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Determination of GTI-2040, a novel antisense oligonucleotide, in human plasma by using HPLC combined with solid phase and liquid–liquid extractions

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Abstract

GTI-2040 is a 20-mer phosphorothioate oligonucleotide complementary to the mRNA of the R2 subunit of ribonucleotide reductase (RNR). It is under clinical development as an anti-cancer agent. A reverse phase high-performance liquid chromatograph (HPLC) method was established for the quantitative analysis of GTI-2040 in human plasma. Plasma samples were prepared with an initial solid-phase extraction (SPE) followed by a liquid–liquid extraction step. HPLC analysis was performed with a gradient system on a Waters XTerra®MS C18 column. The mobile phase consisted of acetonitrile–tetrabutyl ammonium hydrogen sulfate (TBAS) buffer (pH 9.0, 20 mM) at a flow rate of 1.0 ml/min, and the detector was set at a wavelength of 260 nm. A cationic pairing reagent, tetrabutyl ammonium hydrogen sulfate was added during plasma sample clean-up with solid-phase extraction, resulting in significant improvement in extraction recovery. In addition, TBAS addition to the mobile phase improved the peak symmetry of GTI-2040. This method was successfully used in the analysis of GTI-2040 in clinical plasma samples.

Keywords: Antisense oligonucleotide; GTI-2040; Solid-phase extraction

1. Introduction

Antisense oligonucleotides are short single-stranded DNAs designed to be complementary to specific mRNA sequences. They form duplexes with the target mRNA, thereby interfering with gene expression and production of target proteins. There have been a growing number of antisense oligonucleotides in clinical development as anti-cancer agents [1–4]. Various analytical methods, such as gel capillary electrophoresis (GCP) [1], high-performance liquid chromatograph (HPLC) [5] and HPLC-mass spectrometry (LC/MS) [6], have been developed and used in quantitating antisense oligonucleotides in patient samples from clinical studies.

One common problem in developing HPLC methods for quantitating antisense oligonucleotides is the extensive sample clean-up required [7]. Antisense oligonucleotides are highly bound to plasma proteins, therefore, proper sample clean-up is critical in separating them from endogenous interferences. Sample extraction with methanol or phenol/chloroform is generally time consuming and causes significant material loss, resulting in low and/or variable extraction recovery [5,7,8]. Solid-phase extractions (SPE) have been used to simplify and improve the efficiency of sample preparation. Early SPE methods generally required a two-step extraction followed by an additional desalting step [9]. More recently, one-step SPE has been described in conjunction with LC/MS [6]. A major limitation of previously described SPE methods is low extraction recovery.

Ribonucleotide reductase (RNR) is an essential enzyme that is required for the reductive conversion of ribonucleoside 5′-diphosphates to corresponding 2′-deoxyribonucleotides, a crucial rate-limiting step during DNA synthesis and repair. RNR is composed of two subunits, R1 and R2, which are encoded on different chromosomes [10]. R2 over-expression is associated with enhanced malignant transformation and drug resistance, hence R2 inhibition has become a promising anticancer

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strategy [11]. GTI-2040 is a 20-mer phosphorothioate oligonucleotide complementary to the mRNA of the R2 subunit. It has shown activity against a broad spectrum of tumor cell lines and tumor xenografts [12]. At present, GTI-2040 is undergoing clinical evaluation in multiple phase II studies in combination with other chemotherapeutic agents. To support its clinical development, there is a need for sensitive and specific analytical methods for quantitating GTI-2040 plasma levels.

Here we report a simple and robust method of quantitating GTI-2040 using a combination of solid phase and liquid–liquid extractions and high-performance liquid chromatography with UV detection.

2. Experimental

2.1. Chemical and reagents

GTI-2040 (Lot# 0600101) was provided by Lorus Therapeutics Inc. (North York, Ont., Canada). The internal standard (IS), a phosphorothiate oligonucleotide composed of 27 thymidine nucleotide residues, was obtained from the Center for Applied Genomics at the Hospital for Sick Children (Toronto, Ont., Canada). HPLC grade methyl *t*-butyl ether (MTBE) was purchased from Fisher Scientific Inc. (Nepean, Ont., Canada). Methanol and acetonitrile were purchased from EM Science (Gibbstown, NJ, USA). Tetraethyl ammonium acetate tetra hydrate (TEAA), tetrabutyl ammonium hydrogen sulfate (TBAS), ethylenediaminetetraacetic acid disodium salt (EDTA), 3-cyclohexylamino-1-propane-sulfonic acid (CPS), tetrabutyl ammonium hydroxide (TBAH) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Purified deionized water was prepared with the Milli-Q Academic Quantum Purification System (Millipore, Milford, MA, USA). Human plasma used for the preparation of controls and standards was obtained from the Canadian Blood Services (Toronto, Ont., Canada).

2.2. Standard stock solutions

GTI-2040 stock solutions were prepared separately in duplication by dissolving 10.0 mg GTI-2040 in 10 ml of purified deionized water to a concentration of 1.0 mg/ml, and stored at −20 °C. The IS stock solution was prepared by dissolving 1.0 mg IS in 5.0 ml of purified deionized water to a concentration of 200 μg/ml, and stored at −20 °C.

Calibration standards were prepared by diluting stock solutions further with blank human plasma each day to obtain GTI-2040 concentrations of 0.20, 0.40, 1.0, 2.0, 4.0, 10.0, 12.0 μg/ml. The internal standard was further diluted with purified deionized water to a final concentration of 40 μg/ml. Calibration curves were computed by linear regression using ratios of peak areas of GTI-2040 to IS. Pools of quality control (QC) samples were prepared by mixing appropriate amounts of stock solutions and pooled blank human plasma to obtain GTI-2040 concentrations of 0.5, 2.0 and 10 μg/ml, and stored in batch at −20 °C for the duration of validation procedure.

2.3. Sample preparation

Plasma samples were thawed at ambient temperature. A 200 μl volume of plasma was transferred to a polypropylene tube (100 mm × 14 mm), and 20 μl of working IS solution was added and mixed for 30 s on a vortex-mixer. The mixture was mixed with 600 μl of CPS buffer (25 mM CPS adjusted to pH 10.75 with 2 mM EDTA) and 800 μl of TBAS buffer (100 mM TBAS adjusted to pH 10.75 with TBAH). The samples were loaded to Waters Oasis® HLB 1 ml (30 mg) extraction cartridge (Waters Co., Milford, MA, USA) conditioned with 2 ml of methanol and 2 ml of a mixture of CPS buffer and TBAS buffer (v/v, 9:1). The cartridges were washed with 3.0 ml of a mixture of CPS buffer and TBAS buffer (v/v, 9:1). Samples were eluted with 1.2 ml of acetonitrile: CPS buffer: water (v/v/v, 50:25:25). After addition of 1.5 ml MTBE to the elution tubes, these tubes were capped, shaken on a shaker (New Brunswick Scientific Corp., Eddison, NJ, USA) for 10 min and centrifuged at 704 × *g* for 10 min at ambient temperature. The organic layer was discarded and the aqueous layer were transferred to 1.5 ml polypropylene microtubes and evaporated at ambient temperature for 1 h using the Savant Universal Vacuum System (Thermo Electron Corp., Milford, MA, USA). After the sample microtubes were centrifuged at 704 × *g* for 30 min at ambient temperature, a volume of 100 μl was transferred to a 250 μl polypropylene auto-sample vial, and 50 μl was injected onto the HPLC system for quantitative analysis.

2.4. Chromatographic conditions

The chromatography system consisted of a LC-10ADvp liquid chromatography equipped with an SPD-10Avp UV detector (Shimadzu Corp., Kyoto, Japan). Separation of the analytes was achieved at ambient temperature using a Waters XTerra®MS C18 reversed-phase analytical column (50 mm × 4.6 mm, 3.5 μm particles) protected by a matching Waters C18 guard column. The detector was set at a wavelength of 260 nm. A gradient elution was applied. The binary mobile phase consisted of mobile phase A: 20 mM TBAS and 30% acetonitrile, pH 9.0 and mobile phase B: 20 mM TBAS and 70% acetonitrile, pH 9.0. The mobile phase was 60% A and 40% B for the first 3.5 min, it was changed gradually to 10% A and 90% B by 13.5 min and kept constant until 22.0 min, at which time point it was changed gradually to 60% A and 40% B by 23.0 min. The flow rate was 1.0 ml/min. Data were collected and analyzed using the Shimadzu Class VP software (Version 7.1.1 SP)

2.5. Method validation

Method validation runs were performed on nine consecutive days, and included calibration curve and QC samples at low, medium and high concentration. The accuracy or percent deviation (DEV) of the assay was calculated as:

$$\mathrm{DEV}(\%) = \left(\frac{\text{observed concentration}}{\text{nominal concentration}} - 1\right) \times 100 \qquad (1)$$

Estimates for the between-run precision (BRP) were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variance. The between-groups mean square (MSbet), the within-group mean square (MSwit) and the grand mean (GM) of the observed concentrations across runs were calculated using S-PLUS (Version, Insightful Corp., Seattle, WA, USA). The BRP was defined as:

$$\mathrm{BRP} = \sqrt{\frac{(\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n}{\mathrm{GM}}} \times 100 \quad (2)$$

where n represents the number of replicate samples within each run. The within-run precision (WRP) was calculated as:

$$\mathrm{WRP} = \sqrt{\frac{\mathrm{MS_{wit}}}{\mathrm{GM}}} \times 100 \quad (3)$$

To determine the extraction recovery of GTI-2040 and IS, at least four QC samples at each concentration were processed as described above. The recovery was determined by comparing areas obtained from QC samples with those spiked solutions of same concentrations, and expressed as a percentage.

The specificity of the method was evaluated using blank human plasma samples from six different donors. Chromatograms were visually inspected for the presence of endogenous interfering peaks. The stability of GTI-2040 in plasma was evaluated using QC samples following two full freeze-thaw cycles. The stability of sample solution as prepared above was evaluated at 6, 12, and 24 h under normal laboratory conditions. In addition, the stability of GTI-2040 standard solutions was evaluated after stored at 4 °C for 24 h, 1 week, and 1 month.

2.6. Optimization of SPE and chromatographic conditions

SPE extraction conditions were evaluated at the nominal concentration of 2.0 μg/ml for GTI-2040 and 4.0 μg/ml for IS, respectively. Recovery of the extraction was determined by comparing the absolute peak areas of processed samples with those of unprocessed spiked samples, expressed as a percentage. The processed samples were prepared by the extraction procedure described in 2.3 except for varying TBAS concentrations at 0, 5, 10, 25, 50, 100, and 150 μM; CPS buffer pH values at 4.5, 7.4, 9.0, 10.75, and 12.50; and the acetonitrile percentage of elution solution at 30, 40, 50, 60, and 70%, respectively.

2.7. Patient samples

Blood samples for steady-state GTI-2040 concentrations were collected at various time intervals from 13 patients participating in a phase I/II study of GTI-2040 combined with docetaxel in patients with advanced non-small cell lung cancer. GTI-2040 was administered as a continuous infusion for 14 days out of every 21 days. Docetaxel was administered intravenously over 60 min at either 60 or 75 mg/m^2 every 21 days. The first three patients were administered GTI-2040 at 3 mg/kg/day, while the rest of patients were treated at 5 mg/kg/day. The protocol was approved by the Ethics Review Boards of participating institutions, and patients gave written informed consent prior to treatment. The specimens were centrifuged at 704 × g for 15 min at room temperature. Plasma was separated and stored at −70 °C until analysis.

3. Results and discussion

3.1. Condition optimization

Early chromatographic methods for quantification of antisense oligonucleotides in biological matrices require a significant amount of sample handling including time-consuming protein digestion, phenol/chloroform extraction and ethanol precipitation [5,8]. Although solid-phase extraction is less time consuming, the recovery rate reported is low, partially due to the fact that a majority of antisense oligonucleotides are irreversibly bound to the reversed phase solid-phase extraction column cartridge [9] (Fig. 1).

To optimize GTI-2040 extraction from plasma samples, we studied the effect of pH and other conditions on GTI-2040 extraction recovery. GTI-2040 was first ionized by adjusting plasma samples to different pHs with CPS buffer. TBAS was then used to entrap GTI-2040 in cationic micelles to protect it from binding with the SPE column. As expected, GTI-2040 recovery was affected by the pH of CPS buffer used (Fig. 2). At pH < 7, GTI-2040 was minimally extracted due to non-ionization. By increasing CPS buffer pH to higher than 7, GTI-2040 was

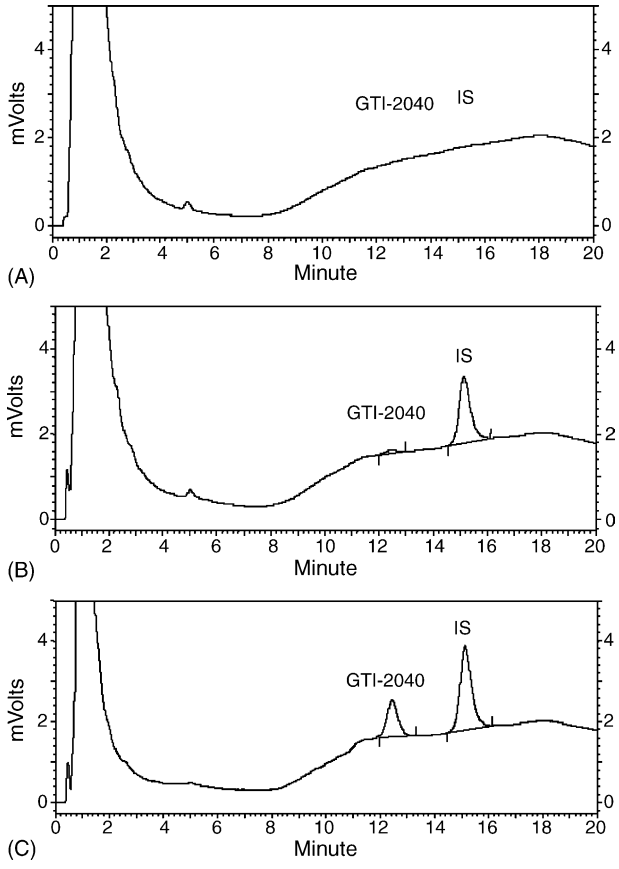

Fig. 1. Chromatograms of blank plasma (A), plasma spiked with GTI2-2040 at LLOQ (B) and a patient plasma sample (C).

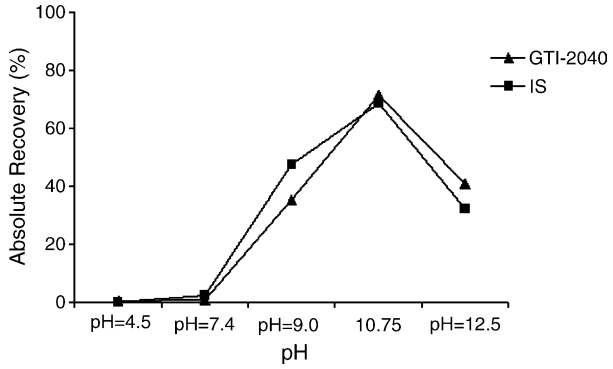

Fig. 2. Effect of CPS buffer pH on the recovery of GTI-2040 and IS.

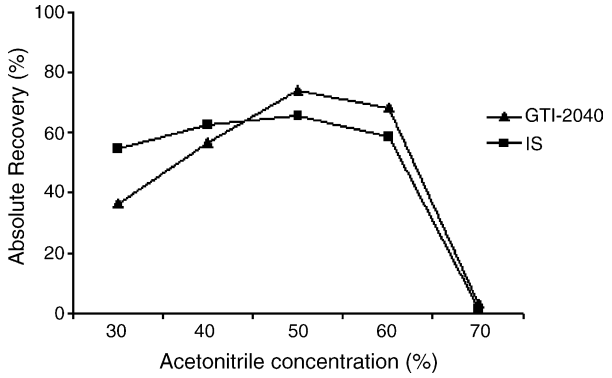

Fig. 4. Effect of acetonitrile concentration on the recovery of GTI-2040 and IS.

gradually ionized and extracted. The maximal recovery was obtained at a pH near 10.75. The recovery started to decrease at pHs greater than 10.75 due to degradation of GTI-2040, an observation consistent with results from studies examining other antisense oligonucleotides. As a result of the high pH required for extraction, general silica-based solid phase cartridges could not be used due to their limited usable pH ranges. The Waters Oasis®HLB sorbent is a macroporus copolymer with pH range of 1–14, making it ideally suitable for the current application.

The recovery of GTI-2040 was also affected by the concentration of the ion-pairing agent, TBAS, used (Fig. 3). The optimal recovery was achieved with TBAS concentration in the range of 50–150 mM. Lastly, GTI-2040 recovery was influenced by the concentration of acetonitrile in the SPE elution solution (Fig. 4). The elution solution with 50% acetonitrile appears to be optimal. Decreasing acetonitrile concentration weakens the eluting capability, while increasing acetonitrile concentration reduces the solubility of GTI-2040, both of which result in lower extraction recovery. As shown in Figs. 2–4, the effects of these conditions on the recovery of IS were similar to those on GTI-2040, confirming that it is a suitable internal standard of GTI-2040. Under these optimized conditions, the recovery of GTI-2040 after a one-step reversed phase SPE was higher than 85% (Table 1).

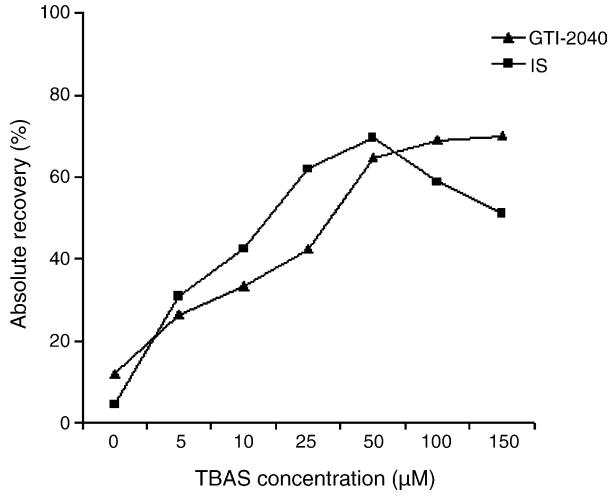

Fig. 3. Effect of TBAS concentration on the recovery of GTI-2040 and IS.

Eluting solutions were usually vacuum evaporated under heated conditions in previously reported analytical methods for quantitation of antisense oligonucleotides. However, GTI-2040 was found to be heat-liable during our study. To prevent and minimize such degradation, various organic solvents were used to concentrate samples. Of all organic solvents tested, MTBE gives the highest yield. Other reagents either caused GTI-2040 to be partially oxidated due to the presence of trace peroxides (e.g., diethyl ether) or caused tailing of the GTI-2040 peak due to the presence of trace organic acids (e.g., ethyl acetate). The extraction recovery of the combination of solid-phase extraction and MTBE liquid–liquid extraction ranged from 65.2 to 73.8% for GTI-2040.

In addition to TBAS, TEAA was investigated as an ion-pairing reagent. Both TEAA and TBAS showed the similar characteristics for improving the recovery of GTI-2040 extraction and its chromatographic behavior. However, there were endogenous interfering substances with TEAA.

3.2. Chromatography

The retention times of GTI-2040 and the internal standard were approximated 12.5 and 15 min, respectively. The total run time for each sample analysis was 30 min. Chromatograms of HPLC analysis of a blank human plasma sample, a sample of human plasma spiked with GTI-2040 at the lower limit of quantitation (LLOQ), and a patient sample were shown in Fig. 1. There were no interferences from endogenous substances.

In human plasma, calibration curves of GTI-2040 were linear over the range of 0.2–12 μg/ml. The mean slope and intercept of the calibration curves were 0.32 ± 0.02 and -0.02 ± 0.02,

Table 1
Validation characteristics of GTI-2040 in human plasma

Nominal concentration (μg/ml)	0.5 ($n \geq 4$)	2.0 ($n \geq 4$)	10 ($n \geq 4$)
Accuracy (%DEV)	9.80	−10.4	5.66
Precision (R.S.D.%)			
Intra	2.13	1.50	0.68
Inter	12.6	6.21	4.26
Extraction recovery (%) (SPE)	91.21	94.72	85.00
Extraction recovery (%) (SPE and MBTE extraction)	65.21	70.48	73.76

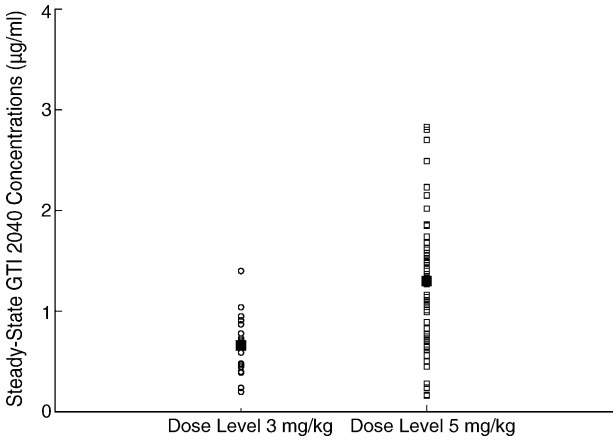

Fig. 5. Steady-state concentrations of GTI-2040.

respectively, with the mean r^2 being 0.9992 ± 0.0009. The LLOQ of GTI-2040 was determined to be 0.2 μg/ml. Accuracy and precision were 10.1 and 15.2% at LLOQ, and −0.55 and 2.27% at upper limit of quantitation (ULOQ) of 12.0 μg/ml, respectively. The between- and within–day precision values of QC samples at three different concentrations range from 0.68 to 12.7%. The accuracy of QC samples for GTI-2040 ranged from −10.4 to 9.8%. These values confirmed that this assay was reproducible and valid.

With the use of ion-pairing reagents such as TBAS and TEAA in the mobile phase to improve the selectivity and the peak symmetry, the optimal pH of the mobile phase was near 9. No pressure buildup was observed during the method development, validation and sample analysis.

There was no significant change for GTI-2040 in plasma through two full cycles of freeze-thaws and for GTI-2040 in sample solutions after 24 h under normal laboratory conditions. However, GTI-2040 standard solution was found to be degraded after one-week storage at 4 °C, therefore the standard solution was prepared daily and was kept from light.

Due to a lack of standards for GTI-2040 metabolites, the interference from these metabolites could not be adequately investigated in this study. Some chromatograms of patient samples showed small peaks near the GTI-2040 peak. However, the effect of these small peaks was estimated to be less than 10%.

3.3. Pharmacokinetic analysis

The method was used to measure steady-state GTI-2040 concentrations in 13 patients who had completed cycle 1 of treatment. The mean steady-state concentration of GTI-2040 was shown in Fig. 5 for the two dose levels studied. Steady-state concentrations are similar to those seen in a previous phase I study of GTI-2040 in patients with advanced solid tumors [1].

4. Conclusion

In conclusion, the method presented here for quantitation of GTI-2040 is robust and sensitive. By varying experimental conditions, the recovery of GTI-2040 from a one-study SPE was optimized, and the recovery was significantly improved. The LLOQ of the current method is similar to that of a previous reported capillary electrophoresis method. However, simplified sample handling in the current method makes analysis considerably less time consuming than capillary electrophoresis. In addition, this method has been successfully applied in measuring plasma samples from patients treated with GTI-2040. Finally, as more antisense oligonucleotides are developed as therapeutic agents, our results should contribute to development of efficient and reproducible analytical methods for quantitation of antisense compounds in clinical samples.

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References

[1] A.A. Desai, R.L. Schilsky, A. Young, L. Janisch, W.M. Stadler, N.J. Vogelzang, S. Cadden, J.A. Wright, M.J. Ratain, Ann. Oncol. (2005).
[2] S. Rao, D. Watkins, D. Cunningham, D. Dunlop, P. Johnson, P. Selby, B.W. Hancock, C. Fegan, D. Culligan, S. Schey, T.C.M. Morris, T. Lissitchkov, J.W. Oliver, J.T. Holmlund, Ann. Oncol. 15 (2004) 1413.
[3] J. Marshall, H. Chen, D. Yang, M. Figueira, K.B. Bouker, Y. Ling, M. Lippman, S.R. Frankel, D.F. Hayes, Ann. Oncol. 15 (2004) 1274.
[4] D.J. Stewart, R.C. Donehower, E.A. Eisenhauer, N. Wainman, A.K. Shah, C. Bonfils, A.R. MacLeod, J.M. Besterman, G.K. Reid, Ann. Oncol. 14 (2003) 766.
[5] A.J. Bourque, A.S. Cohen, J. Chromatogr. B Biomed. Appl. 662 (1994) 343.
[6] J.L. Johnson, W. Guo, J. Zang, S. Khan, S.A. Bardin, A. Ahmad, J.X. Duggan, I. Ahmad, Biomed. Chromatogr. 19 (2004) 272.
[7] R.Z. Yu, R.S. Geary, A.A. Levin, Curr. Opin. Drug Discov. Devel. 7 (2004) 195.
[8] A.J. Bourque, A.S. Cohen, J. Chromatogr. 617 (1993) 43.
[9] J.M. Leeds, M.J. Graham, L. Truong, L.L. Cummins, Anal. Biochem. 235 (1996) 36.
[10] M. Kolberg, K.R. Strand, P. Graff, K.K. Andersson, Biochim. Biophys. Acta 1699 (2004) 1.
[11] H. Fan, C. Villegas, A. Huang, J.A. Wright, Cancer Res. 58 (1998) 1650.
[12] Y. Lee, A. Vassilakos, N. Feng, V. Lam, H. Xie, M. Wang, H. Jin, K. Xiong, C. Liu, J. Wright, A. Young, Cancer Res. 63 (2003) 2802.