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Journal of Chromatography B, 805 (2004) 135–140
JOURNAL OF CHROMATOGRAPHY B
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Determination of SU5416, a novel angiogenesis inhibitor, in human plasma by liquid chromatography

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Received 3 November 2003; received in revised form 5 February 2004; accepted 23 February 2004

Abstract

A high-performance liquid chromatographic (HPLC) assay with UV detection has been developed for the quantitative determination of the antiangiogenic agent SU5416 in human plasma. Sample pretreatment involved a single protein-precipitation step with acetonitrile containing the internal standard, chrysin. Separation of the compounds of interest was achieved on a column packed with HP Zorbax C_8 material (5 μm particle size; length: 150 mm; i.d.: 4.6 mm) using a dual solvent system of 0.01 M aqueous ammonium acetate and acetonitrile delivered as a nonlinear gradient at a flow-rate of 1.00 ml/min. Simultaneous UV detection was performed at 440 nm (SU5416) and 268 nm (chrysin). The calibration graph was fit to log-transformed response-concentration data over a range of 10–5000 ng/ml. Values for accuracy and precision, obtained from six quality controls analyzed on different days in replicates of 3 or 6, ranged 92.9–109 and 0.8–6.2%, respectively. The developed method was successfully applied to study the pharmacokinetics of SU5416 in a cancer patient receiving the drug as a 1 h infusion.

Keywords: SU5416; Angiogenesis inhibitor

1. Introduction

Angiogenesis is required for tumor growth and metastasis, and is characterized by excessive neovascularization. Hence, it represents a potential target for the treatment of cancer [1]. A wealth of data accumulated during the past 10 years indicates that vascular endothelial growth factor (VEGF)-mediated angiogenesis is a key process in the growth of malignant tumors. The biological effects of VEGF are mediated by two distinct receptors, VEGF receptors (VEGFR) 1 and 2, whose expression are largely limited to the vascular endothelium and which are often over-expressed in tumors. Several different strategies have been used to inhibit VEGF, including treatment with anti-VEGF antibodies (e.g., bevacizumab) and inhibition of the cognate tyrosine kinase insert domain-containing receptor [1]. Various small molecule kinase-domain containing receptor kinase inhibitors have progressed to the clinical testing stage. One of these, SU5416 (*Z*-3-[(2,4-dimethylpyrrol-5-yl)-methylidenyl]-2-idolinone; Fig. 1A), is a lipophilic, highly protein bound agent, that not only inhibits VEGFR-1 and VEGFR-2 [2], but also c-kit [3] and both wild-type and mutant FLT-3 [4], thereby blocking several intracellular signaling pathways and affecting angiogenesis [5]. Phase II clinical trials of SU5416 suggest that the drug might be an effective treatment option either alone or in combination with standard therapies in a number of malignant diseases, including renal cell carcinoma [6] and acute myeloid leukemia [7–9].

There is large inter- and intra-subject variation in the peak concentration of SU5416 and in the area under the curve (AUC) after the same dose administered to cancer patients [10–12], which presumably relates to inter-individual variability in the expression of Phase I and II enzymes involved in SU5416 elimination [13]. In support of further clinical pharmacologic studies with SU5416, we describe here the development and validation of a specific, sensitive, accurate, and precise analytical method for the determination of SU5416 in human plasma samples. The assay method requires only a single-step protein precipitation step with

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doi:10.1016/j.jchromb.2004.02.036

acetonitrile, followed by high-performance liquid chromatographic (HPLC) assay with UV detection, and can be implemented easily for routine analysis.

2. Experimental

2.1. Chemicals and reagents

SU5416 was provided by Sugen, Inc. (South San Francisco, CA, USA) through the Cancer Therapy Evaluation Program of the National Cancer Institute (Bethesda, MD, USA). HPLC-grade acetonitrile, methanol and the internal standard, chrysin (Fig. 1B) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium acetate was purchased from Mallinckrodt (Paris, KY, USA). De-ionized water was obtained from a Milli-Q-UV Plus water system (Millipore, Milford, MA, USA). Pooled blank plasma was obtained from the Blood Bank at the Warren Grant Magnuson Clinical Center (Bethesda, MD, USA).

2.2. Chromatography

A Hewlett Packard 1100 HP Series (Agilent, Wilmington, DE, USA) was utilized, which included a HP 1100 series pump, vacuum degasser, autosampler, thermostat and photodiode array detector. Signal acquisition and peak integration were performed on a HP Intel Pentium III using the Chemstation software (Agilent). A HP Zorbax C_8 reverse-phase column (5 μm particle size; length: 150 mm; i.d.: 4.6 mm) protected by a C_{18} guard column, both from Waters Corp. (Milford, MA, USA), was used for analysis. The mobile phase consisted of 0.01 M aqueous ammonium acetate and acetonitrile with a gradient from 70% 0.01 M aqueous ammonium acetate to 90% acetonitrile at a flow-rate of 1.00 ml/min (Table 1). Absorbance was measured at 440 nm for SU5416 and 268 nm for the internal standard. Ratios of the peak area of SU5416 to the internal standard versus nominal concentrations of the standard were used to construct the calibration curve for quantitative computations.

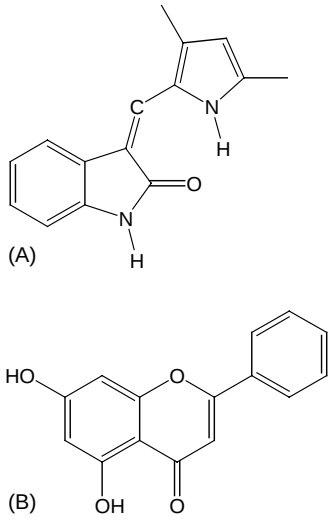

Fig. 1. Chemical structures of SU5416 (A) and the internal standard, chrysin (B).

2.3. Preparation of standard pools and quality controls

Stock solution A was prepared by accurately weighing 10 mg of SU5416, and dissolving in 10 ml of absolute methanol yielding a (bright yellow) 1 mg/ml solution. Working solutions B (400 μg/ml), C (100 μg/ml), D (40 μg/ml), E (10 μg/ml), and F (4 μg/ml) were prepared by serial dilutions of the stock solution A using absolute methanol. Spiked plasma samples used for the calibration standards were prepared by addition of the appropriate volume of working standard solutions (A, B, C, D, E or F) to 10 ml of pooled blank plasma. SU5416 plasma standard concentrations used for generating the calibration curves were 10, 25, 50, 100, 250, 500, 1000, 2500, and 5000 ng/ml. Quality control samples of SU5416 were prepared at concentrations of 30, 75, 200, 400, 2000, and 4000 ng/ml. Portions of prepared standards and quality controls were stored at −70 °C. Separate stock solutions of A were used to prepare the standards and the quality controls.

2.4. Sample preparation

All frozen samples were allowed to thaw at room temperature and homogenized by vortex-mixing. Acetonitrile containing the internal standard at a concentration of 500 ng/ml was used directly as a protein precipitating solvent. Two hundred and fifty microliters of spiked sample standards, quality control samples or unknown plasma samples were placed into a snap-cap centrifuge tube, and deproteinized with 1 ml of acetonitrile by vigorous vortex-mixing for 30 s, followed by centrifugation at 10,000 rpm at 4 °C for 5 min. The supernatant was placed into a disposable glass tube and evaporated to dryness under a gentle stream of air at 40 °C. The dried residue was reconstituted in 200 μl of a mixture of 0.01 M aqueous ammonium acetate and acetonitrile (70:30 (v/v)), vortex-mixed for 20 s and then transferred to limited-volume injection vials. To minimize the isomerization of SU5416, sample preparation was done under light-protected

Table 1
Gradient profile

Step	Time (min)	Duration (min)	Flow rate (ml/min)	Solvent A[a] (%)	Solvent B[b] (%)
1	0.00	0.00	1.00	70	30
2	4.00	4.00	1.00	70	30
3	4.10	0.10	1.00	10	90
4	7.00	3.00	1.00	10	90
5	14.0	7.00	1.00	10	90

[a] 0.01 M aqueous ammonium acetate.
[b] Acetonitrile.

conditions, and HPLC vials were placed in the vial chamber protected from light for 18 h before injection.

2.5. Validation

The assay performance was assessed in terms of linearity, accuracy, precision, sensitivity, specificity, and freeze–thaw stability. On each validation day, calibration curves were analyzed in duplicate along with quality control samples. The procedure was performed on 3 (75, 200, 400, and 2000 ng/ml) or 6 different days (30 and 4000 ng/ml) using six replicate determinations for each concentration on each day.

2.5.1. Response function

Calibration curves were constructed by least-squares linear regression analysis of peak area ratios of SU5416 to the internal standard versus the SU5416 concentration of the nominal standards with and without weighting, and by using various log-transformed nonlinear power-fit models. To establish the optimal quantification method (with internal standard or without) and weight factor, the correlation coefficient, and accuracy and precision of back-calculated calibration concentrations were taken into consideration.

2.5.2. Accuracy and precision

Accuracy and precision were determined by analyzing quality control samples with SU5416 concentrations in the low, mid and high concentration ranges of the calibration curves. Accuracy (RE%) was defined as percent difference between the observed concentration and the nominal concentration:

$$\mathrm{RE\%} = \frac{[\text{observed}] - [\text{nominal}]}{[\text{nominal}]} \times 100\%$$

The precision of the assay was assessed by the between-run and within-run precision. Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across run days were calculated using the NCSS 2001 package (J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT, USA). The between-run precision (BRP) was defined as

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

where n represents the number of replicates within each validation run. The within-run precision (WRP) was calculated as

$$\mathrm{WRP} = \frac{\sqrt{\mathrm{MS_{wit}}}}{\mathrm{GM}} \times 100\%$$

2.5.3. Lower limit of quantification

The lower limit of quantitation was defined as the lowest concentration of SU5416 that could be reliably and reproducibly measured with values for accuracy, and precision of less than 20%, with concentration determinations performed in replicates of at least 6.

2.5.4. Specificity

Pooled blank samples were used to determine whether endogenous matrix constituents co-eluted with SU5416 or the internal standard. Blank samples were also obtained from 12 different patients prior to SU5416 administration, and were analyzed to determine if other drugs taken by these patients eluted around the retention times of SU5416 or the internal standard.

2.5.5. Freeze–thaw stability

The stability of SU5416 in plasma subjected to three consecutive freeze–thaw cycles was tested by triplicate analysis of quality control samples containing 30 ng/ml, 200 ng/ml, or 4000 ng/ml. The calculated SU5416 concentrations were compared to control portions that were assayed immediately.

2.6. Pharmacokinetic analysis

The patient studied was a female with a histologically confirmed diagnosis of advanced cancer, who received chemotherapeutic treatment with single-agent SU5416 at a twice-weekly dose of 110 mg/m^2 (Sugen). The drug was administered as a 1 h intravenous infusion. The current experiment was approved by the National Cancer Institute Institutional Review Board, and the patient signed informed consent before study entry.

A total of eight blood samples (7 ml each) were obtained and collected in 10 ml glass tubes containing heparin as an anticoagulant. These samples were obtained before the first dose of SU5416 was administered, and then again at the following time points: 30 min after the start of infusion; immediately before the end of infusion (∼1 h); and at 15 and 30 min, and 1, 2, 3, 4, 6, 8, and 24 h after the end of infusion. Specimens were immediately centrifuged at 3000 rpm for 5 min to separate the plasma, which was then stored at −70 °C. Plasma concentration-time data of SU5416 were analyzed by noncompartmental methods using the software package WinNonlin Version 4.0 (Pharsight Corporation, Mountain View, CA).

3. Results and discussion

3.1. Chromatography and detection

Typical chromatograms resulting from the HPLC–UV analysis of extracts of 250 μL plasma from a pre-dose control blank sample (A), a control plasma sample spiked to contain SU5416 at a concentration of 500 ng/ml (B), and a

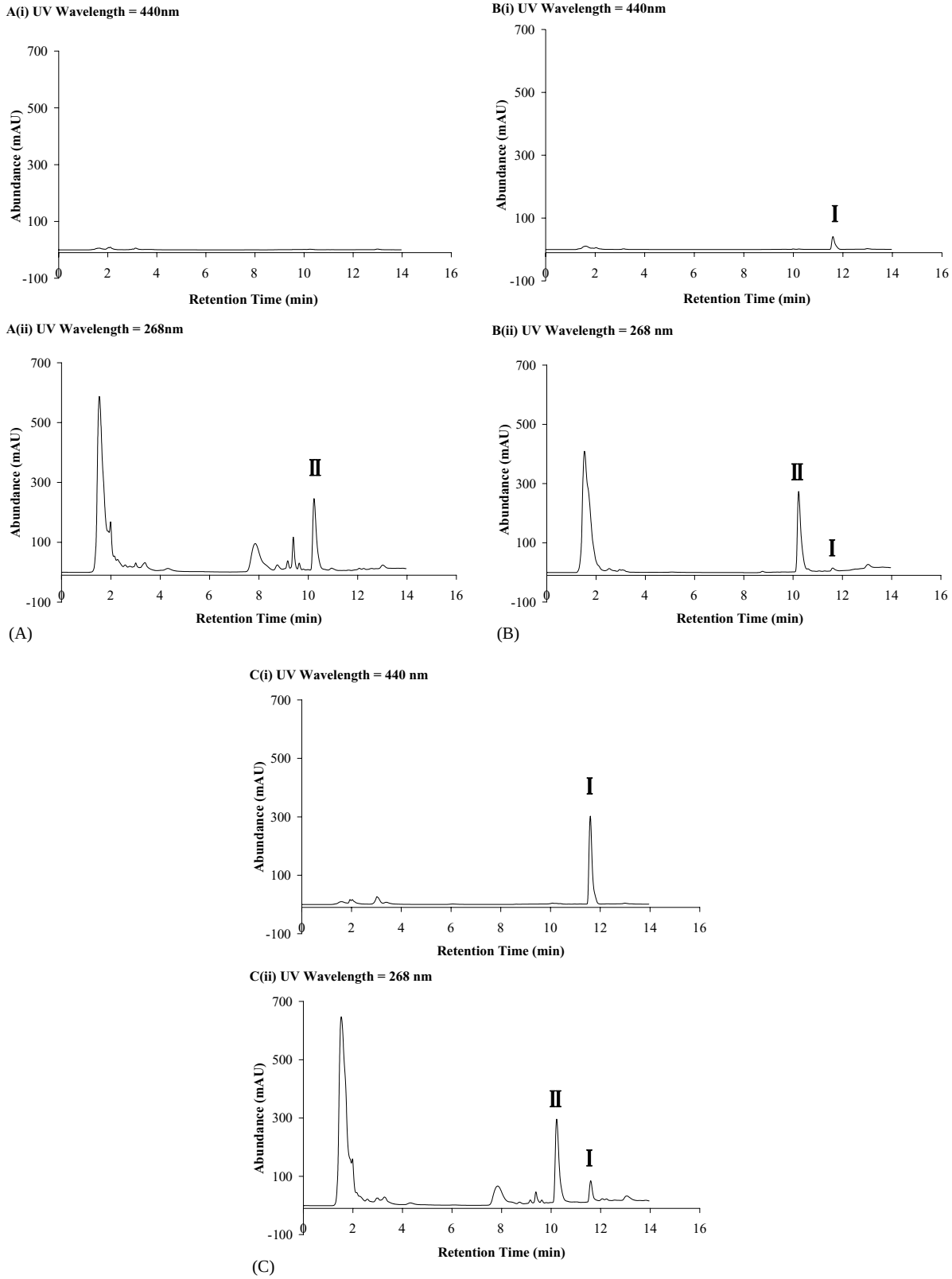

Fig. 2. Typical reverse-phase HPLC–UV chromatograms of a blank human plasma sample (A), a sample spiked with SU5416 at a concentration of 500 ng/ml (B), and a plasma sample obtained from a patient with cancer at the end of a 1 h intravenous infusion of SU5416 (dose, 110 mg/m^2) (C). The top and bottom chromatograms were obtained using the optimal UV absorption wavelengths for SU5416 (440 nm) and the internal standard chrysin (268 nm), respectively. The labeled chromatographic peaks indicate SU5416 (I), and the internal standard chrysin (II), respectively.

Table 2
Back calculated concentrations from calibration curves

Nominal	GM (ng/ml)	S.D. (ng/ml)	RE (%)	R.S.D. (%)	*n*
10	10.6	0.811	6.0	7.7	12
25	24.2	0.585	−3.2	2.4	12
50	47.8	1.11	−4.4	2.3	12
100	101	2.19	0.9	2.2	12
250	247	3.42	−1.3	1.4	12
500	502	9.59	0.4	1.9	12
1000	1005	11.1	0.5	1.1	12
2500	2589	30.2	3.6	1.2	12
5000	4930	132	−1.4	2.7	12

Abbreviations: GM, grand mean; S.D., standard deviation; RE, relative error from nominal value; R.S.D., relative standard deviation; *n*, number of replicate observations within each validation run (i.e., 2 samples at each concentration were run on six separate occasions, for a total of 12 samples at each concentration).

Table 3
Assessment of accuracy and precision from quality-control samples

Nominal	GM (ng/ml)	S.D. (ng/ml)	RE (%)	WRP (%)	BRP (%)	*n*
30	30.7	2.29	+2.4	3.58	6.20	6
75	69.6	1.29	−7.2	1.56	1.35	3
200	208	4.31	+4.0	2.17	[a]	6
400	372	4.47	−7.1	0.796	1.22	3
2000	2103	62.2	+5.1	3.17	[a]	3
4000	4378	170	+9.5	1.04	3.54	6

Abbreviations: GM, grand mean; S.D., standard deviation; RE, relative error from nominal value; WRP, within-run precision; BRP, between-run precision; *n*, number of replicate observations within each validation run.
[a] No additional variation was observed as a result of performing the assay on different days.

sample from a patient obtained 1 h after intravenous administration of SU5416 (C) are displayed in Fig. 2. SU5416 and the internal standard peaks were well resolved under the optimized conditions. The retention times of SU5416 and the internal standard chrysin were 11.6 and 10.2 min, respectively. The UV wavelengths used for optimal detection of SU5416 and chrysin were 440 and 268 nm, respectively. The total separation time was determined to be 14 min.

3.2. Validation

The lowest and most constant bias across the concentration range investigated were obtained following regression analysis of the data to a power fit with log-transformed data for the peak area ratio of SU5416 to chrysin and a log-transformed nominal drug concentration (data not shown). For each analytical run, a nine-point plasma standard curve was constructed, and was shown to be linear over the tested range of 10–5000 ng/ml. The mean (±standard deviation) regression equation obtained during the method validation, obtained in duplicates on six separate occasions, showed an intercept of 0.989 ± 0.00747 and an *x*-coefficient of -3.156 ± 0.0417 ($r^2 = 0.9996 \pm 0.00048$; $n = 12$).

Using this procedure, the lower limit of quantitation was determined to be 10 ng/ml, with a precision of 7.65% and a RE% from the nominal standard of +5.97%. Preliminary pharmacokinetic analysis of clinical samples indicated that plasma concentrations of SU5416 were always lower than 5000 ng/ml, and hence the upper level of the curve was set at this concentration. Over the entire concentration range of the standard curve, the mean observed percent deviation was between −4.4 and +6.0%, at a precision of less than 7.7% (Table 2).

The assay performance data for the determination of independent quality control samples of SU5416 in plasma are presented in Table 3. The between-run precision and within-run precision ranged from 1.22 to 6.20 and 0.796 to 3.58%, respectively, for the various concentrations tested. At the same concentrations, the values for accuracy were always between −7.2 and +9.5%, which is well within the guidelines of the US Food and Drug Administration (i.e., precision ≤ 15%, and accuracy ≤ 15%) for bioanalytical method validation.

Pooled blank plasma from different individuals showed no interfering endogenous compounds. In addition, pretreatment plasma obtained from patients receiving SU5416 also did not show any chromatographic peaks around the retention times of SU5416 and/or the internal standard.

Three repeated freeze–thaw cycles had no apparent influence on the stability of plasma samples containing 30 ng/ml, 200 ng/ml or 4000 ng/ml. After the third freeze–thaw cycle, SU5416 plasma concentrations had deviations from the nominal values within the range of −3.5 and +4.4%, irrespective of the tested plasma concentrations as determined by a nonparametric Kruskal–Wallis test ($P > 0.05$). Processed plasma samples were found to be stable at room temperature upon standing in the autosampler tray for at least 18 h, allowing for overnight analysis of extracted samples.

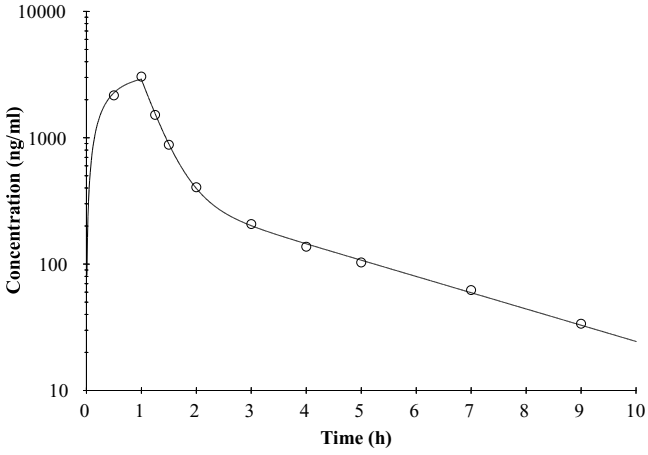

Fig. 3. Plasma concentration-time profile of SU5416 following a 1 h intravenous infusion of the drug at a dose of 110 mg/m^2 to a patient with cancer.

3.3. Application of the method

The developed procedure was applied to samples obtained from a single cancer patient treated with a SU5416 administered as a 1 h intravenous infusion. The resulting concentration-time profile of SU5416 is shown in Fig. 3. The peak concentration of SU5416 was 3050 ng/ml, and the area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was 4168 μg·h/ml, which is similar to values previously reported in an adult population [10].

4. Conclusion

In conclusion, a simple and rapid assay method was developed and validated for the determination of SU5416 in human plasma. The performance criteria for sensitivity, accuracy, precision, linearity, stability, and specificity were acceptable, indicating that the method can be used for determination of SU5416 in plasma samples obtained from patients treated with the drug. The method is currently being used to study the pharmacokinetic profile of SU5416 in patients diagnosed with recurrent high-grade astrocytoma or mixed glioma treated on a North American Brain Tumor Consortium (NABTC) trial.

Acknowledgements

This work was funded in part by the North American Brain Tumor Consortium (NABTC) Grant Number CA62399.

References

[1] L.S. Rosen, Cancer Control 9 (2002) 36.
[2] Y. Kasahara, R.M. Tuder, L. Taraseviciene-Stewart, T.D. Le Cras, S. Abman, P.K. Hirth, J. Waltenberger, N.F. Voelkel, J. Clin. Invest. 106 (2000) 1311.
[3] G.W. Krystal, S. Honsawek, D. Kiewlich, C. Liang, S. Vasile, L. Sun, G. McMahon, K.E. Lipson, Cancer Res. 61 (2001) 3660.
[4] T. Itokawa, H. Nokihara, Y. Nishioka, S. Sone, Y. Iwamoto, Y. Yamada, J. Cherrington, G. McMahon, M. Shibuya, M. Kuwano, M. Ono, Mol. Cancer Ther. 1 (2002) 295.
[5] D.B. Mendel, A.D. Laird, B.D. Smolich, R.A. Blake, C. Liang, A.L. Hannah, R.M. Shaheen, L.M. Ellis, S. Weitman, L.K. Shawver, J.M. Cherrington, Anticancer Drug Des. 15 (2000) 29.
[6] B.C. Kuenen, M. Levi, J.C. Meijers, A.K. Kakkar, V.W. van Hinsbergh, P.J. Kostense, H.M. Pinedo, K. Hoekman, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 1500.
[7] R.M. Mesters, T. Padro, R. Bieker, M. Steins, M. Kreuter, M. Goner, S. Kelsey, P. Scigalla, W. Fiedler, T. Buchner, W.E. Berdel, Blood 98 (2001) 241.
[8] F.J. Giles, A.T. Stopeck, L.R. Silverman, J.E. Lancet, M.A. Cooper, A.L. Hannah, J.M. Cherrington, A.M. O'Farrell, H.A. Yuen, S.G. Louie, W. Hong, J.E. Cortes, S. Verstovsek, M. Albitar, S.M. O'Brien, H.M. Kantarjian, J.E. Karp, Blood 102 (2003) 795.
[9] W. Fiedler, P. Staib, R. Kuse, U. Duhrsen, M. Flasshove, F. Cavalli, D.K. Hossfeld, W.E. Berdel, Cancer J. 7 (Suppl. 3) (2001) S129.
[10] A. Stopeck, M. Sheldon, M. Vahedian, G. Cropp, R. Gosalia, A. Hannah, Clin. Cancer Res. 8 (2002) 2798.
[11] B.C. Kuenen, L. Rosen, E.F. Smit, M.R. Parson, M. Levi, R. Ruijter, H. Huisman, M.A. Kedde, P. Noordhuis, W.J. van der Vijgh, G.J. Peters, G.F. Cropp, P. Scigalla, K. Hoekman, H.M. Pinedo, G. Giaccone, J. Clin. Oncol. 20 (2002) 1657.
[12] D.B. Mendel, R.E. Schreck, D.C. West, G. Li, L.M. Strawn, S.S. Tanciongco, S. Vasile, L.K. Shawver, J.M. Cherrington, Clin. Cancer Res. 6 (2000) 4848.
[13] L. Antonian, H. Zhang, C. Yang, G. Wagner, L.K. Shawver, M. Shet, B. Ogilvie, A. Madan, A. Parkinson, Drug Metab. Dispos. 28 (2000) 1505.