ELSEVIER

Journal of Chromatography B, 755 (2001) 237–243

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Measurement of the novel antitumor agent 17-(allylamino)-17-demethoxygeldanamycin in human plasma by high-performance liquid chromatography

Elizabeth B. Agnew[a], Richard H. Wilson[a], Jean L. Grem[a,*], Leonard Neckers[b], Daoqin Bi[a], Chris H. Takimoto[a]

[a]*Developmental Therapeutics Department, Medicine Branch, Division of Clinical Sciences, National Cancer Institute, Bethesda, MD 20889, USA*

[b]*Cell and Cancer Biology Department, Medicine Branch, Division of Clinical Sciences, National Cancer Institute, Rockville, MD 20850, USA*

Received 28 August 2000; received in revised form 1 February 2001; accepted 1 February 2001

Abstract

A sensitive HPLC assay has been developed to determine the concentration of 17-(allylamino)-17-demethoxygeldanamycin (AAG) in human plasma over the concentration range of 12.5 to 2500 n*M* (7.33 to 1465 ng/mL). After the addition of 1000 n*M* geldanamycin as the internal standard, 1 mL samples of human plasma were subjected to solid-phase extraction, via Bond-Elut C_{18} cartridges, followed by analysis using an isocratic reversed-phase HPLC assay with UV detection. A Phenomenex Kingsorb, 3 micron, C18, 150×4.60 mm column and a Phenomenex Security Guard pre-column, C_{18} (ODS, Octadecyl), were used to achieve separation. AAG and GM were monitored at 334 and 308 nm, respectively, on a Hewlett-Packard 1050 Diode-Array Detector. The mobile phase, run at a flow-rate of 1 mL/min, was composed of 50% (v/v) 25 m*M* sodium phosphate (pH 3.00) with 10 m*M* triethylamine and 50% acetonitrile. HPLC effectively resolved AAG with retention times of 14.60 $\pm$ 0.54 min and the internal standard geldanamycin at 10.72±0.38 min ($n = 15$). This assay was able to measure plasma concentrations of AAG, the lower limit of quantitation being 12.5 n*M*, at a starting dose of 10 mg/m^2 infused intravenously over 1 h in a Phase I clinical trial in adult patients with solid tumors. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: 17-(Allylamino)-17-demethoxygeldanamycin

1. Introduction

17-(Allylamino)-17-demethoxygeldanamycin (AAG) (NSC-330507) is a benzoquinone ansamycin derivative of geldanamycin, a potent herbicidal agent isolated from the broth of the soil organism *Streptomyces hygroscopicus* (Fig. 1A and B) [1]. In preclinical studies, these agents were cytotoxic for a variety of murine tumor cell lines [1]. Mechanistic studies revealed that geldanamycin, and the related compound herbimycin, reversed the transformation of mammalian cells by the Rous sarcoma virus by

*Corresponding author. Medicine Branch, National Cancer Institute, Building 8, Room 5101, National Naval Medical Center, 8901 Wisconsin Avenue, Bethesda, MD 20889-5105, USA. Tel.: +1-301-435-5382; fax: +1-301-480-1683.

E-mail address: jgrem@helix.nih.gov (J.L. Grem).

0378-4347/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(01)00113-X

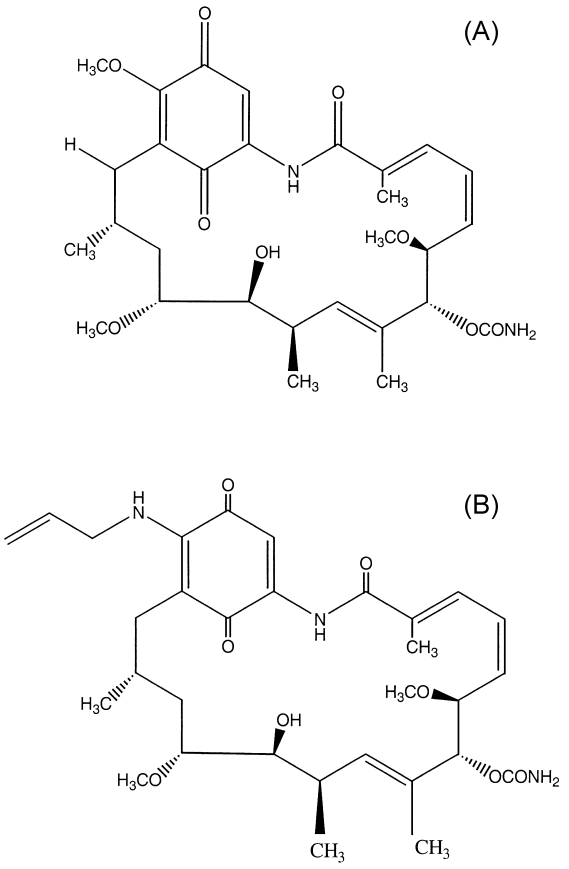

Fig. 1. Structures of geldanamycin (GM) (A) and 17-(allylamino)-17-demethoxygeldanamycin (AAG) (B).

inhibiting the *src* protein tyrosine kinase activity [2]. Later studies also demonstrated that these agents could broadly inhibit a variety of different tyrosine kinases and that they were cytotoxic to many different human tumor cell lines in vitro [3].

More recent pioneering work by Whitesell and Neckers identified heat shock protein 90 (hsp90) as the actual molecular target of geldanamycin [4]. Tight binding of geldanamycin and its analogs, such as AAG, to the ATP binding site of hsp90 results in the loss of the heat shock protein's chaperone function. This property is essential for the proper folding and normal functioning of multiple different cellular proteins including key cell signaling kinases, such as Raf-1 kinase, lck, erbB2, and v-fps, and for various hormone receptors including the glucocorticoid, estrogen, androgen, progesterone, and retinoid receptors.

In preclinical toxicity studies, geldanamycin caused severe hepatic toxicity and was dropped from clinical development [5]. However, the geldanamycin derivative AAG was still biologically active, and was better tolerated in preclinical animals models [6]. Consequently, AAG was selected for further clinical development and Phase I clinical trials of intravenously administered AAG were recently initiated. In conjunction with an ongoing Phase I study, we developed a sensitive, reproducible method of measuring AAG in human plasma using solid-phase extraction and UV detection. The lower limit of quantitation of AAG in plasma is 12.5 n*M*.

2. Experimental

2.1. Chemicals and solvents

17-(Allylamino)-17-demethoxygeldanamycin (AAG), MW 586 g/mol (CAS number 75747-14-7), was obtained from the Pharmaceutical Resources Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD, USA). Geldanamycin (GM), MW 560 g/mol (CAS number 30562-34-6), and all other chemicals were reagent grade and were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified. All solvents were HPLC-reagent grade and were purchased from Fisher (Fair Lawn NJ, USA).

2.2. Solid-phase extraction

Bond-Elut, 100 mg, C_{18} solid-phase extraction cartridges (Varian, Harbor City, CA, USA) were conditioned by passing 1 mL of methanol followed by 1 mL of water through the cartridge packing. Blood samples were drawn into heparinized tubes, transported on ice, and immediately centrifuged at 800 *g* for 10 min at 4°C. A volume of 1 mL of plasma supernatant was combined with 10 μL of geldanamycin 100 μ*M* internal standard in a 1.5 mL polypropylene microcentrifuge tube and vortex-mixed for 15 s. The sample was loaded at a rate of 0.25 mL/min onto a solid-phase extraction cartridge and washed twice with 1 mL of water followed by 1 mL of 100% methanol to elute the AAG and internal standard. Samples were evaporated to dryness using

a Zymark TurboVap (Hopkinton, MA, USA) with filtered compressed air and a water bath temperature of 50°C. The residue was resuspended in 250 μL of HPLC mobile phase (described below) and transferred to 500 μL low volume, crimp-top glass vials (Agilent, Palo Alto, CA, USA) for HPLC analysis.

2.3. High-performance liquid chromatography

HPLC separation was achieved using a reversed-phase Phenomenex Kingsorb, 3 μm, C_{18}, 150×4.60 mm column with a Phenomenex Security guard cartridge system, C_{18} (ODS, Octadecyl), as the pre-column (Torrance, CA, USA). A Hewlett-Packard 1050 HPLC system (Wilmington, DE, USA) was used to deliver an isocratic mobile phase of 50% (v/v) acetonitrile–25 m*M* sodium phosphate buffer, pH 3.00, containing 10 m*M* triethylamine at a flow-rate of 1.0 mL/min. The AAG and geldanamycin internal standard were measured using a HP1050 diode-array ultraviolet detector set at a wavelength corresponding to the maximal absorbance of 334 nm for AAG and 308 nm for geldanamycin with a 16 nm bandwidth for both. Injection of 100 μL of sample was accomplished using an autosampler which was left unattended during the 25 min analytic runs.

2.4. Standard solutions

Stock solutions of 1 m*M* AAG and geldanamycin in DMSO were stored at −20°C in 500 μL single-use aliquots. The AAG standards were stable under these storage conditions for at least 1 month. Geldanamycin stock solutions in DMSO were sensitive to repeated freeze–thaw cycles, so single-use vials were prepared once and discarded after 2 months of storage at −20°C. Plasma calibration standards were prepared by adding 10 μL of 100 μ*M* geldanamycin in DMSO as the internal standard and 10 μL of different stock solutions of AAG in DMSO to 1 mL of plasma to give final drug concentrations of 12.5, 25, 50, 100, 250, 500, 1000, and 2500 n*M* AAG and 1000 n*M* of geldanamycin.

2.5. Calculations

Calibration curves were generated by plotting the ratio of the AAG peak area at 334 nm to geldanamycin peak area at 308 nm on the ordinate and the AAG plasma concentration on the abscissa. These data were fitted using least square linear regression with a weighting factor of $1/(y_{obs})^2$, not including the origin. Standard curves were prepared separately for each HPLC run and unknown sample concentrations were determined using the calibration curve obtained during the same HPLC analysis.

2.6. Absolute and relative recovery

Plasma (1 mL) standards were prepared in triplicate containing 25 n*M*, 250 n*M*, and 2500 n*M* AAG and 1000 n*M* geldanamycin. Samples were extracted, processed and analyzed by HPLC as described above. Relative peak areas of AAG and geldanamycin in these samples were compared to unextracted standards containing the exact same amount of AAG and geldanamycin. The percent absolute recovery was defined as: (peak area of the plasma standard/peak area of the unextracted standard)×100.

2.7. Validation

Analytical method evaluation was performed using the criteria suggested by Shah et al. [7]. Ten distinct calibration curves were analyzed over a 3-month time period and the accuracy and precision of each of the eight calibration standards were determined by calculating the mean value and relative standard deviation (RSD) at each concentration. The within-run and the between-run precision and accuracy were determined by preparing three quality control samples of AAG at 25, 250 and 2500 n*M* in human plasma and analyzed in quintuplicate and determined on five separate occasions using five different sample calibration curves.

2.8. Stability of stored plasma samples

The stability of AAG was examined in plasma containing 1000 n*M* AAG stored at −80°C for up to 1 month. The effect of blood processing delays was also analyzed by spiking fresh blood and plasma with 1000 n*M* AAG and incubating on ice and at room temperature (25°C). The analysis of AAG was performed at 0, 1, 2, and 4 h as described above.

2.9. Analysis of patient samples

Fifteen plasma samples were drawn from each of nine adult cancer patients treated with 1 h infusions of 10 to 56 mg/m^2 (17–96 μmol/m^2) of AAG formulated in 2.0 mL dimethylsulfoxide and diluted in 48 mL of 2% egg phospholipids and 5% dextrose in Water for Injection, USP. Blood was collected into lithium-heparin tubes and immediately centrifuged at 800 *g* for 10 min at 4°C to isolate plasma. Plasma samples were stored frozen at −80°C until analysis.

3. Results

3.1. High-performance liquid chromatography

High-performance liquid chromatography effectively resolved AAG with retention times of 14.60±0.54 min and the internal standard geldanamycin at 10.72±0.38 min ($n = 15$). The peak corresponding to a plasma concentration of 552 n*M* in a patient receiving intravenous AAG at 40 mg/m^2 over 1 h is shown in Fig. 2A. UV detection was sufficiently sensitive to detect 12.5 n*M* AAG in 1 mL of plasma with a signal-to-noise ratio of 3.4:1 (Fig. 2B). The optimal UV detector response for AAG was at 334 nm; however, the use of a second wavelength of 308 nm to monitor the geldanamycin internal standard increased the detector response of this peak by over 3.5-fold (Fig. 3A and B).

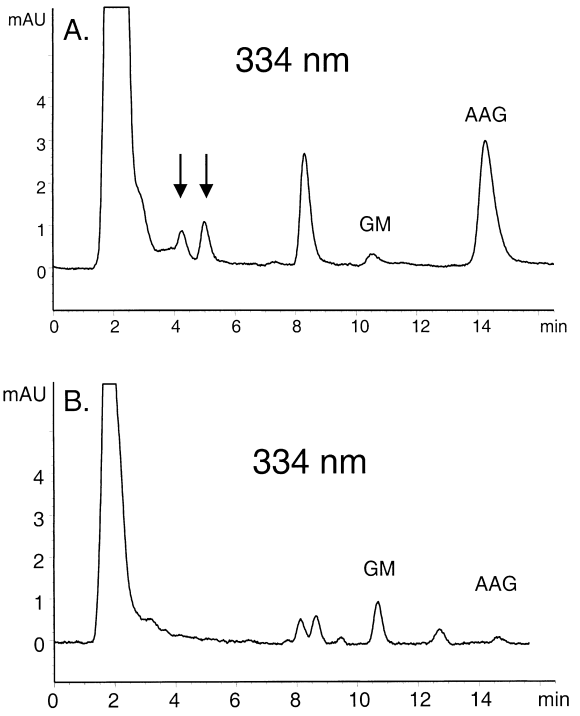

Fig. 2. Chromatography and UV detection of 17-(allylamino)-17-demethoxygeldanamycin (AAG) in the plasma of a patient 90 min after the start of a 1 h infusion of 40 mg/m^2 of AAG (A), and a plasma standard containing 12.5 n*M* of AAG (B). Identified peaks include AAG and geldanamycin (GM). Suspected metabolite peaks are indicated by the black arrows.

3.2. Recovery from plasma

Absolute recovery of AAG from plasma was compared to unextracted samples. The recovery of AAG from 1 mL of plasma containing 25 n*M*, 250 n*M* and 2500 n*M* AAG was reproducible with overall recoveries of 76.0±12.7% ($n = 6$), 71.1±4.4% ($n = 3$), and 69.6±2.6% ($n = 3$), respectively. The absolute recovery of the 1000 n*M* geldanamycin internal standard from plasma was 92.6±3.4% ($n = 6$).

3.3. Measurement of AAG by HPLC

Assay reproducibility was examined by analyzing calibration curves containing eight standard concentrations of AAG at 12.5, 25, 50, 100, 250, 500, 1000, 2500 n*M* and geldanamycin at 1000 n*M*. Analysis of 10 different plasma standard curves examined over a 3-month period resulted in a correlation coefficient (mean±SD, $n = 10$) of 0.993±0.011 with a *y*-intercept of 0.030±0.057 which was not significantly different from zero (Students' *t*-test, $P = 0.2$). The RSDs ranged from 1.6 to 11.6% over the concentration range of 12.5 to 2500 n*M* (Table 1).

The within- and between-run accuracy and precision were determined by analyzing five separate plasma standards at concentrations of 25 n*M*, 250 n*M* and 2500 n*M* AAG. The RSDs for the within-run analysis ($n = 5$) were 7.8, 1.3, and 7.4% respectively, while the between-run RSDs were 10.3, 4.3, and 10.8%, respectively, for these three concentrations (Table 2). The deviation of the measured concentration from the theoretically true AAG concentration was less than 15.6% in all cases (Table 2).

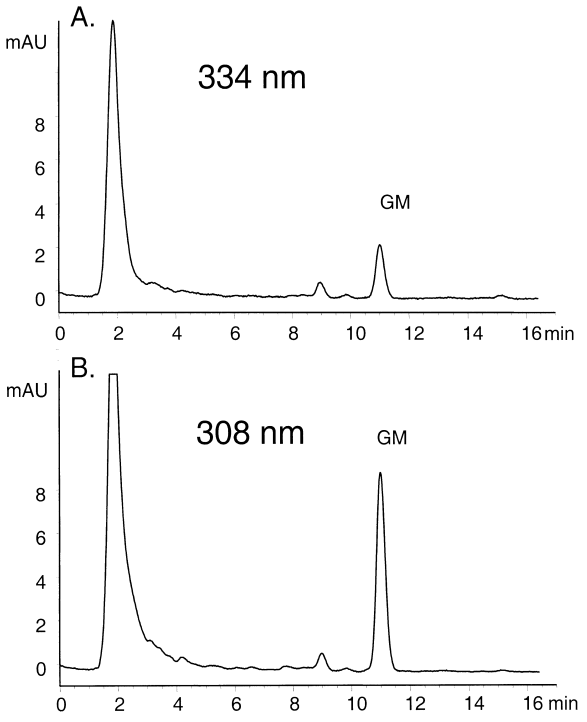

Fig. 3. Chromatographs of a plasma sample containing 1000 n*M* of geldanamycin internal standard monitored by UV absorbance at 334 nm (A) and at 308 nm (B).

3.4. Lack of conversion of AAG to geldanamycin

Because geldanamycin was used as the internal standard, human plasma from patients treated with the highest dose of AAG currently administered in our ongoing Phase I study (56 mg/m^2 intravenously over 1 h) was examined in the absence of the addition of any internal standard. Plasma samples were examined at the end of the infusion and up to 24 h after the start of the infusion in three separate patients. No evidence of a peak corresponding to the retention time of geldanamycin was detected in any plasma sample (data not shown).

Table 1
17-(Allylamino)-17-demethoxygeldanamycin (AAG) calibration curves

Standard conc. (n*M*)	Measured conc. (mean±SD, $n = 6$) (n*M*)	Relative standard deviation (%)
12.5	13.9±1.6	11.6
25	25.5±1.1	4.3
50	51.0±2.2	4.2
100	96.3±3.8	4.0
250	245.6±5.1	2.1
500	505.0±8.1	1.6
1000	985.6±34.2	3.5
2500	2533.6±173.5	6.8

3.5. Stability of AAG in plasma and whole blood

The stability of AAG in fresh blood and plasma was determined in order to establish the optimal conditions for sample processing and storage. Incubation of 1000 n*M* AAG in either whole blood or plasma at 0°C (on ice) for up to 4 h prior to solid-phase extraction resulted in no discernable change in the AAG signal intensity (Fig. 4). However, when the spiked samples were incubated at room temperature (25°C), the signal intensity decreased by 13 and 25% in plasma and whole blood, respectively. For this reason, blood samples were kept on ice immediately after collection and were never allowed to warm to room temperature during processing.

Plasma samples containing 1000 n*M* AAG were stable for up to at least 1 month when stored at −80°C prior to sample processing (*t*-test, $P = 0.3$). No discernable changes in the measured concentration of 1000 n*M* AAG were observed when plasma samples were analyzed in triplicate after one ($P = 0.8$) and two freeze–thaw cycles ($P = 0.5$).

3.6. Patient plasma samples

Plasma AAG concentrations were measured in patients participating in our Phase I clinical trial of intravenously administered AAG infused at 10 to 56 mg/m^2 daily over 1 h each day for 5 days every 3 weeks. Plasma samples were obtained during the infusion and at various time points after the end of the infusion. The plasma concentration versus time curve for a representative patient treated at 40 mg/m^2 is shown in Fig. 5. Preliminary analysis suggests the pharmacokinetic data is best described by a two-compartment, open model. Endogenous peaks interfering with the measurement of AAG or geldanamycin have not been identified in any patient currently studied ($n = 9$).

Table 2
Within- and between-run accuracy and precision for measuring 17-(allylamino)-17-demethoxygeldanamycin (AAG) in human plasma

Theoretical conc. (n*M*)	Measured conc. (n*M*)	Percentage of the theoretical concentration	Standard deviation (n*M*)	Relative standard deviation (%)
Within-run ($n = 5$)				
25 n*M* AAG	28.9	115.6	2.3	7.8
250 n*M* AAG	251.3	100.5	3.3	1.3
2500 n*M* AAG	2490.9	99.6	183.8	7.4
Between-run ($n = 5$)				
25 n*M* AAG	24.4	97.6	2.5	10.3
250 n*M* AAG	253.1	101.2	10.9	4.3
2500 n*M* AAG	2639.6	105.6	284.5	10.8

In all patients, two drug treatment-associated HPLC peaks were detected using UV absorbance at 334 nm with retention times of 4.17 ± 0.08 and 4.96 ± 0.03 min ($n = 12$ separate injections) (Fig. 2A). Both peaks appeared during the infusion, peaked at the end of the infusion, and declined roughly in concert with the AAG peak. The peak areas of both these unknowns were always smaller in size than the AAG peak, ranging in size from 0.1 to 0.75 times the AAG peak area. Based upon animal pharmacokinetic and metabolic studies and upon similar retention times, it is likely that one of these unidentified peaks represents the 17-aminogeldanamycin metabolite [6]. Further characterization of these unknown metabolites is in progress. No other suspected metabolites have been observed in human plasma.

4. Discussion

Analytic methods for measuring geldanamycin and AAG in murine plasma have been previously published [3,6], but to our knowledge this is the first

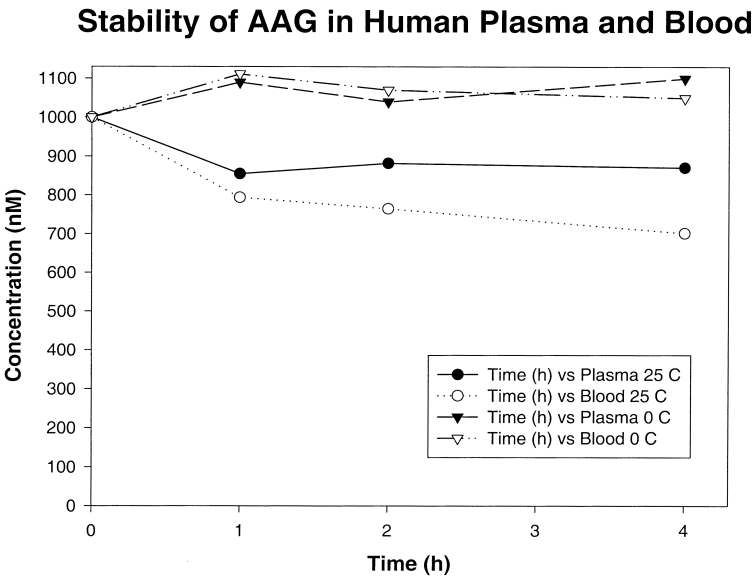

Fig. 4. Stability of 17-(allylamino)-17-demethoxygeldanamycin (AAG) in whole blood and plasma. AAG at 1000 n*M* was added to fresh whole blood and fresh human plasma and incubated for up to 4 h at 0 and 25°C.

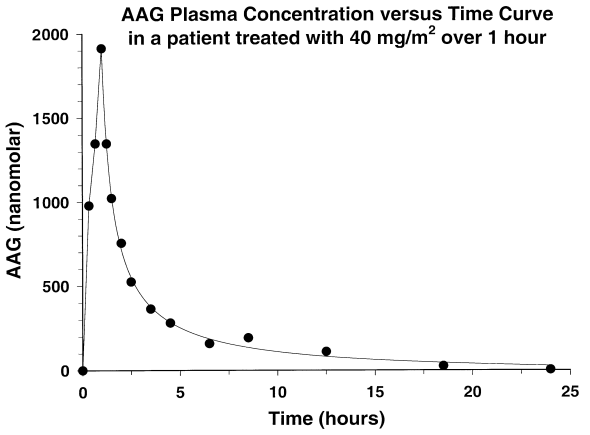

Fig. 5. Plasma concentration versus time curve of 17-(allylamino)-17-demethoxygeldanamycin (AAG) in an adult cancer patient treated with a 1 h drug infusion at a dose of 40 mg/m^2.

report of a validated analytic method for AAG in human plasma. Solid-phase extraction of AAG from human plasma combined with HPLC analysis and UV detection allowed us to successfully analyze plasma concentrations of AAG over the range of 12.5 to 2500 n*M*. Use of two different monitoring wavelengths, 334 nm for AAG and 308 nm for geldanamycin, optimized the detection of both compounds during the same run (Fig. 3A and B). The lower limit of quantitation of 12.5 n*M* for AAG allowed us to accurately measure plasma concentrations in patients until a minimum of 8 h after the start of a 1 h drug infusion even at the lowest drug doses administered to our patients. The drug was stable on ice in human blood and plasma for at least 4 h, allowing for batched sample processing. Once frozen, plasma specimens were generally measured within 1 week of the collection; however, these samples were stable for up to 1 month when stored at −80°C.

Preclinical pharmacokinetic studies of AAG in mice demonstrate hepatic metabolism of AAG to various metabolites, some of which are present in plasma. The most prominent was 17-aminogeldanamycin which was rapidly formed in mouse hepatic microsomes, but was a less prominent substrate in human in vitro drug metabolism studies [6]. In our patient samples, two small peaks in plasma samples were detected in patient samples with retention times of 4.17±0.08 and 4.96±0.03 min. These peaks appeared early during the 1 h drug infusion and they were consistently smaller in area than the AAG drug peak. Additional studies are ongoing to identify whether one of these peaks truly represents the dealkylated 17-AG derivative. No other potential metabolite peaks have been identified in patients specimens to date (data not shown). Furthermore, no interconversion of AAG to geldanamycin, which was used as the internal standard for this assay, was observed.

The plasma concentration versus time curve from a representative patient treated at 40 mg/m^2/1 h is shown in Fig. 5. Preliminary analysis suggests pharmacokinetic data is best described by a two-compartment, open model. The sensitivity of this assay allowed us to characterize drug kinetics in human plasma following a 1 h infusion of AAG at the starting dose level in our Phase I study of 10 mg/m^2. Currently, patients are being treated with an infusion of 56 mg/m^2 of AAG, and pharmacokinetic monitoring is continuing.

In conclusion, we have developed a simple, sensitive and validated analytic method for measuring AAG in human plasma. This assay utilizes solid-phase extraction followed by analysis using isocratic reversed-phase HPLC separation with UV detection. Our assay is currently being used to measure the plasma drug concentrations of AAG in our ongoing Phase I clinical trial of AAG.

References

[1] C. DeBoer, P.A. Meulman, R.J. Wnuk, D.H. Peterson, J. Antibiot. (Tokyo) 23 (1970) 442.
[2] Y. Uehara, Y. Murakami, K. Suzukake-Tsuchiya, Y. Moriya, H. Sano, K. Shibata, S. Omura, J. Antibiot. (Tokyo) 41 (1988) 831.
[3] J.G. Supko, R.L. Hickman, M.R. Grever, L. Malspeis, Cancer Chemother. Pharmacol. 36 (1995) 305.
[4] L. Whitesell, E.G. Mimnaugh, B. De Costa, C.E. Myers, L.M. Neckers, Proc. Natl. Acad. Sci. USA 91 (1994) 8324.
[5] T.W. Schulte, L.M. Neckers, Cancer Chemother. Pharmacol. 42 (1998) 273.
[6] M.J. Egorin, D.M. Rosen, J.H. Wolff, P.S. Callery, S.M. Musser, J.L. Eiseman, Cancer Res. 58 (1998) 2385.
[7] 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. McDowall et al., Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249.