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Determination of CC-5013, an analogue of thalidomide, in human plasma by liquid chromatography–mass spectrometry

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Abstract

A high-performance liquid chromatographic assay with MS detection has been developed for the quantitative determination of the anti-angiogenic agent CC-5013 in human plasma. Sample pretreatment involved liquid–liquid extraction with acetonitrile/1-chlorobutane (4:1, v/v) solution containing the internal standard, umbelliferone. Separation of the compounds of interest was achieved on a column packed with Waters C_{18} Nova-Pak material (4 μm particle size; 300 mm × 3.9 mm internal diameter) using acetonitrile, de-ionized water, and glacial acetic acid in ratios of 20:80:0.1 (v/v/v) (pH 3.5) delivered at an isocratic flow rate of 1.00 ml/min. Simultaneous MS detection was performed at *m*/*z* 260.3 (CC-5013) and *m*/*z* 163.1 (umbelliferone). The calibration curve was fit to a linear response–concentration data over a range of 5–1000 ng/ml using a weighting factor of 1/*x*. Values for accuracy and precision, obtained from four quality controls analyzed on three different days in replicates of five, ranged from 98 to 106% and from 5.5 to 15.5%, respectively. The method was successfully applied to study the pharmacokinetics of CC-5013 in a cancer patient receiving the drug as single daily dose.

Keywords: CC-5013; LC–MS; Pharmacokinetics; Angiogenesis inhibitor

1. Introduction

Angiogenesis is the development of new blood vessels from pre-existing vessels [1]. It is involved not only in normal physiological processes [2] but also in pathological conditions such as tumor growth and metastases. The sequence of events that occur during angiogenesis in response to pro-angiogenic factors include degradation of the extracellular matrix, endothelial cell migration and proliferation, alignment and fusion of newly formed vessels, and blood flow initiation [3,4]. These distinct stages are all potential targets for anticancer treatment. In light of this, previous studies have suggested that the inhibition of angiogenesis at any one of these stages may be an effective strategy in the treatment of various human malignancies [5].

Thalidomide has been shown to be effective in a variety of tumor types [6], and it was found that thalidomide inhibits tumor growth at least in part by anti-angiogenic mechanisms. More recently, analogues of thalidomide have been developed that are more potent inhibitors of angiogenesis [7,8]. CC-5013 [α-(3-aminophthalimido) glutarimide; lenalidomide], an immunomodulatory thalidomide analogue, has demonstrated higher potency than thalidomide in the HUVEC (human umbilical vein endothelial cells) proliferation and tube formation assays (unpublished data). These assays also indicated a dose-dependent decrease in HUVEC proliferation and tube formation with increasing concentration of

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CC-5013. Other in vitro studies have indicated anti-migratory effects of CC-5013 and the inhibition of tumor growth in vivo [9]. Immodulatory properties of CC-5013 include the inhibition of cytokines such as VEGF, bFGF, IL-6 and TNF-α. These cytokines are key regulators of tumor growth and angiogenesis [10]. Phase I and II clinical studies indicate that CC-5013 does not exhibit sedation as a major side effect, but rather results in occasional neurotoxicity and myelosuppression. In a recent phase III study, the use of CC-5013 for advanced and refractory multiple myeloma has been associated with significant biological responses in M-protein [11]. In support of further clinical pharmacologic studies with CC-5013, we describe here the development and validation of a specific, accurate, precise, and sensitive analytical method for the determination of CC-5013 in human plasma.

2. Experimental

2.1. Chemicals and reagents

CC-5013 was provided by Celgene Corporation (Warren, NJ, USA). HPLC-grade acetonitrile, 1-chlorobutane, dimethyl sulfoxide (DMSO) and the internal standard, umbelliferone (Fig. 1B) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Glacial acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ, 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 NIH's Warren Grant Magnuson Clinical Center (Bethesda, MD, USA) and centrifuged at 2400 rpm for 10 min to remove fibrous material prior to use.

2.2. Liquid chromatography–mass spectrometry

A HP 1100 LC–MS (Agilent Technology, Palo Alto, CA, USA) was utilized for analysis, which included a HP 1100 G1312A binary pump, vacuum degasser, G1329A injection temperature controlled column compartment, and a HP 1100 MSD single quadrupole mass spectrometer equipped with an electrospray source. Signal acquisition and peak integration were performed on an HP Intel Pentium III using the Chemstation software (Agilent). A Waters C_{18} Nova-Pak reverse-phase analytical column (4 μm particle size; 300 mm × 3.9 mm internal diameter) from Waters Corp. (Milford, MA, USA), was used for analyte separation. The mobile phase consisted of HPLC-grade acetonitrile, de-ionized water, and glacial acetic acid in ratios of 20:80:0.1 (v/v/v) (pH 3.5). The mobile phase was delivered isocratically at a flow rate of 1 ml/min. The analytical column was kept at ambient temperature. The sample injection volume was 80 μl. Total sample run time was 8 min. The effluent was interfaced to the ESI-MS system without splitting. The MS system was operated in the positive ion mode and the conditions were optimized to generate maximum analyte signal. The MS conditions were as follows: drying gas flow 13 l/min, nebulizer pressure 55 psi, drying gas temperature 350 °C, capillary voltage 3000 V, fragmentor 70 V, and multiplier gain 1. Nitrogen was used as a nebulizing and drying gas. Selected ion monitoring was accomplished at *m/z* 260.3 for CC-5013 and *m/z* 163.1 for the internal standard, umbelliferone, as protonated molecular ions. Both ions had a dwell time of 289 ms and were monitored in the high-resolution mode.

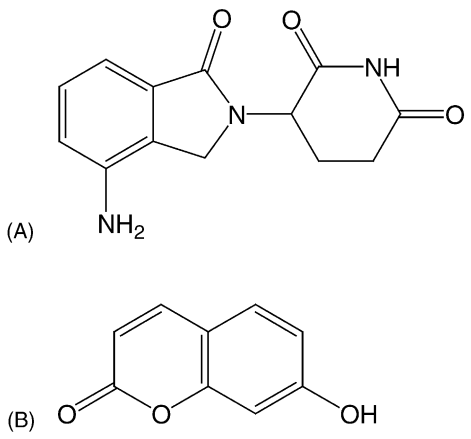

Fig. 1. Chemical structures of CC-5013 (A) and the internal standard, umbelliferone (B).

2.3. Standards preparation

Stock solution A was prepared by accurately weighing 10 mg of CC-5013, and dissolving in 10 ml of DMSO, to yield a concentration of 1 mg/ml solution. Working standard solutions B (50 μg/ml), C (10 μg/ml), and D (2 μg/ml) were prepared by serial dilutions of the stock solution A using HPLC-grade acetonitrile. For each analytical run, spiked plasma samples used for the calibration standards were prepared in duplicate by addition of the appropriate volume of working standard solutions (A, B, C, or D) to pooled blank plasma. CC-5013 standard concentrations used for generating the calibration curves were 5, 10, 25, 50, 100, 250, 500, and 1000 ng/ml. Quality control (QC) samples of CC-5013 were prepared at the following concentrations; three times the lower limit of quantitation (LLOQ) (the low-level QC at 15 ng/ml), at 15% of the highest point of the standard curve (150 ng/ml), at 40% of the highest point of the standard curve (mid-level QC at 400 ng/ml) and at the 80% of the highest point of the standard (high-level QC at 800 ng/ml). Aliquots of prepared QC samples were stored at −80 °C until analysis. A working internal standard solution was prepared by accurately weighing 10 mg of umbelliferone, and dissolving in 10 ml of DMSO, to yield a concentration of 1 mg/ml. A volume of 125 μl of umbelliferone (1 mg/ml) solution was placed into 1000 ml of a mixture of acetonitrile/1-chlorobutane (4:1, v/v). This acetonitrile/1-chlorobutane (4:1, v/v) solution containing 125 ng/ml of

umbelliferone was used for the liquid–liquid extraction process.

2.4. Sample preparation

All frozen standards and samples were allowed to thaw at room temperature and homogenized by vortex-mixing. Aliquots of 600 μl of spiked sample standards, QC samples or unknown plasma samples were placed into a plastic 15-ml Cellstar tube, to which 2.4 ml of acetonitrile/1-chlorobutane solution were added containing internal standard. This mixture was vortex-mixed for 1 min, and centrifuged for 10 min at 2400 rpm. The organic layer was transferred to a glass tube and evaporated to dryness under a gentle stream of air at 40 °C. The dried residue was reconstituted in 125 μl of mobile phase, vortex-mixed for 20 s, and then transferred to micro-centrifuge tubes in which they were centrifuged for 10 min at 13,000 rpm. Finally, the supernatant was transferred to limited-volume injection vials, and 80 μl were injected into the LC–MS system.

2.5. Validation

2.5.1. Validation procedure

The method was assessed in terms of specificity, accuracy, precision, recovery, sensitivity, linearity of detector response and stability. On each validation day, calibration curves were analyzed simultaneously with QC samples containing CC-5013. The procedure was performed on three different days using six replicates per standard concentration (5, 10, 25, 50, 100, 250, 500, and 1000 ng/ml) and five replicates per QC concentration on each day.

2.5.2. Specificity

Twenty-six blank samples from different subjects who were on other commonly used drugs were analyzed for chromatographic interference. Possible matrix effects were investigated by infusing a 2 μg/ml solution of CC-5013 (in mobile phase) into the MS post-column, using a syringe pump via a tee. After a constant response was established, six different blank plasma samples were extracted and reconstituted, as detailed in Section 2.4, and were injected into the LC–MS system.

2.5.3. Accuracy, precision and recovery

Accuracy and precision were evaluated by determination of CC-5013 in five replicates at four different concentrations of QC samples analyzed over three different days. Each run consisted of blank plasma samples without internal standard in duplicate, blank plasma samples with internal standard in duplicate, calibration standards in duplicate, and QC samples in replicates of five; this run was done daily for 3 days to evaluate the assay performance. Accuracy (DEV) was defined as percent difference between the mean observed concentration and the nominal concentration:

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

Non-LLOQ standards and QC samples were considered reliable if at least two out of every three estimates of the accuracy were within the range of 85–115%.

The precision of the assay was determined by the between-run (intermediate precision) and within-run precision (repeatability), respectively. 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:

$$\text{BRP} = \frac{\sqrt{(\text{MS}_{\text{bet}} - \text{MS}_{\text{wit}})/n}}{\text{GM}} \times 100\%$$

where n represents the number of replicates within each validation run.

The within-run precision (WRP) was calculated as:

$$\text{WRP} = \frac{\sqrt{\text{MS}_{\text{wit}}}}{\text{GM}} \times 100\%$$

In order to perform the assay with reliable intermediate precision and repeatability, both the BRP and WRP should not exceed a 15% limit.

In order to establish the extraction efficiency of CC-5013 from plasma using the liquid–liquid extraction method described in Section 2.4, two groups (A and B) of QCs were prepared. In group A, 30 μl of working standards were spiked into tubes containing 570 μl of pooled blank plasma for each QC (i.e. 15, 150, 400, and 800 ng/ml), and in group B, 30 μl of working standards were spiked into tubes containing 570 μl of mobile phase. Group A tubes were extracted as described in Section 2.4, then centrifuged, and the supernatant dried under air, reconstituted with mobile phase, and centrifuged again before the supernatant was injected into the LC–MS system. A volume of 125 μl unprocessed solution of group B tubes was transferred into injection vials directly, which was then injected into the LC–MS system. The peak area of unprocessed solutions in group B was adjusted, multiplied by 4.8, to correct for dilution difference. Recovery was determined by comparing the peak area of the QC samples (15, 150, 400, 800 ng/ml) with that of the corrected unprocessed solutions of corresponding concentrations:

$$\%\text{Recovery} = \frac{E}{U} \times 100\%$$

where E is the mean area count of test samples for one concentration level after sample extraction, and U is the mean area count of unprocessed samples. Each concentration was run in six replicates.

As a guideline, the recovery of CC-5013 need not be 100%, but should not vary by more than 15%.

2.5.4. Calibration curve

2.5.4.1. Lower limit of quantification. The LLOQ was defined as the lowest concentration of CC-5013 that could be reliably and reproducibly measured with concentration determinations performed in replicates of at least five. To determine the LLOQ, pooled plasma samples were spiked to contain 5 ng/ml, and were run on four different days. The CC-5013 peak was to be distinct from noise peaks and for verification of LLOQ, the peak area and peak height in a chromatogram from a pretreated CC-5013 plasma sample containing 5 ng/ml was compared with the noise signal. The LLOQ had to have a precision of $\leq$20% and a signal-to-noise ratio $\geq$3.

2.5.4.2. Response function. Calibration curves were constructed by least-squares linear regression analysis where an eight-point calibration curve by plotting peak area ratio (y) of CC-5013 to internal standard *versus* the CC-5013 nominal concentration (x) without weighting, or by using $1/x$ or $1/x^2$ as optional weighting factors. Calibrator response functions and choice of regression analysis were investigated by calculating correlation coefficients and the percent deviation for all standard concentrations (%DEV). In order to establish the best standard curve for quantification (with or without IS), the simplest model tested with the lowest bias was used for further analysis.

2.5.5. Stability

Stability tests were performed to verify the stability of CC-5013 during handling procedures. Samples were assayed at the four QC concentrations (i.e. 15, 150, 400, and 800 ng/ml). The samples were subjected to three freeze–thaw cycles with each freeze cycle lasting at least 12 h. The concentration of the drugs after each storage period was related to the concentration of freshly prepared samples in the same analytical run. The stability of drug in the injection vials pending analysis was performed, where samples were prepared and placed in injection vials for 24 h before injection into the LC–MS system.

Analytes were considered stable if the concentration deviated less than $\pm$20% from the concentrations of freshly prepared samples.

2.6. Pharmacokinetic analysis

The suitability of the method for pharmacokinetic purposes was evaluated using plasma samples obtained from a 42-year-old patient with a histologically confirmed diagnosis of glioblastoma multiforme, who received CC-5013 treatment as a single oral agent once-daily dose of 2.5 mg/m^2. This experiment was approved by the National Cancer Institute Institutional Review Board, and the patient signed informed consent before participation.

A total of nine pharmacokinetic blood samples was obtained and collected in 10-ml glass tubes containing heparin as an anticoagulant. These samples were obtained before the first dose of CC-5013 was administered, and then again at the following time points: 0.5, 1, 2, 4, 6, 8, 24, and 48 h post-dose. Specimens were immediately centrifuged at 2400 rpm for 5 min to separate the plasma, which was then stored at $-80\,^{\circ}$C.

3. Results and discussion

3.1. Chromatography and detection

Typical chromatograms resulting from the LC–MS analysis of extracts of 125 μl plasma from a pre-dose control blank sample (A), a control plasma sample spiked to contain CC-5013 at a concentration of 5 ng/ml (B), and a sample from a patient obtained 1 h after administration of CC-5013 (C) are displayed in Fig. 2. CC-5013 and the internal standard peaks were well resolved under the optimized conditions. Thalidomide and aminoglutethimide were the first choices for internal standards for this method based on their structural similarities to CC-5013. Unfortunately, the assay method developed for CC-5013 was not appropriate for thalidomide (i.e. poor peak shape), and aminoglutethimide may be used in prostate and breast cancer patients hence possible chromatographic interference from aminoglutethimide. Another compound tested for use as an internal standard was chrysin, but using the assay method developed for CC-5013 yielded very poor peak shape and long retention time for chrysin. Umbelliferone, though not structurally similar in structure to CC-5012 is (1) commercially available and (2) can be quantitated using the assay developed for CC-5013. The retention times of CC-5013 and the internal standard, umbelliferone, were 3.2 and 6.3 min, respectively. The total sample run time was set to 8 min.

3.2. Validation

3.2.1. Specificity

Analysis of blank plasma samples obtained from multiple patients concurrently on other common drugs did not show any chromatographic interference around the elution area of CC-5013 and/or the internal standard. A slight matrix effect was noted at retention times ranging from 3.0 to 4.2 min. However, despite possible matrix effects, the accuracy and precision were within accepted guidelines (FDA guidelines). This indicates that this method is good and robust for the determination of CC-5013 in human plasma.

3.2.2. Accuracy, precision and recovery

The assay performance data for the determination of independent QC samples of CC-5013 in plasma are presented in Table 1. The between-run precision and within-run precision ranged from 5.5 to 15.5% and from 9.9 to 13.8%, respec-

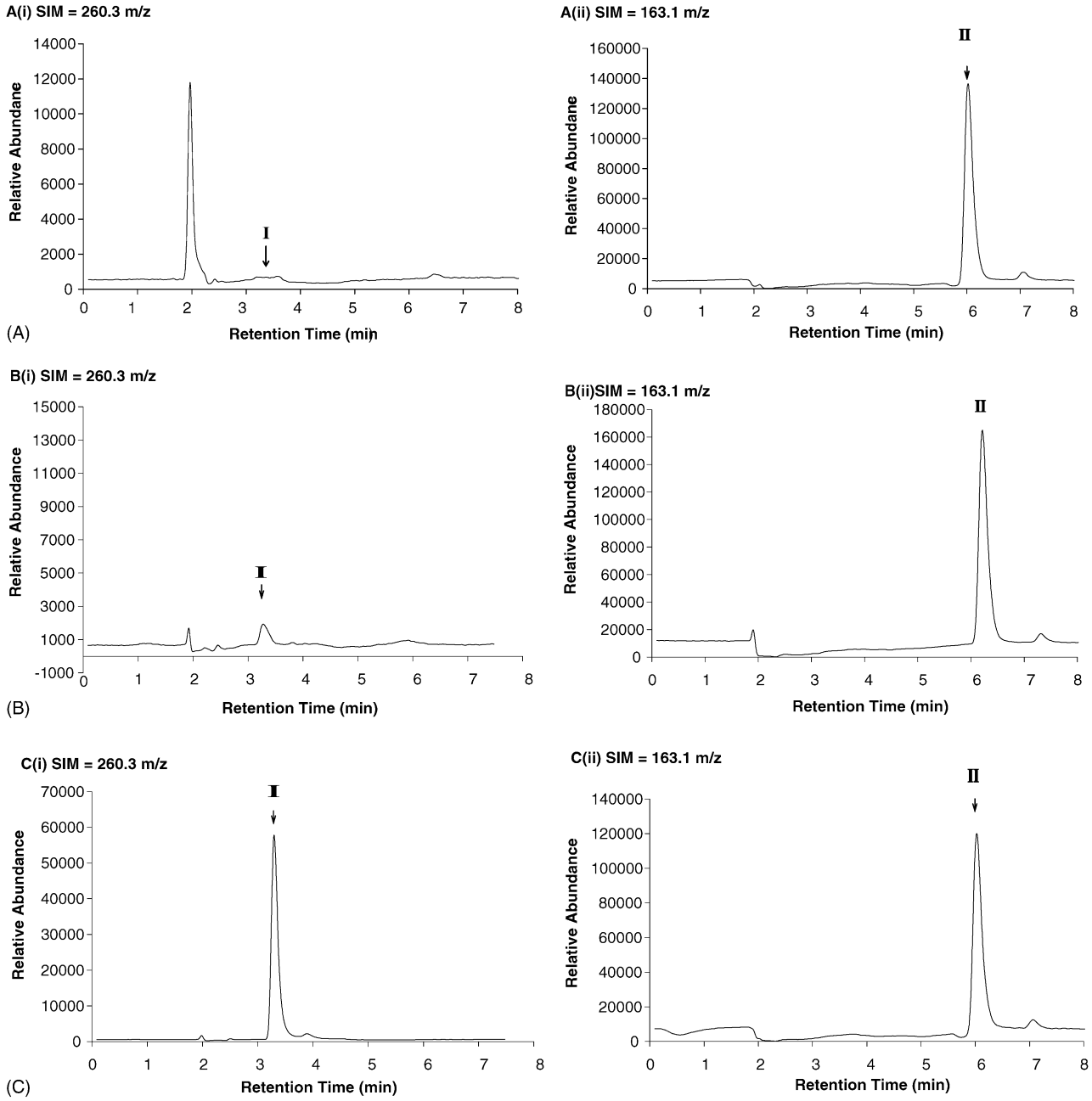

Fig. 2. Typical reverse-phase LC–MS chromatograms of a blank human plasma sample (A), a sample spiked with CC-5013 at a concentration of 5 ng/ml (B), and a plasma sample obtained from a patient with cancer 1 h after drug administration (oral dose, 2.5 mg/m^2/day) (C). The left and right chromatograms were obtained using the optimal molecular ion for CC-5013 (*m*/*z* 260.3) and the internal standard umbelliferone (*m*/*z* 163.1), respectively. The labeled chromatographic peaks indicate CC-5013 (I), and the internal standard umbelliferone (II).

tively, for the various concentrations tested. At the same concentrations, the deviation from nominal concentration (accuracy) ranged between −1.6 and +5.7%. The mean recovery for QC samples was determined to be 72.4%, with a coefficient of variation (percent CV) ranging from 0.7 to 15%. Given the low variation in recovery, and acceptable accuracy and precision the extraction efficiency was considered acceptable.

3.2.3. Calibration curve

3.2.3.1. Lower limit of quantification. The LLOQ was determined to be 5 ng/ml. The percent CV was 14.9% and the percent DEV from the nominal standard was +16.2%, which is within acceptable limits. Preliminary pharmacokinetic analysis of clinical specimens indicated that plasma concentrations of CC-5013 were always higher than 5 ng/ml at the doses and sampling times specified in the study protocol.

Table 1
Assessment of accuracy and precision from quality-control samples

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	DEV (%)	WRP (%)	BRP (%)	*n*
15	15.9	2.76	+5.7	9.9	15.2	12
150	147.7	28.41	−1.6	11.3	15. 5	11
400	417.78	55.02	+4.4	13.8	[a]	13
800	806.7	107.27	+0.8	12.3	5.5	13

Abbreviations: GM, grand mean; S.D., standard deviation; DEV (%) relative deviation 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.

3.2.3.2. Response function. The model with the least total bias across the concentration range investigated was obtained using the reciprocal of the concentration ($1/x$) as the weighting factor. For each calibration curve, the calibrators were back-calculated from the response factor and the intercept. The deviation for all concentrations from the nominal concentrations was between −16.5 and 16.2%, whereas the percent CV values ranged from 1.0 to 15.5% (Table 2). For each analytical run, an eight-point plasma standard curve was constructed, and was shown to be linear over the tested range of 5–1000 ng/ml. The mean (±standard deviation) regression equation obtained during the method validation, obtained in duplicates on three separate days, showed an intercept of -0.00037 ± 0.00027 and a slope of 0.00031 ± 0.00003 ($r^2 = 0.99478 \pm 0.00338$; $n = 6$).

3.2.4. Stability

Three repeated freeze–thaw cycles had no apparent influence on the stability of QC samples containing CC-5013 at concentrations of 15, 150, 400, or 800 ng/ml. After the third freeze–thaw cycle, CC-5013 plasma concentrations had deviations from the nominal values within recommended guidelines, irrespective of the tested plasma concentrations. The stability of the analyte in injection vials was assessed after 24 h at ambient temperature, and the observed percent CV was 7.85, 22.83, 11.95, and 4.26% for samples containing 15, 150, 400, or 800 ng/ml, respectively.

Table 2
Back-calculated concentrations from calibration curves

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	CV (%)	DEV (%)	*N*
5	5.8	0.9	14.9	16.2	6
10	9.8	1.0	10.2	−2.2	6
25	26.8	1.6	5.9	−7.9	6
50	45.9	4.3	9.3	−8.1	6
100	83.5	0.9	1.0	−16.5	6
250	259.4	40.1	15.5	3.8	6
500	528.3	48.1	9.1	5.7	6
1000	9756	79.4	8.1	−2.4	6

Abbreviations: GM, grand mean; S.D., standard deviation; DEV (%), relative deviation from nominal value; CV (%), coefficient of variation; *n*, number of replicate observations within each validation run, i.e. two samples at each concentration were run on three separate occasions, for a total (*N*) of six samples at each concentration.

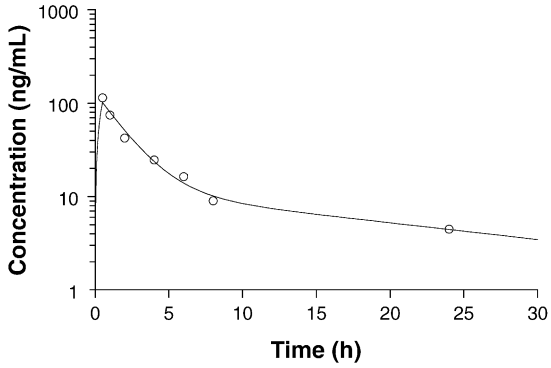

Fig. 3. Plasma concentration–time profile of CC-5013 in a patient with advanced glioma treated at a dose of 2.5 mg/m^2.

3.2.5. Application

After completion of the validation process, the assay was applied to determine serial CC-5013 plasma concentrations in a single patient treated with CC-5013 in a phase I clinical trial. Concentration–time data for CC-5013 in plasma from this patient who received the drug orally at a dose of 5 mg (2.5 mg/m^2) is presented in Fig. 3. Plasma concentration data of CC-5013 were analyzed by non-compartmental methods using the software package WinNonlin v4.0 (Pharsight Corporation, Mountain View, CA). The peak concentration for CC-5013 was 114.2 ng/ml, the area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was 393.3 ng h/ml, and the terminal half-life ($t_{1/2}$) was 2.7 h.

4. Conclusion

CC-5013 is an analogue of thalidomide that is currently undergoing evaluation in phase I–II clinical trials. The agent has also obtained orphan drug status from the FDA but no simple, accurate and precise analytical method for the determination of CC-5013 concentrations in plasma has been published. In the current report, the development and validation of an analytical assay is described for the determination of CC-5013 in human plasma samples. The performance criteria for specificity, accuracy, precision, recovery, sensitivity, linearity, and stability have been assessed and accepted as being within the recommended guidelines of the FDA, indicating that the method can be used for determination of CC-5013 in human plasma. The method is currently being used to further study the pharmacokinetic profile of CC-5013 in patients with cancer.

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