Published in final edited form as:

J Chromatogr B Analyt Technol Biomed Life Sci. 2009 May 15; 877(0): 1460–1464. doi:10.1016/j.jchromb.2009.03.015.

Validation and implementation of a liquid chromatography/tandem mass spectrometry assay to quantitate aminoflavone (NSC 686288) in human plasma

Richard Wiegand, Jianmei Wu, Xianyi Sha, Patricia LoRusso, Elisabeth Heath, and Jing Li^{*}
Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201

Abstract

A reverse-phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method was developed and validated for determination of aminoflavone (AF) in human plasma. Sample preparation involved a liquid-liquid extraction by the addition of 0.25 mL of plasma with 1.0 mL ethyl acetate containing 50 ng/mL of the internal standard zileuton. The analytes were separated on a Waters X-TerraTM MS C₁₈ column using a mobile phase consisting of methanol/water containing 0.45% formic acid (70:30, v/v) and isocratic flow at 0.2 mL/min for 6 minutes. The analytes were monitored by tandem-mass spectrometry with electrospray positive ionization. Linear calibration curves were generated over the AF concentration range of 5–2000 ng/mL in human plasma. The lower limit of quantitation (LLOQ) was 5 ng/mL for AF in human plasma. The accuracy and within- and between-day precisions were within the generally accepted criteria for bioanalytical method (<15%). This method was successfully applied to characterize AF plasma concentration-time profile in the cancer patients in a phase I trial.

Keywords

Aminoflavone; High performance liquid chromatography; Mass spectrometry; LC-MS/MS; Pharmacokinetics

1. Introduction

Aminoflavone (AF; 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one; NSC 686288) (Figure 1a) is a novel diaminoanthraquinone analogue that has shown potent antiproliferative activity against selected human breast and renal cancer cell lines *in vitro* and *in vivo* [1–4]. AF displays a unique COMPARE pattern of antiproliferative activity against the panel of the National Cancer Institute (NCI) 60 human cancer cell lines, suggesting a novel mechanism of action. Its antiproliferative activity involves the induction of DNA-protein cross-links, replication-associated DNA double-strand breaks, oxidative DNA damage, S-phase checkpoint activation, and apoptosis [3,5]. AF undergoes extensive phase I metabolism by multiple cytochrome P450 (CYPs) as well as phase II metabolism by sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs) [6]. Metabolic activation of AF by human CYPs and SULTs, in particular, CYP1A1 and SULT1A1, has

^{*}Requests for reprints should be addressed to: Jing Li, Ph.D., Karmanos Cancer Institute, 4100 John R, HWCRC, Room 523, Detroit, MI 48201, USA. Phone: 313-576-8258; Email: lijn@karmanos.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

been implicated as an underlying mechanism for the unique pattern of tumor sensitivity to AF [1,4]. Specifically, AF is metabolized by CYP1A1 to form N-hydroxy AF compounds that are readily conjugated by SULT1A1, therefore forming N-sulfoxy-AF metabolites that are unstable and further converted into highly reactive nitrenium ions capable of reacting with cellular DNA and protein [4]. AF itself also induces its own metabolism by activation of transcription of CYP1A1 and SULT1A1 through the AhR pathway [1,2]. AF is highly lipophilic. In an effort to improve its solubility, AFP464 (NSC 710464, aminoflavone prodrug), which is a lysine derivative of AF, has been synthesized. The prodrug is rapidly converted to AF in plasma by nonspecific plasma esterases. AFP464 is currently being evaluated in a Phase I clinical trial in patients with advanced or refractory solid tumors at several institutions including the Karmanos Cancer Institute at Wayne State University (HIC# 045606MP2F).

To evaluate the pharmacokinetic behavior of AF in patients, a specific, sensitive, accurate, and reproducible method for the quantitation of AF was necessary. In this report, we developed and validated a high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method for determination of AF in human plasma.

2. Experimental

2.1. Chemicals and reagents

AF (NSC # 686288) was provided by National Cancer Institute. The internal standard, zileuton [N-(1-benzobthien-2-ylethyl)-N-hydroxyurea] was obtained from Rhodia Pharma Solutions Ltd., Northumberland, UK. All other chemicals and reagents were HPLC grade. Water was filtered and deionized with a US Filter PureLab Plus UV/UF system (Siemens, Detroit, MI, USA) and used throughout in all aqueous solutions. Drug-free (blank) human plasma from healthy donors was obtained from Harper Hospital blood bank (Detroit, MI).

2.2. Stock solutions and standards

Stock solutions of AF and zileuton (internal standard) were prepared in methanol at a concentration of 1 mg/mL, and stored in brown glass vials at -20°C . Working stock solutions were prepared fresh on each day of analysis as serial dilutions in methanol. The calibration curves were constructed by spiking AF in blank plasma at the concentrations of 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL. Quality control (QC) samples were prepared in blank plasma at AF concentrations of 5 (lower limit of quantitation, LLOQ), 40, 400, and 1600 ng/mL. All standards and QC samples were prepared fresh daily. For long-term and freeze-thaw stability, QC samples were prepared as a batch and stored at -80°C .

2.3. Sample preparation

Prior to extraction, frozen samples were thawed in a water bath at ambient temperature. A 250 μL aliquot of plasma was added to a 1.5-mL polypropylene eppendorf tube followed by spiking 1 mL of ethyl acetate containing internal standard zileuton (50 ng/mL). The mixture was vortex-mixed for approximate 1 minute, and centrifuged at 14,000 rpm for 5 minutes at ambient temperature. The top layer was transferred to a 1.5-mL polypropylene screw top tube, and evaporated to dryness under a stream of nitrogen in a water bath at $50^{\circ}\text{C} \pm 5^{\circ}\text{C}$. An aliquot of 100 μL of reconstitution solution (methanol/0.45% formic acid in water, 70:30, v/v/) was added into the tube. The mixture was vortex-mixed for 30 second followed by sonication for another 30 seconds, and was centrifuged at 14,000rpm for 5 min. The supernatant was transferred into a 250- μL polypropylene autosampler vial, sealed with a Teflon crimp cap. A volume of 10 μL was injected into the HPLC instrument using a temperature-controlled autosampling device (set at 4°C).

2.4. Chromatographic and mass-spectrometric conditions

Chromatographic analysis was performed using a Waters Model 2695 separations system (Milford, MA, USA). Separation of the analytes from potentially interfering material was achieved at 30°C using Waters X-Terra™ MS column (50 × 2.1 mm i.d.) packed with a 3.5 μm C₁₈ stationary phase, protected by a guard column (10 × 2.1 mm i.d.) packed with 3.5 μm C₁₈ material (Milford, MA, USA). The mobile phase used for the chromatographic separation was composed of methanol/0.45% formic acid in water (70:30, v/v), and was delivered isocratically at a flow rate of 0.2 mL/min. The column effluent was monitored using a Waters Quattro Micro™ triple quadrupole mass-spectrometric detector (Milford, MA, USA). The instrument was equipped with an electrospray ionization source, and controlled by the Masslynx version 4.1 software, running under Windows XP 2000 on an IBM IntelliStation computer. The samples were analyzed using an electrospray probe in the positive ionization mode operating at a cone voltage of 44 V for AF, and 13 V for internal standard zileuton. Samples were introduced into the ionization source through a heated nebulized probe (350°C). The spectrometer was programmed to allow the [MH]⁺ ion of AF at *m/z* 321.1 and zileuton at *m/z* 237.1 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 48 and 9 eV for AF and zileuton, respectively. The product ions for AF (*m/z*, 109.5) and zileuton (*m/z* 160.8) were monitored simultaneously through the third quadrupole (Q3). Argon was used as collision gas at a pressure of 0.00172 mBar, and the dwell time per channel was 0.5 sec for data collection.

2.5. Method validation

2.5.1. Specificity—The specificity of the method was tested by visual inspection of chromatograms of extracted human plasma samples from 6 different donors for the presence of endogenous or exogenous interfering peaks. The plasma samples without (blank) and with spiking the analyte (AF at the LLOQ) and the internal standard (zileuton at 50 ng/mL) were prepared and extracted. The interfering peak area should not exceed 20% of the analyte peak area at the LLOQ and 5% of the internal standard peak area.

2.5.2. Calibration curve—Linearity was assessed at AF concentration ranging from 5 to 2000 ng/mL. Calibration curves were built by fitting the analyte concentrations of the calibrators versus peak area ratios of the analyte to internal standard using least-squares non-linear regression analysis with different weighting scheme (i.e., 1, 1/x, and 1/x²). The selection of weighting scheme was guided by evaluation of goodness-of-fit criteria including correlation coefficient (*R*²), % recovery of back-calculated calibrators and QCs, and residual plots.

2.5.3. Accuracy and precision—Validation runs for the calibrator standards (in duplicate) and QCs (in quintuplicate) including LLOQ, low, medium, and high were performed on three consecutive days. The accuracy was assessed as the relative percentage of the back-calculated to nominal concentration, which was equal to determined concentration/nominal concentration × 100%. The within- and between-day precisions were estimated by one-way analysis of variance (ANOVA) using the JMP™ statistical discovery software version 4 (SAS Institute, Cary, NC, USA). The between-day variance (VAR_{bet}), the within-day variance (VAR_{wit}), and the grand mean (GM) of the observed concentrations across runs were calculated from ANOVA analysis. The within-day precision (WDP) was calculated as: $WDP = 100 \times (\sqrt{VAR_{wit}}/GM)$. The between-day precision (BDP) was defined as: $BDP = 100 \times (\sqrt{((VAR_{bet} - VAR_{wit})/n)}/GM)$, where n represents the number of replicate observations within each day.

2.5.4. Recovery—Extraction recovery was examined at the AF concentrations of 40, 400, and 1600 ng/mL. Absolute extraction recovery was assessed by comparing peak areas of the analyte obtained from the extracted QC plasma samples versus unextracted standards (in reconstitution solution). Relative extraction recovery was assessed by comparing peak area ratios of the analyte to internal standard obtained from the extracted QC plasma samples versus unextracted standards (in reconstitution solution).

2.5.5. Stability—The short-term (bench-top) stability of AF in methanol (working solution) at the concentration of 100 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ as well as in plasma at the concentration of 40 and 1600 ng/mL was tested at ambient temperature (25°C) for 4 h. The autosampler stability of the analyte in the reconstitution solution (methanol/0.45% formic acid in water, 70:30, v/v) was examined at 4°C for 9 h after the QC plasma samples were processed. The freeze-thaw stability of AF in plasma was assessed at the concentrations of 40 and 1600 ng/mL through three freeze-thawing cycles. The long-term stability of AF in stock solution (at 1 mg/mL) and in plasma (at 40 and 1600 ng/mL) was investigated up to 2 months. All QCs were run in triplicate.

2.6. Pharmacokinetic analysis

AFP464 is currently being evaluated in a Phase I clinical trial in patients with advanced and refractory solid tumors. It was administered as a 3-hour intravenous infusion on Days 1 and 8 every 21 days. The dose levels evaluated ranged from 14 to 103 mg/m². The protocol was approved by the Institutional Review Board of the participated institution. All patients provided a written informed consent.

Blood samples were collected in tubes with heparin as anticoagulant at the following time points (in units of hours from the start of the 3-h infusion) on days 1 and 21: 0 (pretreatment), 2.0 (during infusion), 3 (immediately at the end of infusion), 3.5, 4, 6, 8, and 24 hours. The blood samples were immediately placed in an ice bath and then centrifuged at 3000 RPM at 4°C for 10 minutes. Plasma was separated and split into two aliquots, and stored at –80°C until analysis.

The concentrations of AF in patient plasma samples were determined using the described validated method. The pharmacokinetic parameters of AF in individual patients were estimated using noncompartmental analysis with the computer software program WinNonlin version 5.0 (Pharsight Corporation, Mountain View, California).

3. Results and discussion

3.1. Detection and chromatography

The mass spectrum of AF showed protonated molecules ($[\text{MH}^+]$) at m/z 321.1. The collision energy fragmented the analyte into several fragments. The major fragment observed was at m/z 109.5 and was selected for subsequent monitoring in the third quadrupole (Fig. 1a). The internal standard, zileuton, had protonated molecules ($[\text{MH}^+]$) at m/z 237.1 and produced a major fragment at m/z 160.8 (Fig. 1b).

Representative chromatograms of blank and spiked human plasma samples with AF and internal standard that were monitored at m/z 321.1 $\rightarrow$ 109.5 (for AF) and m/z 237.1 $\rightarrow$ 160.8 (for zileuton) are shown in Figs. 2 and 3. The mean ($\pm$ standard deviation) retention times for AF and zileuton under the optimal conditions were at 3.3 ± 0.2 , and 1.5 ± 0.1 min, respectively, with an overall chromatographic run time of 6 minutes (Fig. 3). The selectivity for the analysis was shown by symmetrical resolution of the peaks, with no significant chromatographic interference around the retention times of the analyte and internal standard in the chromatograms of blank plasma from six different donors when monitored for AF and

zileuton (data not shown). During implementation of this assay, pre-treatment plasma samples from the cancer patients were analyzed with this assay with no interferences noted.

3.2. Calibration Curves

The calibration curves were established over the nominal AF concentration range of 5 to 2000 ng/mL. The relationship between peak area ratios of AF to the internal standard versus the nominal AF concentrations was best fitted by a linear equation, expressed as $y = a \cdot x + b$, where y is peak area ratio, x is the analyte concentration, a and b are fitted parameters. A weighting factor, which is inversely proportional to the variance at the given concentration level (x^2), was used. This weighting factor was chosen compared to uniform weighting and weighting by $1/x$ because the weighting factor of $1/x^2$ produced the best goodness-of-fit in terms of the R^2 value, intercept closest to a zero value, percent recovery of calibrators and QCs, and distribution of residues. A mean least-squares linear regression correlation coefficient (R^2) of > 0.99 was obtained in all analytical runs. The distribution of residuals was random, normally distributed, and centered on zero (data not shown). For each calibrator standard (in duplicate each day for 3 days, $n = 6$) on the calibration curves of AF, the average accuracy in terms of percent recovery of the back-calculated relative to nominal concentration ranged from 96.5% to 105.3%, and the within- and between-day variations (expressed as the percentage of relative standard deviations) were less than 10% (Table 1).

3.3. Accuracy, precision, and recovery

The LLOQ for AF was established at 5 ng/mL, at which the mean signal-to-noise ratio was 60 from 20 observations. For the QC samples prepared by spiking human plasma with AF at the concentrations of 5 (LLOQ), 40, 400, and 1600 ng/mL, the average accuracy (expressed as percent recovery of the back-calculated relative to nominal concentration) ranged from 90.5% to 107.8%, and within- and between-day precisions (expressed as the percentage of relative standard deviations) were all less than 5.0% (Table 2).

For the triplicate QCs at the AF concentrations of 40, 400 and 1600 ng/mL, the average absolute extraction recovery of AF from human plasma was determined as 85.1%, 86.1%, and 88.4%, respectively; the average relative extraction recovery was determined as 109.9%, 113.8% and 115.8%, respectively. The average absolute extraction recovery for the internal standard zileuton from plasma was determined as 76.6% ($n=9$) at the concentration of 50 ng/mL.

3.4. Stability

The short- and long-term stability of AF was demonstrated in Table 3. At ambient temperature ($\sim 25^\circ\text{C}$), AF were stable for at least 4 h in methanol working solution at the concentrations of 1 and 100 $\mu\text{g/mL}$. It was stable for at least 4 h in human plasma at the concentrations of 40 and 1600 ng/mL (Table 3). In the autosampler (set at 4°C), AF was stable for at least 9 h in the reconstitution solution (methanol/0.45% formic acid in water, 70:30, v/v), allowing the assay to be performed continuously overnight for a large number of samples (Table 3). Freeze-thaw stability, which was assessed at AF concentrations of 40 and 1600 ng/mL, showed no significant ($<10\%$) degradation of the compound through three full cycles of freeze-thaws (Table 3). The long-term stability tests suggested that AF was stable in methanol (stock solution, 1 mg/mL) at -20°C for at least 1 year (with degradation less than 10%) and it was stable in human plasma at -80°C up to 2 months (with degradation less than 15%) (Table 3).

3.5. Plasma concentration–time profile

This LC-MS/MS method was successfully applied to study the pharmacokinetics of AF in a dose-escalation phase I trial, in which the patients received AFP464 as a 3-h intravenous infusion at dose levels ranging from 14 to 103 mg/m². Because of the rapid conversion of AFP464 to AF in plasma by nonspecific plasma esterases, AFP464 was only detectable during the infusion (e.g., at 2 h) and at the end of infusion (e.g., at 3 h) in the majority of patients (data not shown). The maximum plasma concentration of AF was generally achieved at the end of infusion. AF pharmacokinetic profile was characterized by a biexponential disposition with a first rapid disposition phase followed by a slower disposition phase. Fig. 4 shows the representative plasma concentration–time profiles of AF in two patients who received AFP464 as a 3-h infusion at the dose of 14 and 74 mg/m². In these two patients, the maximum plasma concentration of AF achieved at the end of infusion were 92 and 640 ng/mL, respectively; the AUC_{last} was 222 and 1089 h*ng/mL, respectively.

4. Conclusion

In summary, a sensitive and reliable LC-MS/MS method was developed and validated for the determination of AF in human plasma. The LLOQ for AF was determined at 5 ng/mL in plasma. Linear calibration curves were generated over the AF plasma concentration range of 5 to 2000 ng/mL. This method was successfully applied for quantitation of AF in patient plasma samples over a wide dose range in a dose-escalation phase I trial.

Acknowledgments

This work was supported by NIH U01-CA062487 and by Tigris Pharmaceuticals (Bonita Springs, FL).

References

1. Kuffel MJ, Schroeder JC, Pobst LJ, Naylor S, Reid JM, Kaufmann SH, Ames MM. Mol Pharmacol. 2002; 62:143. [PubMed: 12065765]
2. Loaiza-Perez AI, Kenney S, Boswell J, Hollingshead M, Alley MC, Hose C, Ciolino HP, Yeh GC, Trepel JB, Vistica DT, Sausville EA. Mol Cancer Ther. 2004; 3:715. [PubMed: 15210858]
3. Meng LH, Kohlhausen G, Liao ZY, Antony S, Sausville E, Pommier Y. Cancer Res. 2005; 65:5337. [PubMed: 15958581]
4. Meng LH, Shankavaram U, Chen C, Agama K, Fu HQ, Gonzalez FJ, Weinstein J, Pommier Y. Cancer Res. 2006; 66:9656. [PubMed: 17018623]
5. McLean L, Soto U, Agama K, Francis J, Jimenez R, Pommier Y, Sowers L, Brantley E. Int J Cancer. 2008; 122:1665. [PubMed: 18059023]
6. Chen C, Meng L, Ma X, Krausz KW, Pommier Y, Idle JR, Gonzalez FJ. J Pharmacol Exp Ther. 2006; 318:1330. [PubMed: 16775196]

Fig. 1. Product mass spectrum of a) AF with monitoring at m/z 321.1 $\rightarrow$ 109.5. and b) the internal standard zileuton with monitoring at m/z 237.1 $\rightarrow$ 160.8.

Fig. 2. Chromatograms of blank plasma that was monitored at a) m/z 321.1 $\rightarrow$ 109.5 (for AF), and b) m/z 237.1 $\rightarrow$ 160.8 (for zileuton). The arrows indicate the retention time of AF and the internal standard zileuton.

Fig. 3.

Chromatograms of plasma spiked with a) AF (5 ng/mL) and b) internal standard zileuton (50 ng/mL) as well as chromatograms of a plasma sample collected at the end of infusion (3 h) in one patient who received AFP464 as a 3-h infusion at the dose of 74 mg/m^2 (c and d). The retention times for AF and zileuton were 3.3 ± 0.2 and 1.5 ± 0.1 min, respectively.

Fig. 4. Representative plasma concentration-time profiles of AF in two cancer patients who received AFP464 as a 3-h intravenous infusion at the dose of 14 mg/m² (▲) and 74 mg/m² (□).

Table 1

Accuracy, within- and between-day precisions of calibrator standards ^a in the calibration curve of AF

Nominal concentration (ng/mL)	Determined concentration (ng/mL)	Average Accuracy (%)	Within-day precision (%)	Between-day precision (%)
5 (LLOQ)	5.0 ± 0.2	100.7	5.0	<i>b</i>
10	9.7 ± 0.5	96.5	1.8	5.5
20	19.7 ± 1.6	98.3	5.3	6.5
50	50.4 ± 3.4	100.9	7.4	<i>b</i>
100	97.0 ± 6.5	97.0	4.3	5.6
200	196.1 ± 16.2	98.1	5.0	7.4
500	503.1 ± 36.0	100.6	6.9	2.3
1000	1053.0 ± 45.3	105.3	4.0	1.8
2000	2016.3 ± 126.2	100.8	4.6	4.7

^aEach calibrator standard was evaluated in duplicate on three consecutive days.

^bNo additional variation was observed as a result of performing assay in different days.

Table 2

Assessment of accuracy, within- and between-day precision for the AF QC samples

Nominal concentration (ng/mL)	Determined concentration (ng/mL)	Average accuracy (%)	Within-day precision (%)	Between-day precision (%)
5 (LLOQ)	4.5 ± 0.2	90.5	4.0	3.8
40	37.4 ± 1.6	93.4	3.5	3.0
400	862.0 ± 31.3	107.8	3.1	2.2
1600	1690.0 ± 70.0	105.6	2.8	3.6

^aPerformed in quintuplicate on three consecutive days.

Table 3Assessment of stability of AF^a

	AF concentration (ng/mL)	
	40	1600
Short-term stability (in plasma) (25°C) ^b		
1.0 h	95.6	96.4
2.0 h	87.0	92.6
3.0 h	98.7	91.0
4.0 h	89.7	87.1
Autosampler stability (in reconstitute solution) (4°C) ^c		
2.0 h	94.4	104.2
9.0 h	88.4	101.8
Freeze-thaw stability (in plasma) (−80°C) ^b		
Cycle 1	89.1	85.8
Cycle 2	91.7	85.2
Cycle 3	96.5	94.8
Long-term stability (in plasma) (−80°C) ^b		
1 month	104.2	92.6
2 month	91.5	85.9

^aStability data were expressed as mean percentage of the analyte concentration determined at certain time point relative to that at time zero (nominal concentration)

^bEach concentrations were assessed in triplicate.

^cInjected repeatedly for 9 h with one sample.