ELSEVIER

Journal of Chromatography B, 666 (1995) 307–314

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic method for the determination of AG-331, a novel anti-cancer agent, in human serum and urine using solid-phase extraction and photodiode-array detection

Mingxin Qian, Peter J. O'Dwyer, James M. Gallo*

Department of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA

First received 14 September 1994; revised manuscript received 6 December 1994; accepted 20 December 1994

Abstract

A reversed-phase isocratic high-performance liquid chromatographic method has been developed for the determination of AG-331, a novel thymidylate synthase inhibitor, in human serum and urine. The method involves a solid-phase extraction from C_{18} cartridges without addition of an internal standard. The methanol eluent is evaporated under nitrogen at 40°C, and reconstituted in mobile phase, acetonitrile–water (35:65, v/v) containing 25 m*M* ammonium phosphate. Separation of AG-331 was obtained on a C_{18} column at a flow-rate of 1 ml/min. Chromatographic signals were monitored by a photodiode-array detector at a primary wavelength of 457 nm with a bandwidth of 4.8 nm. Standard curves are linear in the range of 22–2175 ng/ml in plasma and 44–2175 ng/ml in urine, respectively. The extraction recovery ranged from 92.9–102.4%. Intra-day coefficient of variation was less than 9.5%, and inter-day coefficient of variation was less than 14.3% for an AG-331 concentration of 44 ng/ml. This method has been used to characterize the pharmacokinetics of AG-331 in cancer patients as part of ongoing Phase I trials.

1. Introduction

N^6 - [4 - (Morpholinosulfonyl)benzyl] - N^6-methyl - 2,6 - diaminobenz[*cd*]indole glucuronate (AG-331, I, Fig. 1), is a novel lipophilic inhibitor of thymidylate synthase (TS), the rate-limiting step in the biosynthesis of thymidine and hence of DNA. This compound was rationally designed based on knowledge of the three dimensional structure of the TS active site as determined by X-ray crystallography. The tight binding molecular structure of I was developed by de novo design at the cofactor binding site of the protein, and was optimized in an iterative process by solving co-crystallization and structure determinations of protein–ligand complexes [1]. Compound I has substantial preclinical activity as an anti-tumor agent, and reversal studies associated that cytotoxicity with its potent inhibition of TS [1–3].

Compound I does not possess a glutamate moiety, as do other folate-based TS inhibitors (e.g. D1694 and CB3717), and so does not undergo polyglutamylation. Polyglutamylation

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00589-3

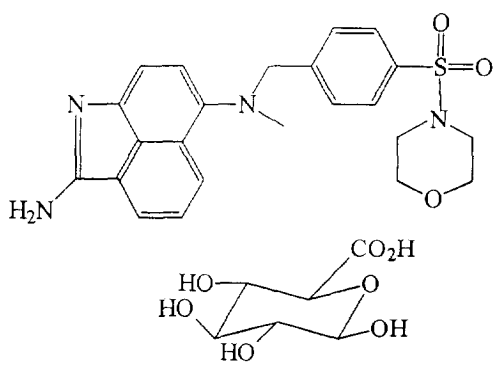

Fig. 1. Chemical structure of AG-331, N^0-[4-(morpholinosulfonyl)benzyl]-N^0-methyl-2,6-diaminobenz[*cd*]indole glucuronate (I).

can enhance cellular retention but may also contribute to toxicity. The lipophilic nature of I may facilitate its penetration into solid tumor masses. In vitro and in vivo investigations have demonstrated the potential of I as an anticancer agent [1–3] and provided a basis to undertake Phase 1 clinical trials. The purpose of the current study was to develop an HPLC method for I in serum and urine samples obtained from ongoing clinical studies that could be used in its pharmacokinetic characterization.

2. Experimental

2.1. Chemicals and reagents

Compound I and internal standard, 4,4-aminodiphenyl sulfone, were obtained from Agouron Pharmaceuticals (San Diego, CA, USA). Acetonitrile and methanol were HPLC grade and purchased from Baxter (Burdick and Jackson Division, Muskegon, MI, USA). Ammonium phosphate was obtained from Sigma (St. Louis, MO, USA). Distilled water was deionized and filtered through a Millipore (Bedford, MA, USA) Milli-Q ultra-pure water system. Bond Elut C_{18} cartridges (300 mg) were obtained from Varian (Harbor City, CA, USA). Blank human serum and urine were obtained from healthy volunteers.

2.2. Preparation of standard and quality control samples

Solutions of I were prepared in mobile phase, acetonitrile–water (35:65 v/v), with 25 m*M* ammonium phosphate buffer (pH 3.5) over the concentration range 1.1–217.5 μg/ml. Ten microliter of each working solution were combined with blank human serum (1 ml) or urine (3 ml) to yield calibration standards over the concentration range 11–2175 ng/ml. Serum and urine quality control (QC) samples were similarly prepared at low, middle and high concentrations from different stock solutions prior to sample analysis.

2.3. Sample preparation

Calibration standards, quality control and human serum or urine samples were processed by a solid-phase extraction procedure on 3-ml Bond Elut C_{18} cartridges. The cartridges were first conditioned with 3 ml of methanol followed by 3 ml of Dulbecco's phosphate buffered saline (PBS, pH 7.2) under gentle vacuum. Each serum or urine sample was loaded onto a cartridge and washed twice with 3 ml of Dulbecco's PBS. After the cartridges were suctioned to near dryness under vacuum, the analytes were eluted into glass tubes with 3 ml of methanol. The eluants were evaporated to dryness under vacuum (Virtis Vac-Spin, Gardiner, NY, USA) or by nitrogen gas (TurboVap LV Evaporator, Zymark Co. Hopkinton, MA, USA) and then reconstituted in 200 μl of the mobile phase. The samples were transferred to vials used in the autoinjector and centrifuged at 15 000 *g* for 5 min. Aliquots (100 μl) of each sample were injected onto the HPLC system.

2.4. Chromatographic system

The HPLC system consisted of a Model 510 pump, a Model 717 WISP autoinjector, and a Model 996 photodiode-array detector (Waters Assoc., Milford, MA, USA). An Adsorbsphere HS C_{18} (5 μm, 250 × 4.6 mm I.D.) HPLC analytical column (Alltech Assoc., Deerfield, IL,

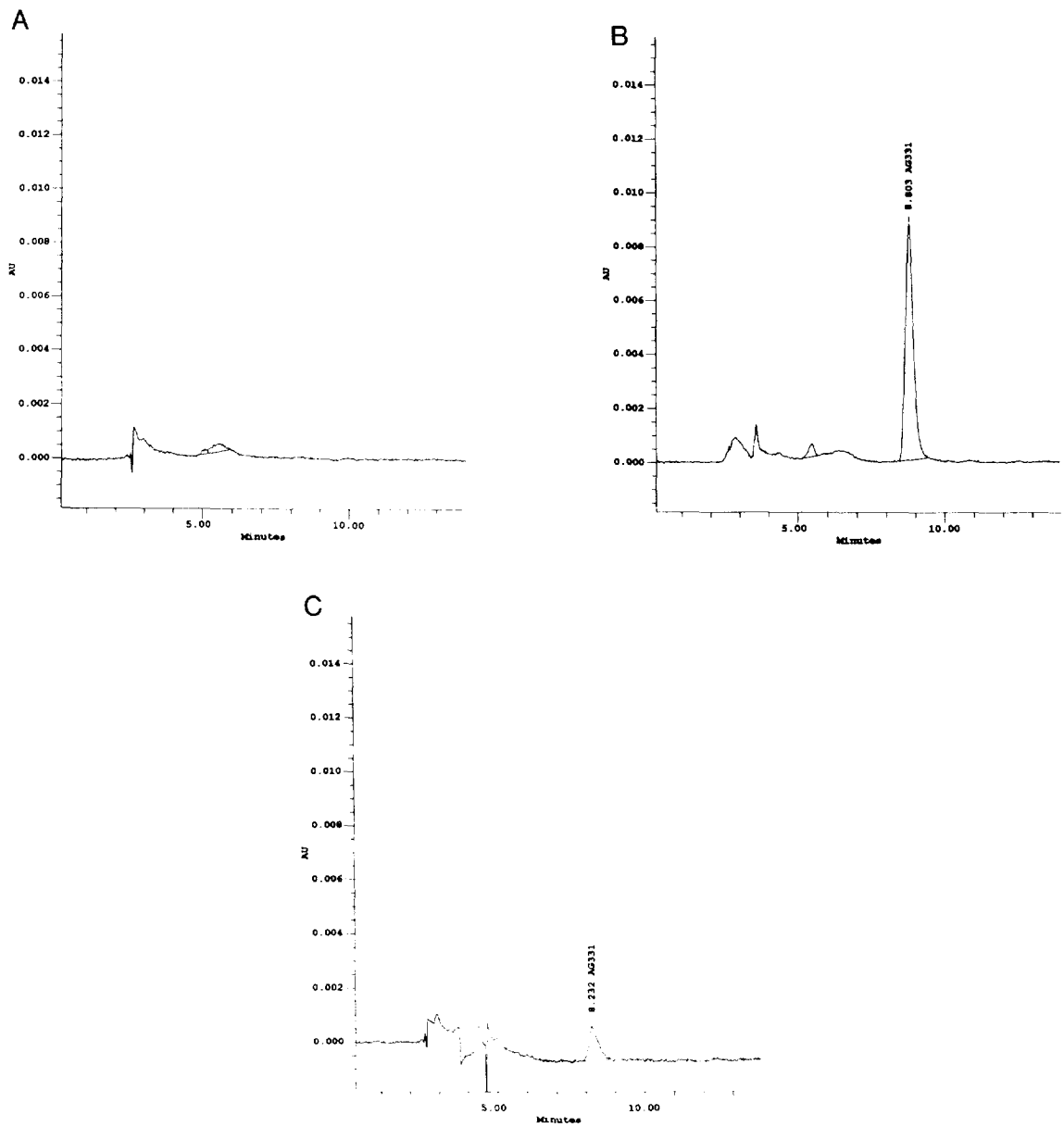

Fig. 2. Representative chromatograms of processed human serum. (a) Pooled blank serum, (b) pooled serum spiked with 435 ng/ml AG-331, (c) serum of a patient who received 100 mg/m^2 of AG-331 per day as a constant-rate infusion over 5 days.

USA) was used preceded by a guard column cartridge with the same solid phase. The mobile phase was prepared by mixing 350 ml of acetonitrile with 650 ml of deionized water, containing 25 m*M* ammonium phosphate; pH was adjusted to 3.5 with H_3PO_4. The mixture was filtered through a 0.2-μm nylon filter (Gelman Science, Ann Arbor, MI, USA) under vacuum. The chromatographic analyses were performed at ambient temperature at a flow-rate of 1 ml/min, with detection at 457 nm. Detector output was analyzed with Millennium 2010 Chromatography Manager (Waters Assoc.). In the early development of the method, spectral data over 220–475

nm were also monitored. Calibration curves were obtained by linear regression of peak height vs. concentrations of I in serum and urine, which were then used to calculate concentrations of I in the QC and unknown samples.

2.5. Assay validation

Stability

Chemical stability of I in stock solution stored at −20°C was examined over a five-week period.

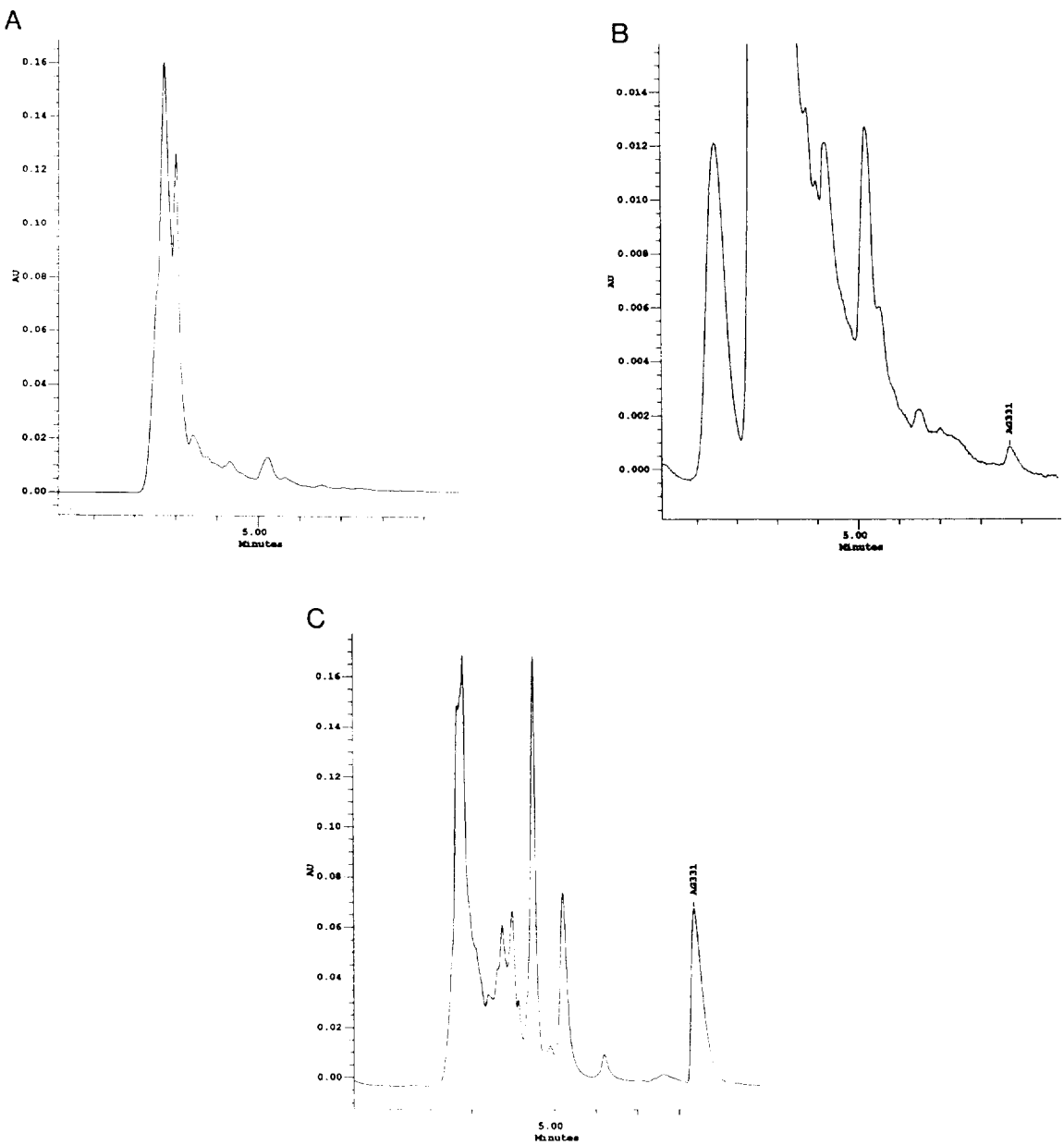

Fig. 3. Representative chromatograms of processed human urine. (A) Pooled blank urine, (b) pooled urine spiked with 50 ng/ml AG-331, (c) urine of a patient who received 200 mg/m^2 of AG-331 per day as a constant-rate infusion over 5 days.

The stability of I was determined in mobile phase stored at room temperature for six days. Storage stability of I in frozen human serum at concentrations of 21.8, 217.5 and 2175 ng/ml ($n = 3$ for each level) was also examined at −80°C over a 3.5-month period. All samples were prepared and analyzed by the HPLC procedure indicated above.

Limit of quantitation (LOQ)

Human serum and urine from volunteers were spiked with 10 μl of working solution of I ($n = 4$) to produce final concentrations of 4.4, 10.9 and 21.8 ng/ml. The samples were prepared and analyzed according to the stated method. The precision, accuracy and confidence interval were used to determine the LOQ.

Accuracy and precision

The accuracy and precision of the method were determined by analyzing serum and urine at low, middle and high concentrations on the same day (intra-assay, $n = 5$) and at all concentrations on different days (inter-assay, $n = 4$–9). Accuracy was expressed as the percentage bias and was calculated as shown in Table 2, and precision was expressed as the percentage coefficient of variation (C.V.%) of the means from these runs.

Recovery

Blank serum and urine were processed as described following addition of I at concentrations of 21.8, 217.5 and 1087.6 ng/ml for serum and 43.5, 108.8 and 1087.6 ng/ml for urine. The extraction recovery was assessed by comparison of the peak heights obtained by direct injection of I, equivalent to the quantity added to serum and urine samples, to peak heights obtained from serum and urine samples that contained known amounts of I.

2.6. Phase I clinical trial

Serum concentrations of I were determined in cancer patients receiving I in 5% dextrose as a 120-h continuous infusion. Blood and urine samples were collected at specified times, serum was harvested by centrifugation for 5 min at 2500 *g*, and then samples were frozen at −80°C until analyzed. Resultant serum concentration of I were analyzed by noncompartmental method [4].

3. Results and discussion

3.1. Chromatographic separation

Representative chromatograms from human serum and urine, are presented in Figs. 2 and 3, respectively. Peak identification of I was confirmed by 3-D spectral analysis. The retention time of I was 8–9 min, and was accompanied by an endogenous peak at 21–22 min. By setting the run time to 14 min, the potential interference of this late-eluting peak with I in subsequent runs was avoided. As seen in the chromatograms, the peak of I was symmetrical and well separated from endogenous peaks when utilizing 457 nm for detection. In the early stage of assay development, detection of I and an internal standard, 4,4'-aminodiphenyl sulfone was made at various wavelengths of I (see Table 1). However, preliminary validation data under the multiple-signal mode of the photodiode-array detector showed there were endogenous peaks that could interfere with quantitation of I and internal standard in serum and urine (see Fig. 4). These interfering peaks were also shown in the blank

Table 1
Variablity (C.V.%) in detection of AG-331 in human serum (25 ng/ml, $n = 3$) at different wavelengths

Wavelength (nm)	Coefficient of variation (%)[a]		
	AG-331	I.S.[b]	AG-331/I.S.
220	29	10	35
230	25	10	32
265	40	9	52
290	81	9	86
457	13	NA[c]	NA[c]

[a] Based on sample peak height or peak-height ratio.
[b] Internal standard.
[c] Not available.

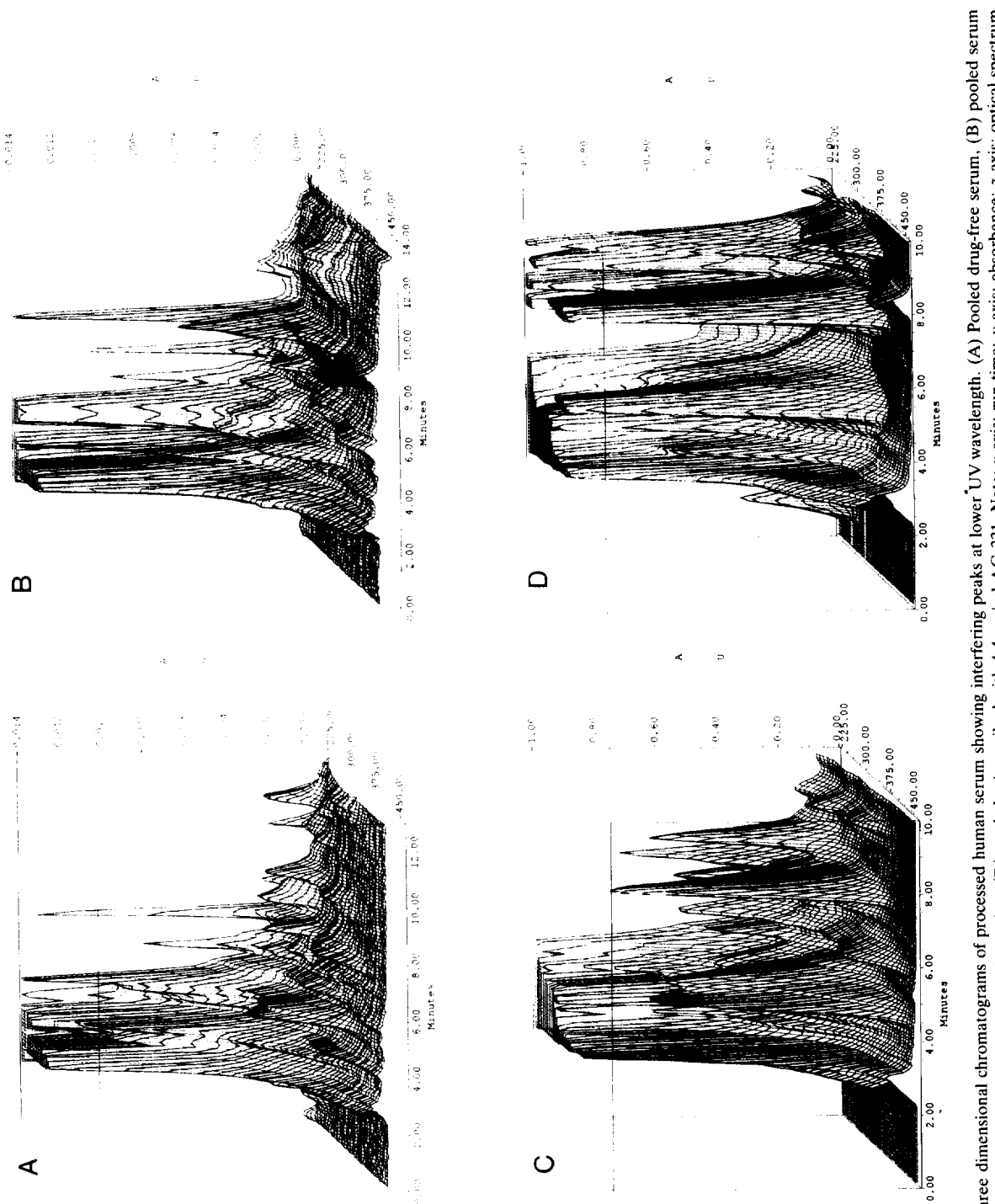

Fig. 4. Three dimensional chromatograms of processed human serum showing interfering peaks at lower UV wavelength. (A) Pooled drug-free serum, (B) pooled serum spiked with 218 ng/ml AG-331, (C) drug-free urine, (D) pooled urine spiked with 4.4 μg/ml AG-331. Note: *x*-axis: run time; *y*-axis: absorbance; *z*-axis: optical spectrum from 210 to 475 nm. Retention time of AG-331 was 8.5 min, as indicated by arrows.

serum from four out of six patients prior to the start of administration of I. It was subsequently decided to utilize 457 nm for further assay development because no interfering peaks were detected at this wavelength. Since I shows a 7-fold lower absorbance at 457 nm compared to 220 nm, an increase from 0.2 ml to 1 ml of serum sample partially compensated for the loss in sensitivity at 457 nm. The internal standard did not appreciably absorb at 457 nm, thus the assay was developed based on external standard methodology. In order to prolong column life, reconstituted samples were centrifuged prior to injection and guard column cartridges were changed every 50 sample injections.

3.2. Extraction recovery

Absolute recoveries of I, using a solid-phase extraction method, ranged from 92.8 to 96.4% in serum and from 96.9 to 103.4% in urine, with a high degree of reproducibility (C.V. $< 8.4\%$, see Table 2). Similar recovery of I was obtained with a 300-μl serum volume and 1-ml solid -phase extraction cartridges. Since a 1-ml sample size was used to increase sensitivity, 3-ml extraction cartridges were used to avoid potential problems in blockage of solvent flow. In the washing steps prior to and after application of sample, use of PBS rather than deionized water drastically increased recovery of I by more than 50%. Liquid–liquid extraction methods, using ethyl acetate or ether, resulted in recoveries of only 75% for I (data not shown). In addition to a high recovery, the solid-phase extraction method offers advantages in purification and concentration of I in urine samples, which proved difficult by direct or dilution injection methods, since only very low levels of I were present.

Table 2
Intra-day recovery, accuracy and precision of AG-331 analysis in human serum and urine

AG-331 added (ng/ml)	AG-331 measured (ng/ml)	Recovery (%)	Bias[a] (%)	C.V. (%)
Plasma				
22	22.9	92.9	5.5	4.5
218	197.6	96.5	−9.2	5.7
1088	1002.5	93.0	−7.8	8.0
Urine				
44	47.2	96.9	8.6	8.4
109	107.6	103.4	−1.0	3.7
1088	984.0	102.3	−9.5	7.0

$n = 5$ for all samples.
[a] Bias = [(measured conc. − added conc.)/added conc.] · 100.

3.3. Assay validation

Based on the recent recommendations made by Shah et al. [5], the LOQ was determined to be 22 ng/ml in serum and 44 ng/ml in urine. Daily standard curves and QC samples were generated for I in serum and urine. The assay was linear over a concentration range of 22 to 2175 ng/ml in serum, and 44 to 2175 ng/ml in urine, with the coefficient of determination (r^2) of greater than 0.993. The validation data of I in human serum and urine are presented in Tables 2 and 3. In most cases, the accuracy and precision was 15% or less. These results indicated that the external standard method was very

Table 3
Inter-day precision and accuracy of AG-331 analyses in human serum and urine

AG-331 added (ng/ml)	AG-331 measured (ng/ml)	Bias (%)	C.V. (%)	n
Serum				
22	22.1	1.7	20.8	9
44	42.2	−3.0	14.3	9
109	106.9	−1.7	8.7	9
218	219.0	0.7	7.7	9
435	431.8	−0.7	3.0	9
1088	1124.3	3.4	7.6	5
2175	2223.7	2.2	4.7	4
Urine				
44	50.2	15.3	3.9	4
109	107.8	−0.9	6.9	4
218	200.0	−8.1	5.0	4
435	402.8	−7.4	8.8	4
1088	1117.4	2.7	4.4	4
2175	2050.5	−5.7	11.3	4

precise and accurate, and thus an effort to identify an internal standard was unnecessary.

3.4. Stability

Stock solutions of I prepared in PBS or in mobile phase, were stable for up to two months at −20°C. Standard solutions, prepared in mobile phase, were stable with less than 1.6% change at 15.8 μM and 158 μM of I at room temperature for at least 144 h, indicating that degradation during sample preparation and autoinjection is negligible. In addition, I was stable in human serum frozen at −80°C over a period of at least 3 months. Between day 1 and 3.5 months, the changes in the peak height values of I were −16.2%, 5.6% and 1.9% ($p > 0.05$) at starting concentrations of 21.8, 217.5 and 2175 ng/ml, respectively.

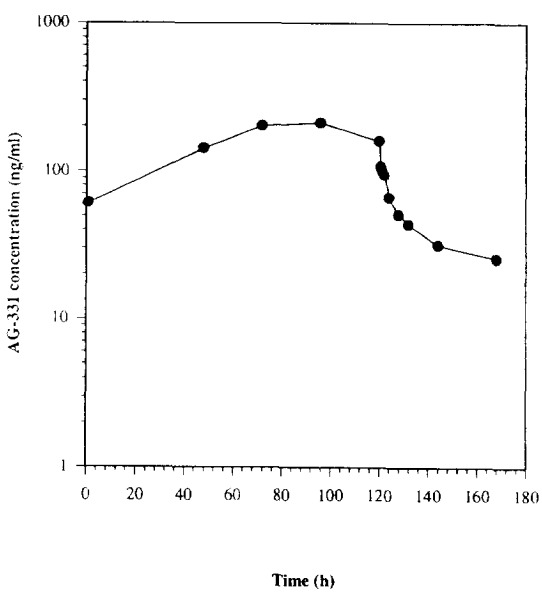

Fig. 5. Representative serum concentration–time profile of AG-331 in one patient who received 100 mg/m^2 of AG-331 per day as a constant rate-infusion over 5 days.

3.5. Clinical pharmacokinetic study

Fig. 5 shows the serum concentration–time profile of I in one patient after i.v. infusion of 100 mg/m^2/d of I for five days. Noncompartmental analysis of the patient's data resulted in a total clearance of 9.9 l/h/m^2, steady-state volume of distribution of 98.3 l/m^2 and elimination half-life of 35.4 h. The fraction of I excreted unchanged in urine was less than 5%, which is consistent with the recent report by Koda et al. [6] in which 3.97% of an i.v. bolus dose of I was found in urine, and indicates that I undergoes substantial non-renal elimination in humans.

Acknowledgement

This work was partially supported by a grant from Agouron Pharmaceuticals (San Diego, CA, USA).

References

[1] M.D. Varney, P.M. Gifford, L.P. Cindy, G.D. Judith, W. Stephanie, M.W. Katherine, J.B. Russell, A.B. Charlotte, A.M. Catharine, L.J.B. Carol, M.H. Steven, F.H. Eleanor, W.W. Ward and W. Jennifer, *J. Med. Chem.*, 35 (1992) 663.

[2] S. Webber, B. Shetty, A. Johnson, K. Welsh, M. Varney, J. Deal, C. Morse and K. Koda, *Proc. Am. Assoc. Cancer Res.*, 33 (1992) 413.

[3] B. Mitrovski, P.G. Johnson and C. Erlichman, *Proc. Am. Assoc. Cancer Res.*, 35 (1994) 300.

[4] M.L. Rocci and W.J. Jusko, *Comp. Prog. Biomed.*, 16 (1983) 203.

[5] V.P. Shah, K.K. Midha, S. Dighe, L.J. Mcgilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, A.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, *J. Pharm. Sci.*, 81 (1992) 309.

[6] R.T. Koda, L. Leichman, S. Webber, B.V. Shetty, C.G. Jeffers and F.M. Muggia, *Proc. Am. Assoc. Cancer Res.*, 35 (1994) 432.