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High-performance liquid chromatographic method for the determination of a novel thymidylate synthase inhibitor, AG 331, in human serum

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Abstract

AG 331 is a novel thymidylate synthase inhibitor currently in Phase I clinical trial. To determine the pharmacokinetic parameters of AG 331 in human subjects, a suitable analytical method was developed using high-performance liquid chromatography. Serum and urine samples were prepared using both solid-phase extraction and solvent extraction. Either 4,4′-diaminodiphenyl sulfone or benz[*cd*]indole-2(1H)-one were used as internal standards for the method. A reversed-phase C_{18} analytical column completely resolved the drug and internal standard peaks from non-specific substances present in biological matrix. The method was validated for precision, accuracy, and reproducibility in serum and was linear over a concentration range of 50–2000 ng/ml, with a limit of detection of 20.0 ng/ml and a quantifiable limit of 50 ng/ml.

1. Introduction

AG 331, N^6-[4-(morpholinylsulfonyl)benzyl]-N^6-methyl-2,6-diaminobenz[*cd*]indole glucuronate (Fig. 1A), is a novel lipophilic thymidylate synthase (TS) inhibitor that is currently under clinical investigation [1]. Unlike classical antifolate TS inhibitors, AG 331 lacks a glutamate moiety thus enhancing the lipophilicity of the molecule facilitating its diffusion across cell membranes. In addition, such a molecule could inhibit intracellular TS in cells that are normally resistant to classical antifolates. AG 331 has shown potent human TS inhibition in vitro (K_{is} = 1.2 nM) [2,3]. High AG 331 inhibitory activity was also reported in murine and human tumor cells in culture. The IC_{50} reported in these studies was in the range of 0.4–0.9 μM [4]. For the purpose of studying clinical pharmacokinetics of AG 331, the development of a reliable analytical method for monitoring drug concentrations in biological fluids was required. This paper describes the development and validation of a

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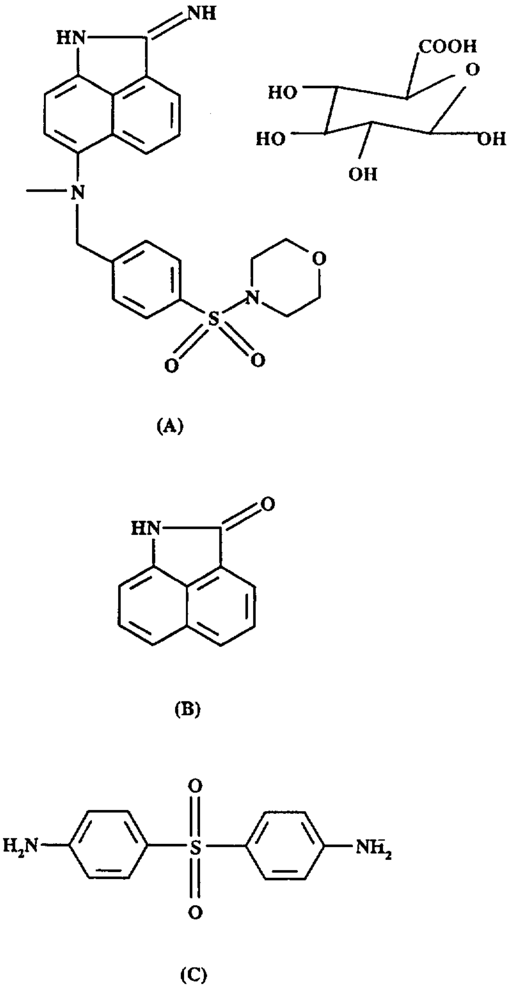

Fig. 1. Chemical structures for (A) AG 331, N^6 - [4 - (morpholinylsulfonyl) - benzyl] - N^6 - methyl - 2,6 - diaminobenz[*cd*]indole glucuronate, and internal standards: (B) benz[*cd*]indol-2(1H)-one, and (C) 4,4′-diaminodiphenyl sulfone.

sensitive, reliable HPLC method for the determination of AG 331 in human serum.

2. Experimental

2.1. Materials and reagents

AG 331 was supplied by Agouron Pharmaceuticals (San Diego, CA, USA). Internal standards, benz[*cd*]indol-2(1H)-one (Fig. 1B) and 4,4′-diaminodiphenyl sulfone (Fig. 1C), were purchased from Aldrich (Milwaukee, WI, USA). Solid-phase extraction column cartridges (C_{18}, 200 mg), ammonium phosphate monobasic, HPLC grade diethyl ether and acetonitrile were purchased from Baxter Scientific Products (Irvine, CA, USA). Reagent grade hydrochloric acid and ammonium hydroxide were obtained from Mallinckrodt Specialty Chemicals, (Paris, KY, USA).

2.2. Instrumentation

The HPLC system consisted of a Perkin Elmer Series 410LC solvent delivery pump, ISS-100 autosampler, LC90 UV spectrophotometric detector, and LCI-100 laboratory computing integrator. Samples (50 μl) were injected by an autoinjector into an Ultrasphere C_{18} analytical column, 250 × 4.6 mm, 5 μm particle size (Beckman, San Ramon, CA, USA). The autosampler injection rack was maintained at 4°C using a Brinkmann Lauda RM6 low-temperature circulator. The absorbance of the effluent was monitored using a variable-wavelength UV detector set at 230 nm. The AG 331 ultraviolet spectra were determined on a Beckman DU-64 UV–Vis spectrophotometer.

2.3. Mobile phase

The mobile phase consisted of acetonitrile–water (30:70) containing 100 m*M* monobasic ammonium phosphate adjusted to pH 3.0 with hydrochloric acid. The mobile phase was filtered through a Nylon 66 membrane filter (Sartorius, Göttingen, Germany) and deaerated with helium gas. Samples were eluted isocratically at a mobile phase flow-rate of 1.5 ml/min at a pressure of 200 bar.

2.4. Preparation of standard solutions

A stock solution of AG 331 was prepared by dissolving an accurately weighed amount of AG 331 in methanol. Appropriate dilutions of this solution were made with methanol to achieve a final stock solution concentration of 200.0 μg/ml. Stock solutions in methanol were stored in tightly capped polypropylene containers at −70°C for up to four months with no significant change in concentration. Prior to preparation of standard solutions of AG 331 in serum, the stock solution was further diluted with mobile phase to concentrations of 20.0 μg/ml and 2.0 μg/ml. Human serum was spiked with these solutions to give final AG 331 concentrations of 50.0, 100.0, 200.0, 500.0, 1000.0 and 2000.0 ng/ml. The two internal standard solutions were prepared by dissolving 15.0 mg of 4,4′-diaminodiphenyl sulfone or 2.5 mg of benz[*cd*]indol-2(1H)-one in 1 ml of methanol. These solutions were further diluted with water to give a final concentration of 15.0 μg/ml for 4,4′-diaminodiphenyl sulfone and 2.5 μg/ml for benz[*cd*]indol-2(1H)-one.

2.5. Sample preparation

All serum samples were sonicated for 5 min prior to sample preparation. Samples were prepared for analysis using either solvent extraction or solid-phase extraction.

Solvent extraction method

To 1 ml of serum sample or serum standard solution in a screwcap tube (16 × 150 mm) was added 50 μl of internal standard solution, 50 μl of concentrated (30%) ammonium hydroxide (final pH 8–8.5) and 5.0 ml of diethyl ether. The tubes were sealed with a Teflon-lined cap and mixed on a mechanical shaker for 10 min, then allowed to sit for 10 min for the solvent layers to separate. The upper ether layer (about 4.2 ml) was carefully aspirated and transferred to a second glass tube (13 × 100 mm) and the ether extract was evaporated to dryness at 30°C using a gentle stream of filtered air. The residue was reconstituted in 150 μl of mobile phase, centrifuged at 13 700 *g* for 5 min. The supernatant was transferred to HPLC injection vials and 50 μl of the supernatant was injected into the HPLC. For the solvent extraction method, either 4,4′-diaminodiphenyl sulfone or benz[*cd*]indol-2(1H)-one could be used as the internal standard since the chromatograms were virtually devoid of any interference from the matrix. The results reported herein used solvent extraction and 4,4′-diaminodiphenyl sulfone as the internal standard.

Solid-phase extraction method

Solid-phase extraction columns (C_{18}, 200 mg) were preconditioned by passing through one column volume of methanol followed by two column volumes of water. The columns were then loaded with 0.50 ml of serum sample or serum standard solutions to which 50 μl of internal standard, benz[*cd*]indol-2(1H)-one, was added. Following loading of the serum samples on the column, the columns were washed with 2 × 1.0 ml of water. The drug and internal standard were eluted from the column using 5 ml methanol. The eluant was evaporated to dryness at 40°C under a stream of dry, filtered air. The residue was reconstituted in 150 μl of mobile phase and centrifuged at 13 700 *g* for 5 min. The supernatant was transferred to the HPLC injection vial and 50 μl was injected into the HPLC.

For urine samples, 0.1 ml of urine was diluted to 1.0 ml with distilled water. The diluted sample was prepared using either the solvent or solid-phase extraction method described above for serum samples. The diluted blank urine was spiked with appropriate volumes of AG 331 stock solution in a manner similar with that used for the preparation of the serum standard curve.

2.6. Effect of pH on UV absorbance

A methanolic solution of AG 331 (200.0 μg/ml) was diluted with either water or phosphate buffers (pH range: 1–10) to give the same final AG 331 concentration (20.0 μg/ml). The UV absorbance spectra of each was determined by scanning the samples from 400 to 200 nm. In addition, to 100 μl of each of the samples, 50 μl of internal standard was added and the samples were injected into the HPLC column to determine the effect of sample pH on the peak-height ratio of drug to internal standard.

2.7. Determination of standard curve and assay validation

Standard curves were generated by plotting the peak-height ratio of AG 331 to internal standard versus AG 331 concentrations. Separate standard curves were generated using mobile phases that differed in pH and ionic strength. The equations describing the standard curves were determined using least-squares regression analysis. Assay reproducibility was determined by assaying six freshly prepared AG 331 concentrations in serum on five separate days. Between-run and within-run assay variability was determined for three serum concentrations (100.0, 1000.0 and 1500.0 ng/ml).

2.8. Recovery and stability of AG 331

Extraction efficiency was determined for the liquid extraction method by comparing the peak heights of standard solutions of AG 331 in serum that have undergone liquid extraction to that of identical concentrations of AG 331 prepared in the mobile phase without extraction.

The stability of AG 331 in aqueous solution and serum was determined at temperatures of −70, 4, 20 and 100°C), and a concentration range of 100.0–1500.0 ng/ml.

3. Results and discussion

The UV absorbance spectra of AG 331 in phosphate buffer at pH 3.0, 7.0 and 10.0 are shown in Fig. 2. The UV spectra were qualitatively similar in the three phosphate buffers although minor changes in absorbance at various wavelengths were observed at pH 10.

The pH of the sample solution injected into the HPLC was found to have a substantial effect on the peak-height ratio of AG 331 to internal standard (Fig. 3.). The decrease in peak-height ratio in samples with elevated pH was not attributed to AG 331 degradation but was likely due to adsorption of AG 331 relative to internal

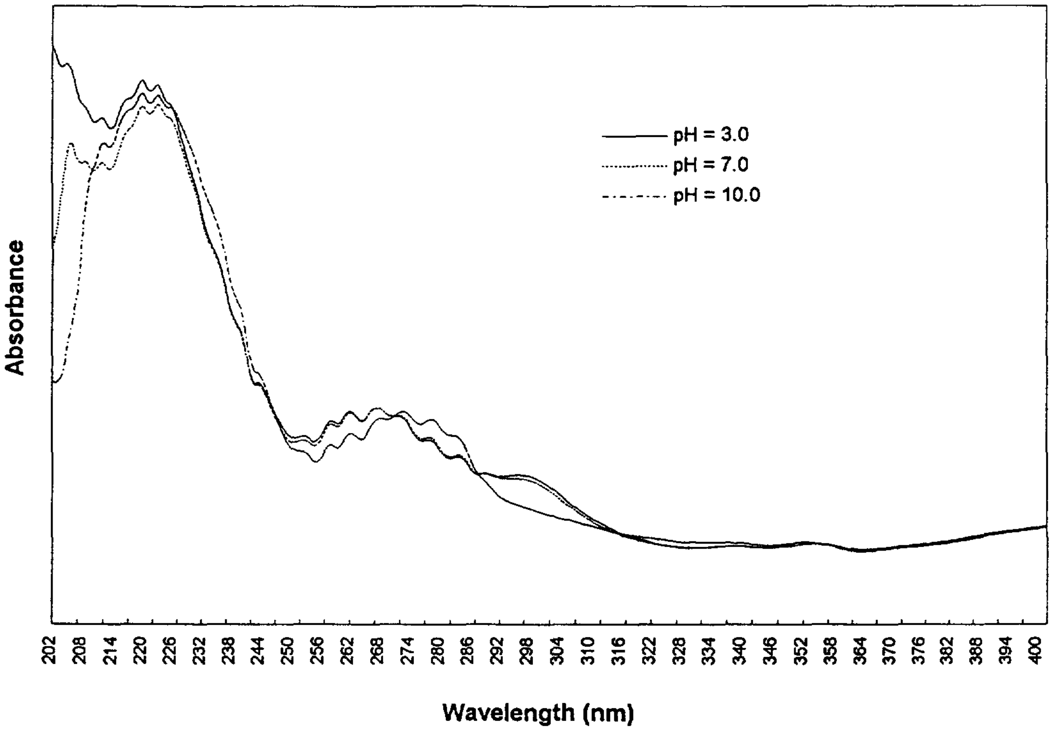

Fig. 2. UV absorbance spectra of AG 331 in solutions (20 μg/ml) buffered at various pH.

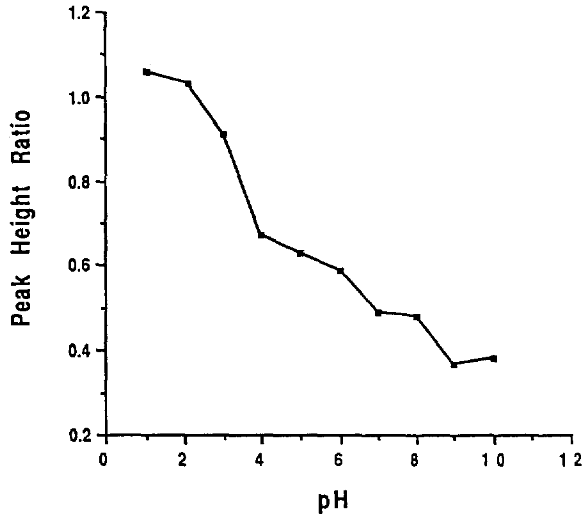

Fig. 3. Effect sample pH on the peak-height ratio of AG 331 to internal standard.

standard to containers or precipitation of AG 331 during sample preparation. This effect was more pronounced in aqueous solutions of AG 331 than in serum samples. Decreases in peak-height ratio observed in neutral or alkaline solutions of AG 331 could be reversed by the addition of mobile phase which resulted in acidification of the sample or by reconstituting the sample with mobile phase following extraction and evaporation. In order to decrease the variability caused by adsorption or precipitation, unnecessary transfer of samples from one container to another was avoided and samples were carefully sonicated before transfer into extraction tubes [5]. An acidic mobile phase was selected to optimize peak-height ratios and to minimize loss. During assay development, several mobile phases were considered. At pH 3.0, a mobile phase containing 100 m*M* monobasic phosphate was found to optimize peak shape and to reduce the retention time of AG 331 and internal standard. The adjustment of pH with HCl resulted in reducing peak tailing. Although assay sensitivity could be improved at pH <2.5, the lower pH was not favorable for column longevity.

Stability studies of AG 331 were conducted at various temperatures. AG 331 stored in methanol or human serum at -70°C in capped polypropylene tubes for up to four months showed no change in concentration. However, serum specimens stored at 4°C for one week resulted in a 5–10% decrease in concentration. In addition, AG 331 solutions stored in phosphate buffer (pH = 7.0) or water at room temperature for one week showed a 25% decrease in AG 331 concentration. Heating AG 331 solutions to 100°C at pH 3.0 and 7.0 for 2 h resulted in a decrease in AG 331 concentration of 10 and 25%, respectively. Degradation of AG 331 at elevated temperatures and surface adsorption (primarily to glass containers) were found to be the major causes of assay variability. Results suggest that serum AG 331 specimens should be stored in polypropylene tubes, frozen at -70°C, prior to analysis.

For the clean-up of AG 331 serum samples, solid-phase extraction using C_{18} solid-phase extraction columns (200 mg) was initially attempted. Recovery using solid-phase extraction was initially found to be poor. However, by increasing the volume of methanol (to 5 ml) used to elute the drug from the extraction column, the recovery of AG 331 and internal standard could be substantially improved. The mean ± S.D. recovery for six AG 331 concentrations ranging from 50 to 2000 ng/ml following solid-phase extraction was $91.4 \pm 4.3\%$ for AG 331 and $90.2 \pm 1.7\%$ for the internal standard, benz[*cd*]indol-2(1H)-one. The recovery using liquid extraction was $78.4 \pm 2.0\%$ for AG 331 and $76.8 \pm 2.5\%$ for the internal standard, 4,4′-diaminodiphenyl sulfone. The recovery efficiency for AG 331 using liquid extraction was not affected by either the volume of ammonium hydroxide used (50–200 μl) or the extraction time.

Typical retention times for AG 331 and the internal standards, 4,4′-diaminodiphenyl sulfone and benz[*cd*]indol-2(1H)-one, were 9.4, 4.7 and 8.4 min, respectively. Chromatograms using liquid extraction appeared to have less interference from the matrix compared to chromatograms using solid-phase extraction. With many interfering peaks eluting between 4 and 5 min with solid-phase extraction, 4,4′-diaminodiphenyl sulfone was not a suitable internal standard. How-

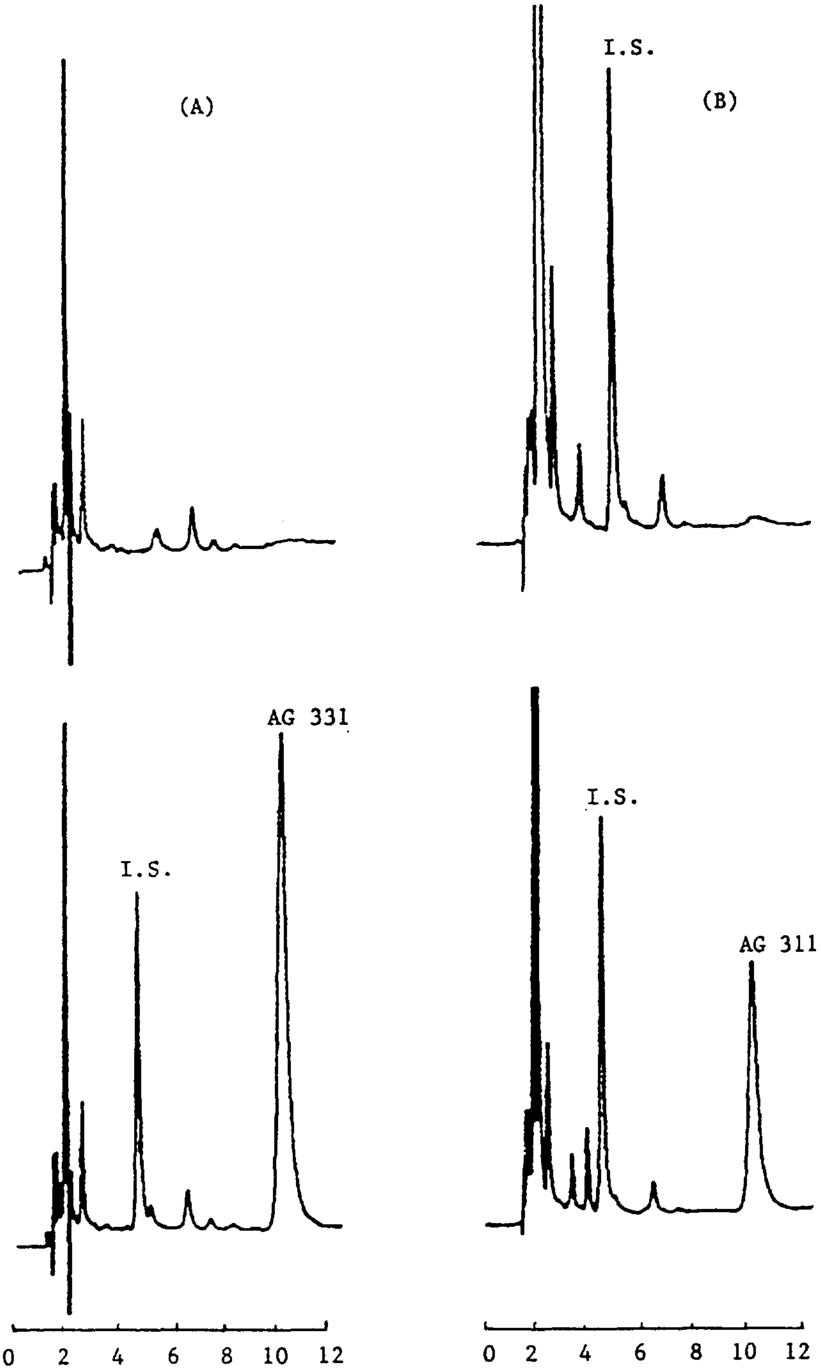

Fig. 4. Typical HPLC chromatograms showing AG 331 and internal standard. (A) Blank normal serum (upper), and spiked with 1000.0 ng/ml AG 331 and 750.0 ng/ml internal standard (I.S.) (lower). (B) Patient serum spiked with 750.0 ng/ml I.S. (upper), and 30 min after i.v. infusion of AG 331 (lower).

Table 1
Day-to-day variability of six AG 331 concentrations measured on five separate days

AG-331 concentration (ng/ml)		C.V. (%)	Error (%)
Actual	Found ($n=5$)		
50.0	51.3 ± 3.0	5.8	2.6
100.0	104.9 ± 9.7	9.2	4.9
200.0	196.0 ± 4.6	2.3	2.0
500.0	503.8 ± 24.7	4.9	0.8
1000.0	979.7 ± 38.0	3.9	2.0
2000.0	2012.5 ± 24.1	1.2	0.6

Standard curve (five separate days): slope (mean ± S.D.) = 0.001207 ± 0.000037; *y*-intercept (mean) = 0.0014799; r^2 (mean) = 0.999

ever, either internal standard was satisfactory when using liquid extraction. For the results reported herein we have used solvent extraction for sample preparation and 4,4′-diaminodiphenyl sulfone as the internal standard.

A typical HPLC chromatogram of 200.0 ng/ml AG 331 and internal standard in human serum is shown in Fig. 4. The assay was validated for day-to-day precision and accuracy by assaying six AG 331 concentrations in serum on five separate days. The results of this study are presented in Table 1. The standard curve was linear over a concentration range of 50.0 to 2000.0 ng/ml, with a mean slope of 0.001207 ± 0.000037 (mean ± S.D.) and a *y*-intercept of 0.001799. The correlation coefficient of the standard curve was 0.999. The within-run and between-run precisions of the method were determined at three pre-selected serum concentrations: 100.0, 1000.0 and 1500.0 ng/ml, reflecting typical low, medium, and high concentrations that were anticipated in patient samples. The results of the within-run and between-run validation studies are shown in Tables 2 and 3. The within-run precision was determined on three AG 331 concentrations stored at −70°C for 24 h after preparation. Within-run validation results show that the found concentrations (mean ± S.D.) for five assays for serum samples containing 100.0 ng/ml, 1000.0 ng/ml and 1500.0 ng/ml were 99.7 ± 5.0, 970.3 ± 35.0 and 1497.4 ± 62.7 ng/ml, respectively, with C.V. ≤5.0% and a percent error ≤3.0%.

Samples used for between-run validation studies were stored at −70°C for 1–6 days. Between-run results show that the mean ± S.D. values for five assays for serum samples con-

Table 2
Within-run precision for AG 331 assay in serum

Sample No.	Concentration found		
	100.0 (ng/ml)	1000.0 (ng/ml)	1500.0 (ng/ml)
1	102.5	1017.2	1440.2
2	106.8	918.8	1471.1
3	95.2	969.6	1476.0
4	98.3	969.0	1495.9
5	95.5	976.8	1603.6
Mean ± S.D.	99.7 ± 5.0	970.3 ± 35.0	1497.4 ± 62.7
C.V. (%)	5.0	3.6	4.2
Error (%)	0.3	3.0	0.2

Table 3
Between-run precision for AG 331 assay in serum

Run No.	Concentration found		
	100.0 (ng/ml)	1000.0 (ng/ml)	1500.0 (ng/ml)
1	106.0	1026.7	1471.0
2	108.8	999.2	1484.5
3	102.0	962.4	1495.9
4	99.7	970.3	1497.4
5	97.0	1028.5	1441.2
Mean ± S.D.	102.7 ± 4.7	997.4 ± 30.8	1478.0 ± 23.1
C.V. (%)	4.6	3.1	1.6
Error (%)	2.7	2.6	1.5

taining 100.0 ng/ml, 1000.0 ng/ml and 1500.0 ng/ml were 102.7 ± 4.7, 997.4 ± 30.8 and 1478.0 ± 23.1 ng/ml, respectively, with C.V. $\leq 4.6\%$ and a percent error $\leq 2.7\%$. The minimum level of detection for the method is 20 ng/ml (peak height four times greater than background noise) and the minimum quantifiable concentration of AG 331 was 50.0 ng/ml. Although the method was not validated for AG 331 in urine, it is likely that the method is applicable for urine specimens in view of the occurrence of substantially higher urinary AG 331 concentrations.

AG 331 is currently being investigated in a Phase I clinical trial. Single escalating doses of AG-331 starting at 12.5 mg/m^2 to 225 mg/m^2 were administered as a 10 min i.v. infusion. Serial blood samples were collected over a period of 72 h. AG 331 serum concentrations were assayed using the method described above and ranged from less than 20 ng/ml to greater than 2500 ng/ml. A typical plasma concentration–time profile for AG 331 from three dose levels (50 mg/m^2, 130 mg/m^2 and 225 mg/m^2) in three individual patients are shown in Fig. 5. The method described herein is rapid and reliable and has proved to be useful in studying the pharmacokinetics of AG 331.

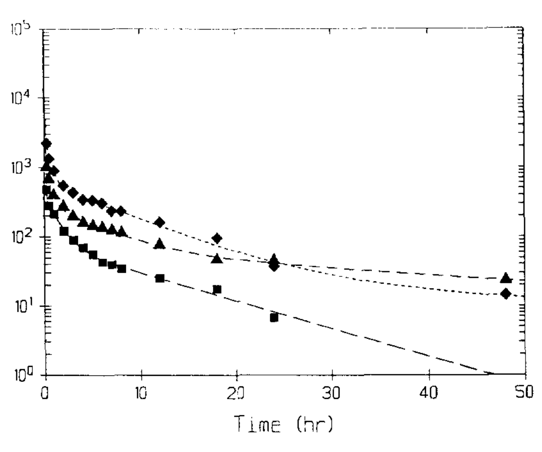

Fig. 5. Typical plasma concentration–time profiles of AG 331 in patients following 10 min i.v. infusions of (■) 50, (▲) 130 and (◆) 225 mg/m^2 of AG 331.

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