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# Development and validation of an HPLC method for AG331 bulk drug substance and lyophilized powder for injection

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**Abstract**

AG331 is a water soluble glucuronate salt of a novel antitumor compound synthesized by protein structure based drug design. A lyophilized powder for injection was developed for clinical studies. During HPLC assay development, AG331 showed an inherent tailing problem due to an amino group in the structure. An optimized reverse-phase gradient HPLC method was developed to minimize the tailing and separate AG331 from its synthetic intermediates (I-1, I-2, I-3, I-4, I-5, I-6, I-8), other impurities and degradation compounds. The method was shown to be linear, precise, accurate, rugged and stability-indicating.


*Keywords:* AG331; Method development; Method validation; Lyophilized powder for injection formulation

## 1. Introduction

AG331, a novel thymidylate synthase (TS) inhibitor, was designed and synthesized based on technology of the three dimensional structure of the TS active site [1]. Classical TS inhibitors, structurally characterized by the presence of a glutamate moiety, have been found to be highly toxic [2]. AG331, a water soluble molecule lacking a glutamate moiety, acts directly towards the TS enzyme and has shown potent anti-tumor activity in vivo against both intraperitoneal and intramuscular tumors. A lyophilized powder for injection was developed for phase I clinical studies.

During HPLC assay development [3], AG331 showed an inherent tailing problem due to an amino group in the structure. The purpose of this study was to develop an efficient, sensitive method for the determination of AG331, its impurities and degradation compounds and minimize the tailing problem. This method was also validated according to good laboratory practice guidelines, and was successfully used to quantitate the stability of AG331 bulk drug substance and AG331 in lyophilized powder for injection for long term stability studies.

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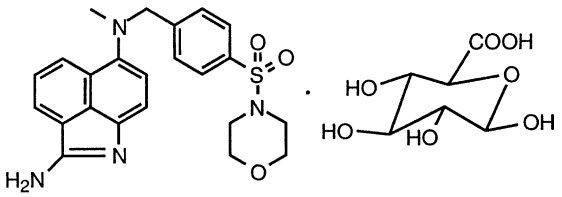


Fig. 1. Structure of AG331.

## 2. Experimental

### 2.1. Materials

The bulk drug substance, AG331, $N^6$-[4-(morpholinosulfonyl)benzyl]-$N^6$-methyl-2,6-diaminobenz[cd] indole glucuronate, was synthesized by Agouron Pharmaceuticals Inc. Its formula is $C_{29}H_{34}N_4O_{10}S$, having a molecular weight of 630.69. The structure of AG331 is shown in Fig. 1. The drug product, a lyophilized product containing 44.4% (w/w) of AG331, 55.6% (w/w) of mannitol and trace of water was developed for parenteral delivery of AG331 at Agouron Pharmaceuticals Inc. [3]. Multiple lots of bulk drug with varying impurity profiles were used for development and validation of the HPLC method.

Acetonitrile, methanol, ammonium phosphate monobasic, triethylamine, *o*-phosphoric acid 85% and water were HPLC grade. Sodium hydroxide and potassium chloride were ACS grade reagents. Hydrochloric acid (37%) was trace metal grade. Acetonitrile and methanol were degassed by sonication. Ammonium phosphate solution was filtered and degassed with 0.46 μm nylon 66 filter. The mannitol powder, USP/NF was purchased from Spectrum chemical Mfg. Corp.

### 2.2. HPLC conditions

A Hewlett-Packard 1050 HPLC equipped with a diode-array detector, vacuum degasser, quaternary pump, and auto-sampler, was used as the primary instrument. The separations were accomplished on a Zorbax® SB-C8 column, 150 mm × 4.6 mm, at a flow rate of 1.1 ml/min at ambient temperature, with an injection volume of 10 μl. The analytical wavelength for peak detection was set at 220 nm.

A gradient reverse-phase high performance liquid chromatographic method was employed with an initial composition of 57% 20 mM ammonium phosphate per 10 mM triethylamine, pH 3.0, 26.5% acetonitrile and 16.5% methanol; for 7.0 min; then ramp up to 45% acetonitrile, 20% methanol in 6.0 min; 35% 20 mM ammonium phosphate per 10 mM triethylamine, pH 3.0, 45% acetonitrile and 20% methanol from 13.0 to 17.0 min.

### 2.3. Preparation of 2.5% mannitol solution (w/v)

The 2.5% mannitol solution was used to prepare AG331 standard solutions. Twenty-five grams of mannitol powder USP/NF was weighed into a 1000 ml volumetric flask and made up to final volume with HPLC grade water.

### 2.4. Standards preparation

Twenty milligrams of AG331 were accurately weighed to the nearest 0.01 mg into a 100 ml volumetric flask. The 2.5% mannitol solution was added and AG331 dissolved with sonication. Dilutions were prepared by pipetting 5, 10, 15, and 20 ml of the AG331 stock solution into 25 ml volumetric flasks and diluting to volume with 2.5% mannitol solution.

## 3. Results and discussion

### 3.1. Choice of detection wavelength

A UV-Vis scan was performed from 190 to 600 nm on an HPLC elution of each intermediate and AG331, using the diode array detector on the Hewlett-Packard HPLC. Absorbance maxima of each intermediate and AG331 were observed between 190 and 250 nm. A compromise wavelength of 220 nm was selected as the optimum wavelength for HPLC analysis because it maximizes the signal response of AG331 and impurity peaks known to be present in AG331, while giving a flat baseline.

### 3.2. Choice of column

The Zorbax® SB-C8, 150 mm × 4.6 mm, 5 μm column was the column selected for AG331, its impurities and degradation compounds. Separation can be achieved with a C18 column, however the tailing

factor of AG331 peak was considerable (>3.2). Using the more polar C8 column gave a more symmetrical AG331 peak and obtained good separation of a main impurity peak which had about 1.5% relative peak area compared to AG331.

### 3.3. Choice of mobile phase

In order to minimize the ion exchange interaction of AG331 with acidic silanols, triethylamine was used as a potential tailing suppressor at 10 mM in 20 mM ammonium phosphate; adjusted pH from 7.0 to 3.0 with *o*-phosphoric acid, 85%. The tailing factor was improved from 2.4 to 1.5. Initially the mobile phase used 20 mM ammonium phosphate per 10 mM triethylamine, pH 3.0:acetonitrile:methanol (57.0:26.5:16.5 (v/v)) at flow rate 1.1 ml/min. resulted in non-satisfactory separation among intermediates I-1, I-3 and I-4. By changing the percentages of acetonitrile and methanol to 45 and 20%, respectively, the polarity of the mobile phase was reduced which improved the separation of I-1, I-3 and I-4 and also shortened the elution time of I-6 and I-8 from 16.4 and 18.6 min, respectively, to 14.2 and 15.4 min, respectively.

Same lots of stability samples were tested to compare the current HPLC method, using a C18 column and two compositions of mobile phase without triethylamine, with the optimized method. The tailing factor was improved from 2.4 to 1.3 and the total impurity/degradant was increased from 1.11 to 2.20% for AG331 drug product and from 0.92 to 2.70% for AG331 drug substance when comparing the current method to the optimized method.

### 3.4. Linearity and precision

Two set of five standard solutions prepared as described in Standards Preparation containing concentrations of AG331 between 40 and 200 μg/ml were assayed using two different lots of columns. Each of the five concentrations was injected sequentially five times from lowest to highest concentration. The parameters of linear regression and peak area versus concentration, were statistically analyzed. The linearity of the method was determined by simple least squares analysis. Correlation coefficients of 0.9999 and 0.9994 were obtained over the concentration range. The relative standard deviations showed acceptable reproducibility, with R.S.D.s ranging from 0.38 to 1.35% and 0.20 to 1.22%, for set I and II, respectively. The results are shown in Table 1.

Table 1
Linearity and precision of standards

| Concentration (μg/ml) | Average peak area (S.D.) (mAU s) | R.S.D. (%) |
|---|---|---|
| 38.88 | 578.66 (6.63) | 1.15 |
| 77.76 | 1228.79 (14.95) | 1.22 |
| 116.6 | 1848.93 (7.26) | 0.39 |
| 155.5 | 2533.33 (17.94) | 0.71 |
| 194.4 | 3229.44 (6.39) | 0.20 |

Results of simple least-squares analysis for AG331 are as follows: intercept, −97.9104; slope, 16.9920; $R^2$, 0.9994.

### 3.5. Limit of quantitation and detection

The limits of detection and quantitation were determined by testing diluents of the lowest concentration of AG331 used for linearity, and then measuring the signal to noise ratio from the resulting chromatograms. The limit of quantitation was found to be 0.23 μg/ml with signal to noise ratio of about 10. The recovery of quantitation level was 124% with an R.S.D. less than 20%, which met the validation criteria for recovery and precision. The limit of detection which was set that the signal to noise level from resulting chromatogram was not less than three was about 0.10 μg/ml.

### 3.6. Accuracy

#### 3.6.1. Bulk drug

The average peak area for each concentration used for linearity and precision was subjected to the linear regression data to obtain calculated concentrations. The calculated concentrations were compared to the theoretical value to obtain percent recovery. The percent recovery between theoretical ($C_{theo}$) and calculated ($C_{calc}$) concentration is derived by the following equation:

$$\text{recovery (\%)} = \frac{C_{calc}}{C_{theo}} \times 100$$

the percent recovery of calculated value versus theoretical value at the extremes of the assay range was 99.0% for 38.54 μg/ml; and 99.8% for 192.7 μg/ml.

*3.6.2. Drug product*

Accuracy method of drug product, prepared solutions of the drug product by spiking into the placebo at three concentration: 42.89, 114.4, and 186.8 μg/ml and injected each solution three times and used linear regression data to calculate actual concentration, was determined by comparing calculated concentration to theoretical concentration. The percent recovery of calculated value versus theoretical value for 42.89 and 186.8 μg/ml were 100.41, 98.49 and 100.42%, respectively.

*3.7. Day to day variation/robustness*

Day to day variation/robustness of the method, prepared three different samples of AG331 near the target concentration of 160.0 μg/ml on three days and injected each sample in triplicate with normalization of different sample weights, was determined by percent difference over 3 days. Day I was 170.6 (peak area/sample weight (mg)) with 1.13% R.S.D., day II was 172.1 (peak area/sample weight (mg)) with 0.84% R.S.D. and day III was 171.1 (peak area/sample weight (mg)) with 0.64% R.S.D. The percent difference over 3 days was 4.2%.

*3.8. Analyst to analyst variation/robustness*

To ensure intermediate precision for an analytical method, a second analyst with knowledge in HPLC methodology performed the linearity and precision testing by preparing a solution of AG331 at 200.4 μg/ml and making dilutions from this solution to obtain concentrations of 160.3, 120.2, 80.16 and 40.08 μg/ml. Each of the five concentrations was injected sequentially five times from lowest to highest. The results are presented in Table 2.

*3.9. Solution stability*

In order to investigate the stability of AG331 solutions, standard solutions were prepared at concentrations of 38.88, 116.6, and 194.4 μg/ml as described in standards preparation section. Peak area responses were determined by assaying a freshly prepared standard solution and another standard solution after aging 24 and 48 h. The percent recovery of AG331 in the stored 24 h standards of 38.88, 116.6 and 194.4 μg/ml were 100.7, 100.6, and 100.3% for standards at room temperature of those found for the freshly prepared standards. The percent recovery of AG331 in the stored 48 h standards of 38.88, 116.6 and 194.4 μg/ml were 100.8, 100.1, and 100.4% for standards at room temperature of those found for the freshly prepared standards.

Table 2
Linearity and precision of standards from second analyst

| Concentration (μg/ml) | Average peak area (S.D.) (mAU s) | R.S.D. (%) |
|---|---|---|
| 40.08 | 693.15 (3.24) | 0.47 |
| 80.16 | 1466.57 (6.82) | 0.47 |
| 120.2 | 2252.45 (10.12) | 0.45 |
| 160.3 | 2988.17 (7.86) | 0.26 |
| 200.4 | 3823.97 (9.72) | 0.25 |

Results of simple least-squares analysis for AG331 are as follows: intercept, −89.9939; slope, 19.4202; $R^2$, 0.9997.

*3.10. Specificity-degradation studies*

Forced degradation studies were performed to provide an indication of the stability-indicating properties of the procedure. Forced degradation was attempted using acid, alkali and heat.

*3.10.1. Forced degradation sample stored at pH1.2/40 °C/121 days*

One milliliter aqueous solution of AG331 with 100 μg/ml concentration in HCl/KCl buffer, pH 1.2, was transferred into Type I clear glass ampoule. The ampoule was flame-sealed and stored at 40 °C stability chamber for 121 days. This sample was allowed to cool to room temperature before it was injected on Hewlett-Packard 1050 HPLC. The resolution between AG331 and its nearest degradation peak was 1.76. (see Fig. 2).

*3.10.2. Forced degradation sample stored at pH12.6/80 °C/91 h*

One milliliter aqueous solution of AG331 with 100 μg/ml concentration in NaOH/KCl buffer, pH 12.6, was placed into Type I clear glass ampoule. The ampoule was flame-sealed and stored at 80 °C stability chamber for 91 h. This sample was analyzed on Hewlett-Packard 1050 HPLC. The resolution between AG331 and its nearest degradation peak was 1.79. (see Fig. 3).

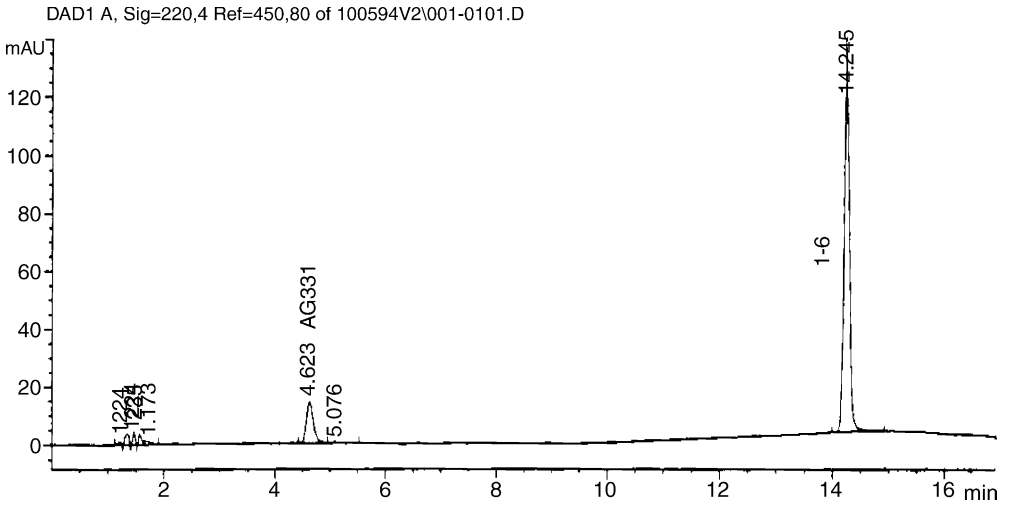


Fig. 2. Chromatogram of forced degradation sample by using acid and heat.

### 3.11. Impurity detection

This HPLC method was also evaluated in order to determine that impurities, intermediates, reagents or starting materials known to be present in AG331 were separated from the main peak in the chromatogram. A blank injection, consisting of 2.5% mannitol solution, was made in order to identify and peaks arising from the mobile phase, which resulted in no observation of peak. Samples of all available synthetic intermediates, starting materials, and synthetic reagents were run separately on the Hewlett-Packard 1150 HPLC. Those analyzed compounds are listed in Table 3, along with the corresponding retention times. The spiked solution containing all intermediates, reagents or starting materials known to be present in AG331 was injected on Hewlett-Packard 1050 HPLC. All of these compounds were observed to be well resolved from the main peak of AG331 with the exception of impurity 9. Since the impurity 9 is the free base of AG331. Therefore, it is impossible to separate AG331 and impurity 9. (see Fig. 4).

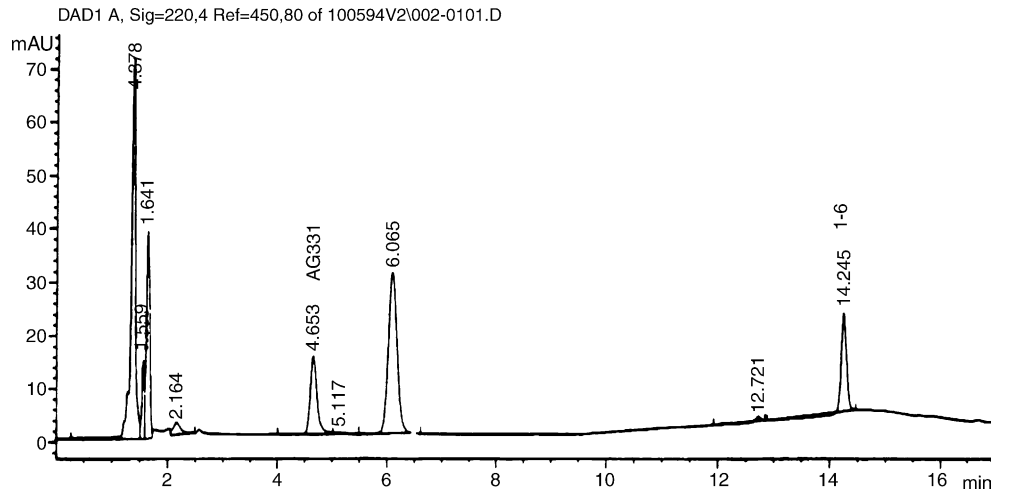


Fig. 3. Chromatogram of forced degradation sample by using alkali and heat.

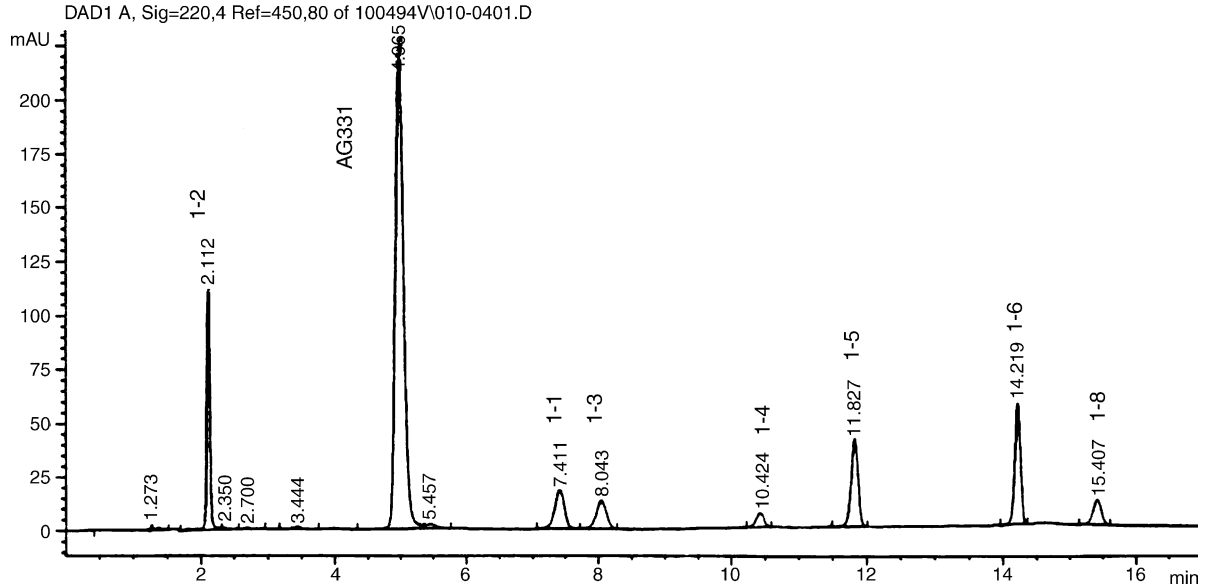


Fig. 4. Chromatogram of mixture of synthetic intermediates, coupling reagents and AG331.

Table 3
Retention time of analyzed compounds

| No. | Analyzed compounds | Retention time (min) |
|---|---|---|
| 1 | Intermediate 1 | 7.29 |
| 2 | Intermediate 2 | 2.11 |
| 3 | Intermediate 3 | 8.13 |
| 4 | Intermediate 4 | 10.50 |
| 5 | Intermediate 5 | 11.87 |
| 6 | Intermediate 6 | 14.24 |
| 7 | Intermediate 8 | 15.41 |
| 8 | Intermediate 9 | 4.95 |
| 9 | AG331 | 4.97 |

## 4. Conclusions

This optimized HPLC method suggests that significant changes have to be made to the current HPLC method for AG331, which include a different column, buffer system and detection wavelength. The improved method eliminated the tailing problem associated with the current method. It also offered significantly improved resolution and detection of impurity/degradation peaks. The optimized HPLC method involving a rapid assay for the determination of AG331 bulk pharmaceutical chemical and AG331 in lyophilized powder for injection and complete separation of impurities and degradation peaks from AG331 peak has been developed and shown to be accurate, linear, precise, reproducible, rugged and stability-indicating. Furthermore, all validation studies meet the predetermined acceptance criteria in accordance with the validation protocol.

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