1.00	1.04 ± 0.19	18.3	4.0
10.00	10.31 ± 0.77	7.5	3.1
18.00	17.67 ± 1.18	6.7	1.8

[a] Coefficient of variation.
[b] [(Found − nominal)/nominal] · 100.

Table 2
Accuracy and precision of the assay for temozolomide in urine

Nominal concentration (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Precision (C.V., %)[a]	Accuracy (deviation, %)[b]
Intra-assay (n = 6)			
4.00	3.52 ± 0.15	4.3	12.0
70.00	71.2 ± 2.27	3.2	1.7
130.00	127.39 ± 2.33	1.8	2.0
Inter-assay (n = 6)			
4.00	3.35 ± 0.47	14.0	16.3
70.00	67.73 ± 3.87	5.7	3.2
130.00	128.95 ± 6.95	5.4	0.8

[a] Coefficient of variation.
[b] [(Found − nominal)/nominal] · 100.

37°C at pH 7.4) [1]. The latter value does not seem to be consistent with in vivo data where the disappearance of the parent drug in plasma is expected to be at least equal or even faster due to the systemic clearance of the drug from the body. For comparison, pharmacokinetic analysis of our first eight patients shows a mean temozolomide $t_{1/2}$ in vivo of 1.65 h ± 0.23, in close agreement with the $t_{1/2}$ of 1.67 h and of 1.81 h ± 0.36 reported by Dhodapkar et al. [11] and Newlands et al. [2], respectively. Nevertheless, because of the lack of stability of temozolomide in plasma, the blood samples should be cooled and acidified immediately after collection. Addition of 1 *M* HCl to blank plasma (1 + 10 of plasma) lowered the pH value of blank plasma from 7.38 to 3.85, indicating that the acidification step was able to stabilize temozolomide in human plasma.

Table 3
Extraction recovery of the temozolomide assay

Nominal concentration (μg/ml)	Recovery (mean ± S.D.) (%)
Plasma (n = 8)	
1.00	86.0 ± 6.9
10.00	88.9 ± 1.7
18.00	90.0 ± 1.5
Urine (n = 8)	
4.00	102.5 ± 3.0
70.00	104.5 ± 2.7
130.00	104.8 ± 3.3

In in vitro studies, the temozolomide concentration in human urine was reduced by 60% at pH 7 after 6 h at 37°C, and by 13% at pH 6. No degradation however could be measured at pH 5 over the same period of time (Fig. 6). These observations prompted us to carefully measure the pH of collected urines and to acidify aliquots immediately after voiding. Neutral urine from patients with reduced diuresis may show a decreased amount of temozolomide due to a prolonged stay in the bladder. However, most of the patients in our study were well hydrated and voided regularly over the period of chemotherapy, thus the degradation of temozolomide in unacidified urine was minimal. The mean 24-h urinary recovery of temozolomide measured in our patients was 6.6%, being in close agreement with the 5.2% value obtained recently by Dhodapkar et al. [11].

Acidified plasma and urine spiked with temozolomide at 10 μg/ml and 70 μg/ml, respectively, were stable for at least three months when stored in the dark at −20°C. The amount of temozolomide in acidified plasma was found to be unchanged after six months of storage under these conditions.

The stability of temozolomide was also assessed in reconstituted samples subjected to

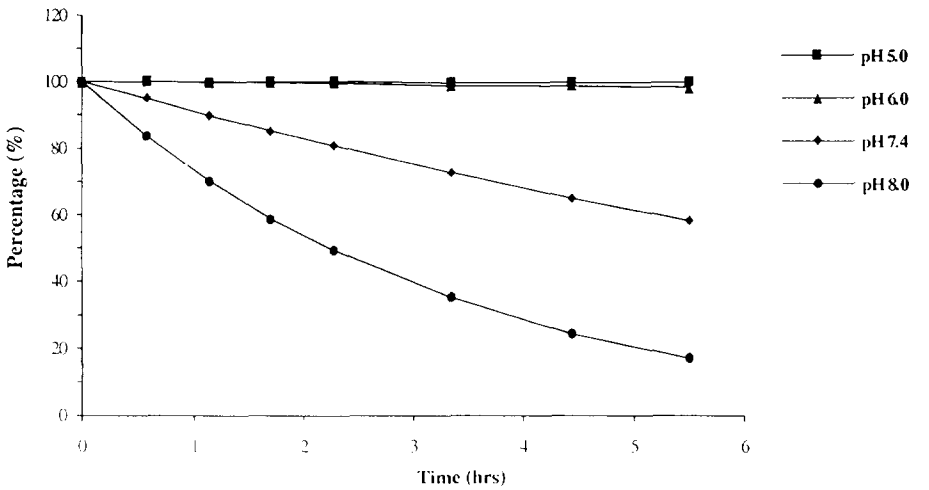

Fig. 4. Stability of temozolomide in phosphate buffers (0.067 *M*) at RT.

HPLC analysis: the temozolomide concentration was unaffected when left at RT over at least 70 h. This observation ensures sample integrity throughout the HPLC run.

Fig. 7 shows the plasma concentration–time profile measured in one patient after a 1-h constant-rate infusion of temozolomide (250 mg/m^2). Pharmacokinetic and pharmacodynamic analysis (O^6-AT as surrogate marker in lymphocytes or directly in excised tumors) are currently carried out in melanoma or glioblastoma patients, which ultimately may allow the establishment of a pharmacokinetic/pharmacodynamic (PK/PD) model [12].

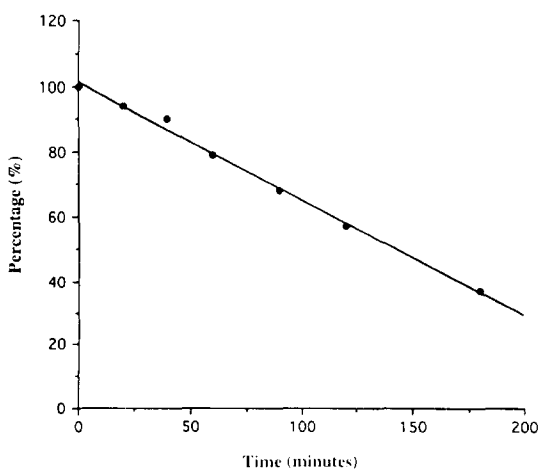

Fig. 5. Stability of temozolomide in human plasma at 37°C. The linear curve is described by $y = 101.25 - 0.36028x$ ($r^2 = 0.995$).

4. Discussion

The concentration-oriented strategy is both rational and efficient for drug evaluation and is particularly well suited for anticancer agents [13]. The quality of pharmacokinetic studies is largely dependent on the reliability of the dosage. The analytical method described in this paper has therefore been fully validated. This is the first report on the quantitation of temozolomide in a biological matrix using SPE as a clean-up procedure. This potentially automatable method fulfils all the desired requirements. It is not only devoid of the drawbacks of repetitive solvents extraction, but also offers a high recovery rate and the advantage of enabling simultaneous processing of a large number of samples. Although not dedicated to routine analysis, our method enables selective and sensitive measurement of temozolomide in a biological matrix. It thus represents a useful tool for better understanding the pharmacological activity of temozolomide in humans and for the

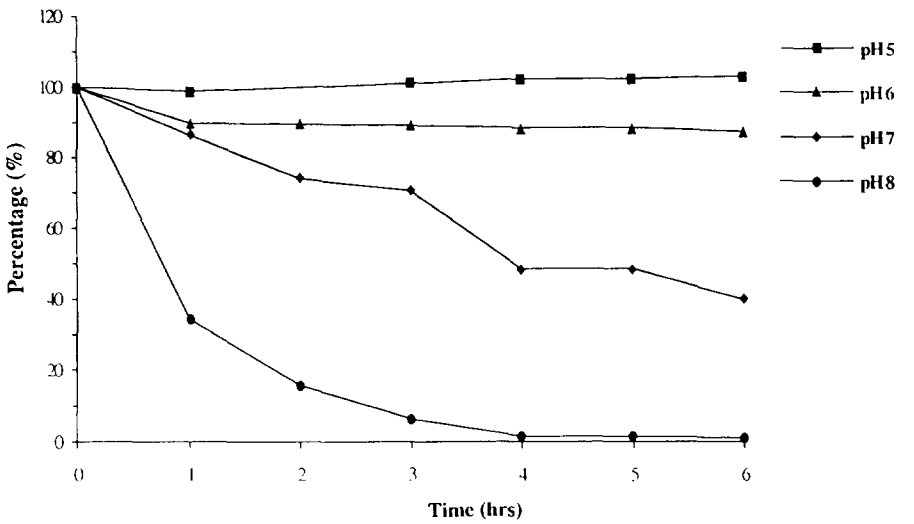

Fig. 6. Stability of temozolomide in human urine at various pH (37°C).

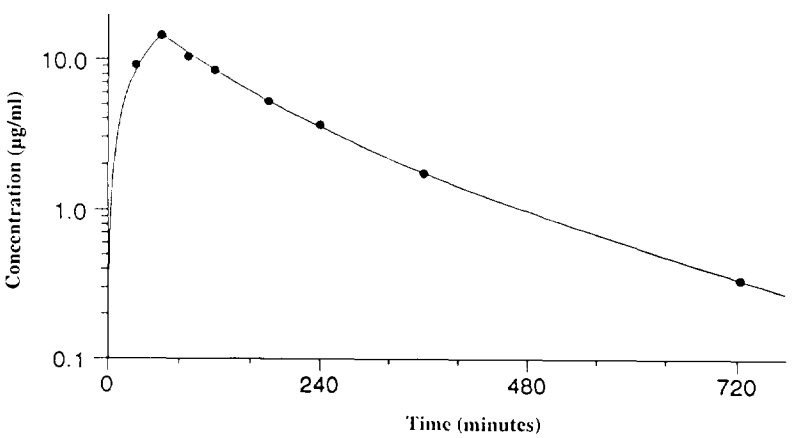

Fig. 7. Concentration–time profile of a patient receiving a 1-h constant-rate infusion of temozolomide (250 mg/m^2).

optimal use of this new agent, for the benefit of patients.

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References

[1] M.F.G. Stevens, J.A. Hickman, S.P. Langdon, D. Chubb, L. Vickers, R. Stone, G. Baig, C. Goddard, N.W. Gibson, J.A. Slack, C. Newton, E. Lunt, C. Fizames and F. Lavelle, *Cancer Res.*, 47 (1987) 5846.

[2] E.S. Newlands, G.R.P. Blackledge, J.A. Slack, G.J.S. Rustin, D.B. Smith, N.S.A. Stuart, C.P. Quarterman, R. Hoffman, M.F.G. Stevens, M.H. Brampton and A.C. Gibson, *Br. J. Cancer*, 65 (1992) 287.

[3] D.B. Smith, E.S. Newlands, G. Blackledge, N.S.A. Stuart, J. Slack and M.F.G. Stevens, *Br. J. Cancer*, 62 (1990) 519.

[4] E.S. Newlands, *Ann. Oncol.*, 5 (Suppl. 5) (1994) 119.

[5] S.M. Lee, N. Thatcher, D. Crowther and G.P. Margison, *Br. J. Cancer*, 69 (1994) 452.
[6] M. Gander, S. Leyvraz, L. Perey, F. Shen, M. Bonfanti, G. Collella, L.A. Decosterd, J. Biollaz, M. D'Incalci and F. Lejeune, *Ann. Oncol.*, 5 (Suppl. 5) (1994) 86.
[7] L.L.H. Tsang, P.B. Farmer, A. Gescher and J.A. Slack, *Cancer Chemother. Pharmacol.*, 26 (1990) 429.
[8] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowwall, K.A. Pittman and S. Spector, *Eur. J. Drug Metab. Pharmacokin.*, 16 (1991) 249.
[9] J.A. Slack and C. Goddard, *J. Chromatogr.*, 337 (1985) 178.
[10] GIBA-GEIGY AG, Basel, *Wissenschaftliche Tabellen Geigy: Teilband Hämatologie und Humangenetik*, GIBA-GEIGY Limited, Basel, 8th edn., Vol. II, 1979, p. 61.
[11] M. Dhodapkar, J. Reid, J. Ames, M.M. Buckner, J.C. Burch and P.A. Rubin, *Proc. Am. Assoc. Cancer Res.*, 35 (1994) A1461, 245.
[12] M. Gander, S. Leyvraz, L. Perey, F. Shen, M. Bonfanti, G. Collella, L.A. Decosterd, J. Biollaz, M. D'Incalci and F. Lejeune, *Proc. Am. Soc. Clin. Oncol.*, 13 (1994) A425, 158.
[13] L. Gianni, *Ann. Oncol.*, 4 (1993) 463.